THE MOLECULAR MECHANISMS OF ACCESSORY CELL-T LYMPHOCYTE INTERACTION

BY

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TO LORNA MY WIFE

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ABSTRACT

By a process of negative selection, lymphoid dendritic cells were isolated to high purity from human tonsils. In comparison with other purified tonsillar accessory cell types these dendritic cells were shown to be potent inducers of T lymphocyte proliferation in the periodate oxidative mitogenesis reaction, autologous MLR and allogeneic MLR.

The tonsillar dendritic cell induced oxidative mitogenesis reaction was used as a model to study the molecular mechanisms of dendritic cell induced T lymphocyte proliferation. For this, panels of antibodies were used as tools to probe different aspects of the cell-cell interaction. The results indicate that dendritic cells induce T lymphocyte proliferation by a complex two stage mechanism. One stage is an early dendritic cell-T lymphocyte clustering stage which is mediated by LFA-1, ICAM-1, the CD2 antigen and LFA-3 and in which the CD45 antigen plays an indirect role. The other stage is a signal transduction stage. LFA-1, ICAM-1, the CD2 antigen, LFA-3 and the CD45 antigen are also implicated at this stage in addition to a variety of other molecules including the CD3, CD4, CD5, CD25, CD26, CD28, CD39, CD44, CD48, CDw70, CD71 and CDw78 antigens and class I and II MHC.

In quantitative assays the molecular events involved in PMA differentiated U937-T lymphocyte clustering were also investigated. This type of clustering is mediated by the same epitopes of LFA-1 and ICAM-1 as dendritic cell-T lymphocyte clustering and again the CD45 antigen is indirectly implicated. There are differences, however, in that the CD2 antigen and LFA-3 are not involved and that the CD44 antigen and class II MHC, too, play an indirect role. Along with evidence that the roles of the CD44 and CD45 antigens in U937-T clustering are distinct, these findings emphasise the heterogeneity of structures and mechanisms involved in accessory cell-T lymphocyte interaction.

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CHAPTER 1

GENERAL INTRODUCTION

Antigen specificity, compartmentalisation and cell co-operativity in vertebrate lymphocytes

One of the hallmarks of the vertebrate immune system must be its ability to recognise antigen specifically. This specific recognition is achieved by two classes of different but related clonally distributed antigen receptors on lymphocytes i.e. T cell antigen receptors (TcR; 1,2) on T lymphocytes and immunoglobulins (Ig; 3,4) on B lymphocytes. Antigen specificity provides the vertebrate immune system with a means of distinguishing between self and non-self antigen. Of particular advantage over invertebrate counterparts, however, is that the response to nominal non-self antigen can be controlled in such a way as to allow for 1) the development of a particular type of immune response that is most appropriate for the removal or innactivation of that antigen and 2) the generation of an appropriate and specific immune memory to that antigen.

Integral to this view of antigen specificity in vertebrate immunity are two concepts. The first is the division of lymphocytes into regulatory (helper/inducer and possibly suppressor T lymphocytes) and effector [cytotoxic T lymphocytes (CTL), delayed type hypersensitivity (DTH) effector T lymphocytes and B lymphocytes] compartments (5). Specific recognition of self antigen by immature autoreactive regulatory T lymphocytes in the thymus leads to the specific deletion or innactivation (cellular suppression or anergy) of these lymphocytes and forms the basis of self/non-self discrimination. Also, at the induction stage of the immune response, specific recognition of non-self antigen allows the preferential activation of those subsets of mature regulatory T lymphocytes that are involved in the mobilisation of the most appropriate effector arm of the immune response; and the expansion of these antigen specific regulatory T lymphocytes as well as of antigen specific effector lymphocytes forms the premise of the

development of an appropriate and antigen specific memory.

The second concept is the concept of cell co-operativity between lymphocytes. Thus, for effector responses in the induction of immunity there is a requirement for effector lymphocytes to co-operate with regulatory T lymphocytes which exert their regulatory influence in the form of the provision of help (soluble factors or cell-cell contact; 6-8). In this context, deletion or innactivation of antigen specific immature autoreactive regulatory T lymphocytes in the thymus abrogates reactivity to self by blocking co-operation between regulatory and effector compartments. In contrast, in the induction of immunity to non-self antigens, antigen specificity permits the development of appropriate immune responses and appropriate memory by eliciting particular pathways of co-operation between regulatory and effector compartments.

Cell co-operativity in T lymphocyte recognition of and response to antigen

In addition to cellular co-operation between regulatory and effector lymphocytes, another example of cell co-operativity in the vertebrate immune system, which also plays an important regulatory role, is in the T lymphocyte specific recognition of and subsequent response to antigen itself. Cellular co-operation is a requirement for T lymphocyte recognition and response to antigen at each of the stages in the life history of these cells in which this event might occur. For example, in the thymus, recognition of self antigen by immature T lymphocytes leading to their selection (positive selection) or deletion/innactivation (negative selection or tolerance induction as outlined above) requires co-operation with thymic stromal accessory cells. Similarly, in the induction of immunity, recognition of non-self antigen by mature T lymphocytes leading to their proliferation and maturation into functional regulatory and effector cells

depends upon their co-operation with peripheral accessory cells; and at the effector stage of the immune response, recognition of non-self antigen by mature effector T lymphocytes leading to the expression of their effector function, too, requires cellular co-operation i.e. with susceptible target cells.

At least for the T lymphocyte recognition of antigen the requirement for cell co-operativity reflects the specificity of the TcR. In contrast to Ig antigen receptors on B lymphocytes, which recognise native antigen alone (3,4), TcR on T lymphocytes recognise self major histocompatability complex (MHC) molecules plus peptides derived from native antigens (9-11). Co-operation in the recognition of ligand, therefore, at each of the developmental, induction and effector stages of T lymphocyte immunity, is a consequence of the need to communicate with cells that can both generate peptides from native antigens and present these peptides upon the cell surface in a complex with self MHC - two events known as antigen processing and presentation respectively (12-14).

As recognition of self MHC plus peptide is essential for the T lymphocyte response to antigen, then the ligand specificity of the TcR also provides a basis for the requirement for cell co-operativity in this process. However, mere recognition of ligand (even if this were to occur above a threshold level that would otherwise permit responses) does not necessarily lead to response at any level in the immune system. Thus, there are additional bases for the requirement for co-operation in the T lymphocyte response to ligand.

For effector T lymphocyte responses at the effector stage of the immune response, for example the exertion of cytotoxic or DTH T lymphocyte effector function, these additional bases may be relatively simple. In essence, they may reflect the requirement for another T lymphocyte surface

molecule-target cell surface molecule interaction, i.e. respectively a CD2 molecule-lymphocyte function associated antigen-3 (LFA-3) interaction, which acts in synergy with the TcR-self MHC plus peptide interaction to convey the response signal (15). For T lymphocyte responses at the induction stage of immunity and possibly also at the developmental stages of immunity, however, in addition to the requirement for a CD2 molecule-LFA-3 interaction, which together with the TcR-self MHC plus peptide interaction constitutes a "first signal", there are still further bases for co-operation. These further bases are the requirement of T lymphocytes to bind accessory cell "second signals" such as the cytokine interleukin-1 (IL-1;16-18).

Antigen processing/presenting cells and accessory cells

Intrinsic to this whole question of induction is the distinction between antigen processing/presenting cells and accessory cells. Antigen processing/presenting cells, are cells that can process native antigens into peptides and present these peptides upon the cell surface in a complex with MHC. Accessory cells, on the other hand, are cells that can induce T lymphocyte responses to MHC plus antigen at the developmental or induction stages of immunity. Antigen processing/presenting ability and accessory cell function are not necessarily equatable. Thus, most nucleated cell types are thought to be capable of processing and presenting antigen in some form or another but few cell types capable of antigen processing/presentation can act as accessory cells. There may be several different reasons for this difference. The first, which applies to the induction stage of the immune response (it is unclear, at present, if it also applies to T lymphocyte development) concerns the "first signal" itself and demands a more detailed description of antigen processing/presentation pathways.

At least two different pathways of antigen processing/presentation are

thought to exist (19,20). The first is a class I MHC processing/presentation pathway in which intracellular cytosolic antigens, including self and non-self antigens such as viruses, are processed into peptides and transported to the cell surface in a complex with class I MHC. The second is a class II MHC processing/presentation pathway. In this pathway, extracellular antigens (again both self and non-self) are first internalised by cells by pinocytosis, receptor mediated endocytosis or phagocytosis. Internalised antigens are then processed into peptides in endosomal, phagosomal, and lysosomal compartments and transported to the cell surface in complex with class II MHC.

Most nucleated cells express class I MHC and it is the class I MHC processing/pathway that most nucleated cells are thought to possess. Indeed, this would seem a requirement rendering most virus infected nucleated cells susceptible to attack by CTL which for the large part recognise peptides in complex with class I MHC (6,21,22). In contrast, most cell types are incapable of processing/presentation via the class II MHC pathway; the pathway involved in helper/inducer T lymphocyte antigen recognition (6,21,22). There may be several reasons for the inability of the majority of cells to process and present via the class II MHC pathway. For example, there may a lack of endocytic activity, an absence of class II MHC processing machinery or an absence of class II MHC itself. Whatever the reason, however, ability or inability to process and present via this route suggests a possible basis for the antigen processing/presenting cell versus accessory cell discrepancy in the induction of immunity. This basis has been alluded to previously and reflects the requirement for regulatory T lymphocyte help in effector T lymphocyte responses. Thus, although most cells can present processed antigen on class I MHC such presentation alone does not afford accessory cell activity as it only allows recognition of

antigen by certain effector T lymphocytes (and possibly suppressor T lymphocytes but which also require help to respond). For accessory cell activity presentation of processed antigen on class II MHC at least, is required, allowing helper/inducer T lymphocyte responses which in turn may also allow the response of effector T lymphocytes.

A second possible basis for the difference between antigen processing/presenting cells and accessory cells concerns the "second signal". Also as alluded to previously, in addition to first signals, second signals are required for T lymphocyte responses to antigen at the induction stage and possibly also developmental stages of immunity. Therefore, lack of accessory cell activity despite antigen processing/presenting ability, whether or not the requirement for class II MHC processing/presenting is satisfied, may reflect an inability to deliver appropriate "second signals" to T lymphocytes.

Finally, it should be made clear that the first and second signal model of accessory cell-T lymphocyte interaction outlined here is a simple one. This model merely focuses upon some of the requirements for T lymphocyte co-operativity with accessory cells in their responses to MHC plus antigen. Recently it has become clear that a whole host of additional cell surface structures may be involved in accessory cell-T lymphocyte interaction suggesting that a third possible reason for the antigen processing/presenting ability versus accessory cell activity difference may be the differential expression or activities of these molecules upon different antigen processing/presenting cell populations.

A family of accessory cells with dendritic morphology

On the question of which cells act as physiological accessory cells in vivo, in time, a variety of different cell types, including mononuclear phagocytes

(16,23,24), B lymphocytes (25,26), endothelial cells (27,28) and epithelial cells (29,30), have been implicated in this role. Recently, however, a large body of evidence has been amassed to suggest that a specialised family of dendritic cells (DC) may be the principal accessory cells of the immune system, whereas the other cell types play only a facultative accessory role. Included in this "DC family" (see Table 1.1) are Langerhans cells, indeterminate cells, veiled cells, interdigitating DC, thymic DC, interstitial DC, peripheral blood DC and splenic marginal zone DC. As well as morphology and accessory function these cells are united as a family on the basis of cell surface marker characteristics and are to be distinguished from other dendritic shaped cells (also see Table 1.1) that are associated with the immune system such as follicular DC (31), Thy-1 positive dendritic epidermal cells (32) and skin keratinocytes (33). In the remainder of this introduction evidence that thymic DC are the principal accessory cells involved in the negative selection of T lymphocytes and that other DC types are the principal accessory cells involved in the induction of T lymphocyte immunity will be discussed. Also, several other aspects of the biology of this important family of accessory cells, including ontogeny and mechanism of action (cellular and molecular) will be considered.

Histology, ultrastructure and markers of the "dendritic cell family" in situ

Langerhans cells. Langerhans cells were first identified as a branched component of human epidermis by Paul Langerhans in 1868 (34) and have since been identified in the epidermis of other species such as mouse (35), rat (36), guinea pig (37), hamster (38) and monkey (39). In healthy body wall, they comprise 2-8% of epidermal cells and occupy a suprabasal position.

A variety of methods have been employed for the visualisation of Langerhans cells in skin biopsies. In early studies Langerhans cells were

Table 1.1 - Dendritic type cells in the vertebrate immune system

Cell type	Location	Function
Langerhans cells	Epidermis (keratinised stratified squamos epithelium?)	Induction of T lymphocyte immunity
Indeterminate cells	Epidermis and dermis	Induction of T lymphocyte immunity?
Thy1+ dendritic epidermal cells	Epidermis	Tumour surveillance
Keratinocytes	Numerous epithelial sites	Source of inflammatory mediators
Veiled cells	Afferent lymph	Induction of T lymphocyte immunity
Interdigitating DC	Tonsillar interfollicular areas, lymph node paracortex, splenic peri-arteriolar lymphatic sheath (PALS)	Induction of T lymphocyte immunity
Marginal zone DC	Splenic marginal zone	Induction of T lymphocyte immunity
Follicular DC	B lymphocyte follicles in peripheral lymphoid organs	Induction of B lymphocyte memory
Thymic DC	Thymic medulla	Tolerance induction in T lymphocytes
Interstitial DC	Various (except brain)	Induction of T lymphocyte immunity
Peripheral blood DC	Peripheral blood	Induction of T lymphocyte immunity

stained with gold chloride (34). More recently advantage has been taken of the expression of the enzymic activities adenosine triphosphatase (ATPase; 40), acid phosphatase (41), aminopeptidase (42), non-specific esterase (43) and prostaglandin D synthetase (36) and an ability of the Langerhans cell to internalise certain neurotransmitters (44). Still further means of identification have involved immune recognition using antibodies. Originally against class II MHC (45,46), CD1a antigen [see Appendix 1 - (1)] (47,48) and the S-100 protein (49), Later against CD1c antigen (50), leucocyte common antigen (LCA/CD45 antigen), CD15c and CD68 antigens (51-53); Fc receptors for IgG (FcRIgG) and Mac-1 alpha chain [also known as CD11b antigen in the human; Mac-1 is complement receptor 3 (CR3), a receptor for inactivated C3b (C3biR); 54]; the NLDC145, MIDC8, F4/80 antigens and Msgp5 antigens (see later). Caution with majority of these different methods, however, is required, given other epidermal cells (resident and inflammatory) are often stained with these reagents (e.g. 55-58).

To date, the most reliable Langerhans cell marker in situ is a cytoplasmic structure known as the Birbeck granule the presence or absence of which is regarded as crucial in the definition of these cells in adult epidermis. First identified in electron microscopic preparations of human vitiliginous skin, by Birbeck et al (59) this organelle is a striated rod or "tennis racket" shaped, membrane encapsulated structure of variable length and position within the cell. It's origin and function are largely unknown. Birbeck granules can often be seen being "pinched off" from the plasma membrane, favouring derivation through an endocytic process (60). However, osmium zinc iodide selectively stains Birbeck granules and the golgi apparatus, favouring derivation from the latter organelle (61,62).

Birbeck granule containing Langerhans cells have been identified at other stratified squamos epithelial sites including the vagina and

ectocervix (63,64), the circumferential margins of the limbic attachments of the cornea to the sclera (38) and the trachea when metaplastic changes in smokers (65) or in patients with vitamin A deficiency (66) result in the transition of the epithelium from a columnar to a stratified squamos form. Identification at these different sites, along with the absence of Langerhans from the cornea itself (38) and the scales of the mouse tail (35), has led to the suggestion that Langerhans cells are a feature only of keratinised stratified squamos epithelium (67). Against this hypothesis, however, is that Langerhans cells have been found in the oral mucosa (68) and in the human oesophagus (69). Moreover, Birbeck granules have been identified in the cells of histiocytosis X (70) and eosinophilic granuloma (71), in which no direct histologic interaction with keratinising epithelial structures occurs.

Several other ultrastructural features of the Langerhans cell are relevant. Thus, Langerhans cells are rich in mitochondria, contain a well developed endoplasmic reticulum and golgi apparatus and exhibit a considerable number of phagocytic inclusions (some containing melanin particles and cellular debris) and lysosomes (59,62,72,73). Also, both microfilaments and microtubules have been observed in Langerhans cells (74,75), suggesting among other things a capacity for movement.

Indeterminate cells. The importance of the Birbeck granule in the definition of the Langerhans cell is underlined by the fact that within the epidermis there exists a population of cells that lack Birbeck granules but exhibit a variety of other Langerhans cell features. These indeterminate cells have been found in a number of different species including humans (76), mice (77) and monkeys (39) where they occupy a basal position.

Shared features with the Langerhans cell include morphology and a large number of mitochondria (78) and expression of class II MHC (79), CD1a

antigen (80), S-100 protein (81) and NLDC145 and Msgp5 antigens (see later). In contrast to the Langerhans cell, however, indeterminate cells have no surface ATPase activity (82) and exhibit fewer lysosomes, endoplasmic reticulum and golgi apparatus (78). Also, unlike Langerhans cells, indeterminate cells express CD1b (50); and p150,95 alpha chain (also known as CD11c antigen in the human) and CD14 antigen expression have also been recorded in situ (83).

Veiled cells. Microscopic examination of lymph from cannulated afferent lymphatics of humans (84), sheep (85), pigs (86), rabbits (87) and dogs (88) reveals a population of large mononuclear cells with actively moving processes. These cells, which are normally absent from efferent lymph, have been aptly named veiled cells. Veiled cells have also been identified in lymph collected from cannulated afferent lymphatics of smaller species such as the rat (89) but in smaller species an alternative experimental approach has commonly been used in the collection of veiled cells. This approach involves the lymphadenectomy of mesenteric lymph nodes allowing union of afferent and efferent lymphatics. Consequently, afferent lymphatic veiled cells drain into thoracic duct lymph which then provides a rich source of these cells (90,91).

From whichever species or source, veiled cells exhibit a number of common characteristics. With respect to morphology, two different appearances have in fact been noted (92,93). The first morphology is the classical morphology, i.e. long thin sweeping veils of cytoplasm extending from the main cell body. In the second morphology, however, blunt pseudopodia rather than veils are seen. It is unclear how stable each of these morphologies are and in this context one suggestion has been that any one veiled cell may interchange between the two .

Ultrastructurally, veiled cells resemble indeterminate cells more than

they do Langerhans cells. Thus, the cytoplasm of veiled cells contains few lysosomes and phagolysosomes and scanty rough endoplasmic reticulum (88,92-94). Cytoplasmic microfilaments (with a postulated role in veil motility, 94) and Birbeck granules (in a minority of veiled cells in lymph draining somatic tissue, not in intestinal lymph, 95,96), however, have been identified and interestingly, in several different species, the number of Birbeck granule containing veiled cells in afferent lymph has been shown to increase following antigenic stimulation (96,97).

Like Langerhans cells, veiled cells have been shown to express non-specific esterase and acid phosphatase (albeit these two enzymes weakly, 86,88) and ATPase (88,92). In addition, peroxidase activity and Feulgen positive cytoplasmic DNA inclusions have been observed in a minority of cells (88,93). Also, like Langerhans cells and indeterminate cells, veiled cells express the NLDC145 and MIDC8 antigens (see later) and strongly express class II MHC (86,88,91,92; including, in the rat, a polymorphic class II MHC determinant detected by the antibody 1F119 - see later). One point of difference, however, with respect to immunological markers, is the expression of FcRIgG and complement receptors. Veiled cells do not express these molecules (86,92,98). In contrast, Langerhans cells express FcRIgG and complement receptors (determined in situ and in vitro - see later) and indeterminate cells have also been shown to express FcRIgG (determined in vitro - see later).

Interdigitating dendritic cells. Pale staining cells in close contact with T lymphocytes and with blunt interdigitating processes, interdigitating DC, were first identified in the lymph node paracortices of rabbits by Veldman in 1970 (99). Before long, interdigitating DC were identified in the lymph node (and tonsillar) paracortices of other species such as humans (100,101) and rats (95,102) and it soon became apparent that, in addition to these

sites, interdigitating DC were present in the spleens of these species (103-107) as well as the spleens of mice (104,108), where they were found in the central PALS of the white pulp.

Morphological and ultrastructural features of interdigitating DC have been described in detail (55,95-97,104,108-113). Apart from interdigitating processes and an intimate contact with T lymphocytes other features include a close contact of interdigitating DC with with reticular fibres and tight junctions (with associated desmosomes) between interdigitating DC and T lymphocytes. Also, large empty vacuoles, a tubular vesicular complex, an irregularly shaped nucleus and a moderate number of mitochondria have been noted as have Birbeck granules in a minority of lymph node interdigitating DC (like veiled cells, the proportion of Birbeck granule containing lymph node interdigitating DC increases upon antigenic stimulation). In contrast to this, however, interdigitating DC have a poorly developed rough endoplasmic reticulum and golgi apparatus and few lysosomes, phagosomes and phagolysosomes (although some have documented the presence of phagocytic inclusions containing cell debris).

In parallel with the progress in the morphological and ultrastructural characterisation of these cells, histological and immunological markers have been studied for their expression upon interdigitating DC in situ. With respect to histological markers, for example the expression of enzymic activities, it has emerged that interdigitating DC closely resemble veiled cells. Thus, there is strong expression of ATPase (105,114) and only weak expression of acid phosphatase (102,109), non-specific esterase (115) and peroxidase (116), with the last three enzyme activities often being absent from interdigitating DC.

Immunological marker-wise, a striking and early recognised feature of interdigitating DC, in all species thus far studied, is their strong

expression of class II MHC (117-119), including the 1F119 determinant (see later) and the RFD1 determinant (a monomorphic human class II MHC determinant - see later). CD1 antigen expression has also been studied. Like Langerhans cells, interdigitating DC express the CD1a and CD1c antigens but not CD1b antigen (50,120). Other shared markers with Langerhans cells, and also with indeterminate cells, include the NLDC145, MIDC8 and Msgp5 antigens (see later) and the S-100 protein (81,121). Unlike Langerhans cells, however, but like veiled cells, interdigitating DC do not express FcRIgG or complement receptors in situ (53,122). Finally, a number of other isolated immunological markers have been noted for their expression upon interdigitating DC. These include CD14 and CD11c antigens (123), CD15 and CD73 antigens (124), CD40 antigen (125), Mac-2 and Mac-3 antigens (52), a unique epitope (detected by the antibody 7F7) of the intercellular adhesion molecule-1 (ICAM-1/CD54 antigen; 126) and controversialy, in humans, a membrane form of IL-1 beta (127).

Thymic dendritic cells. Another DC type, which in many respects is very similar to the interdigitating DC, although has received much less attention, is the thymic DC. Apart from location, thymic DC are considered by many to be identical to interdigitating DC. Here, however, they will be treated separately.

Thymic DC were first recognised in the mouse thymus in 1962 (128). Since this time, thymic DC have been observed in several other species including rats (129), rabbits (99), humans (130) and birds (131). Within thymi, thymic DC are found predominantly in the medulla, particularly at the cortico-medullary junction, and like interdigitating DC in peripheral lymphoid organs, the long cytoplasmic processes of thymic DC are seen to interdigitate with neighbouring T lymphocytes at this site (99,128). Ultrastructurally, thymic DC resemble interdigitating DC including the

possession of Birbeck granules (132,133) and a tubular vesicular complex (133) and an absence of phagosomes, phagolysosomes and lysosomes (133,134).

Some immunological markers have been characterised upon thymic DC in situ. Again like interdigitating DC, thymic DC strongly express class II MHC (130,135; including the 1F119 antigen); and the S-100 protein (121,133), NLDC145, MIDC8 and Msgp5 antigens (see later) are also expressed. Other markers that have been examined and for which thymic DC are positive include the low affinity IL-2 receptor (IL-2R /Tac antigen/CD25 antigen), CD4 antigen and CD45 antigen (136); Mac-1 alpha chain and Mac-2 antigen (137); and TE-3A, T2/30 and MR7 antigens (in humans, 138).

Interstitial dendritic cells. As demonstrated in mice (139), humans (140), rats (141) and dogs (142), cells which stain intensely with class II MHC antibodies and which have a typical dendritic morphology are not confined to skin (or other stratified squamos epithelial sites), lymphatic vessels and lymphoid organs. Indeed, with the exception of brain (141), class II MHC positive, Birbeck granule negative dendritic type cells, or interstitial DC, can be found in virtually every other tissue including the gut (89); lungs (139,143); pancreas (144); kidney, ureter and bladder (145); subcutaneous connective tissue, brain meninges and choroid plexus, liver, skeletal muscle, heart and thyroid (140,141); and even such bizarre locations as teeth (146).

Interstitial DC in rat heart (141), mouse (139) and rat (143) lung and in human liver (140) and pancreas (144) have been examined in some detail for their expression of histological and immunological markers in situ. One common feature of interstitial DC appears to be their lack of expression of the commonly studied histochemical enzymic activities non-specific esterase, beta-glucoronidase, acid phosphatase and ATPase In contrast, there is some degree of heterogeneity in immunological marker profiles. For example, human

liver interstitial DC do not express any of the known FcRIgG, i.e. FcRI-IgG (CD64 antigen, high affinity), FcRII-IgG (CDw32 antigen, intermediate affinity) and FcRIII-IgG (CD16 antigen, low affinity) and the complement receptors CR1 (CD35 antigen, a C3b, C4b, C5b receptor) and CR3 are also absent (some markers which are present include LFA-1, CD14 antigen, CD45 antigen and CD68 antigen). Similarly, human pancreatic interstitial DC do not express CR3. Absence of FcRIgG and complement receptors, however, is not a constant feature of interstitial DC. Thus, rat lung interstitial DC express CR3 and mouse lung interstitial DC express both FcRIgG and CR1.

Other dendritic cell types. Finally, two other DC types have been identified. The first of these is the peripheral blood DC, hitherto identified only in humans (147) and dogs (148). Human peripheral blood DC, as a trace population (representing only 0.1-0.5% of total peripheral blood mononuclear cells, 147,149), exhibit typical dendritic morphology, have an irregularly shaped nucleus, a tubular vesicular complex and numerous mitochondria, and have few pinocytic vesicles, lysosomes and rough endoplasmic reticulum (147). Class II MHC is abundantly expressed but peroxidase and non-specific esterase are absent as determined in situ (147,150). It is likely that similar DC are also present in the peripheral blood of other species, although this has not been examined, owing to the difficulty of obtaining sufficient quantities of peripheral blood from smaller laboratory animals.

The second DC type is found in the marginal zone of the spleen (often adjacent to vessels connecting the red and white pulp) and so far has been identified only in mice (151,152; presumably these cells are also present in the spleens of other species but again this has not been examined). Very few studies have examined these cells in situ and their only known in situ feature (apart from morphology) is expression of the 33D1 antigen (see

later). Possibly, however, these cells have been studied in detail in vitro, as the isolated mouse splenic DC (see later).

The "dendritic cell family" as accessory cells in vivo

Direct evidence that different components of the "DC family" act as accessory cells in normal physiological in vivo immune responses is inherently difficult to obtain. Circumstantial evidence for this claim, however, has been provided by studies of the behaviour of these cells in response to antigen, their accessory function in adoptive transfer experiments and their role in tissue transplantation [see Appendix 1 - (2)]. For thymic DC, additional in vivo experimental approaches have been used to suggest their accessory role in physiological immunity. These approaches will be considered separately at the end of this section.

Behaviour in response to antigen

Antigen uptake. As discussed earlier, a pre-requisite for accessory cell activity (at least in the induction of immunity) is the ability to process and present antigens via the class II MHC pathway. This in turn would seem to demand an ability to localise antigens at the cell surface and/or ingest them by endocytosis. Accordingly, different members of the "DC family" have been examined for these functions in vivo.

Langerhans cells appear to be active in each of the localisation, pinocytosis and phagocytosis of intradermally injected antigen. Localisation and pinocytosis has been demonstrated for a variety of substances including formaldehyde, glutaraldehyde, nickel, cobalt, chromium, mercury, gold, para-phenylenediamine, ethylenediamine and toluenediamine (153-157) and phagocytosis has been demonstrated for ferritin, peroxidase and thorotrast (158-160). By contrast, of the remaining DC types that have been examined,

although rabbit veiled cells (161) and mouse (162) and guinea pig (97) lymph node interdigitating DC have been shown to localise human Ig (HuIg), fluorescein isothiocyanate (FITC) and ferritin respectively following intradermal injection, rat lymph node DC do not pinocytose or phagocytose intradermally administered ferritin or colloidal carbon (163) and rat heart, liver and kidney interstitial DC too fail to ingest colloidal carbon injected intravenously (141).

Despite this, interdigitating DC might be active in the phagocytosis of whole cells. Recently the interdigitating DC has been identified as the principal phagocytic cell which ingests intravenously injected allogeneic thoracic duct lymphocytes 164,165). The physiological significance of this finding is not fully understood. Possibly, however, interdigitating DC similarly phagocytose altered self cells such as virus infected or tumour cells and thus induce immune responses against them and this would be in accord with the occasional identification, within interdigitating DC, of phagocytic inclusions containing cell debris (see page 12). Similarly, such phagocytic inclusions have been observed in Langerhans cells (see page 9) and thymic DC have been shown to ingest necrotic self lymphocytes (following irradiation, 166) and these observations may be relevant to the maintenance of self tolerance as well as to the induction of immune responses to altered self.

In summary then, Langerhans cells readily take up antigens in vivo, consistent with the function of these cells as physiological accessory cells. Other members of the "DC family" that have been tested, however, only poorly take up antigens in vivo (although interdigitating and thymic DC probably take up whole cells). This may seem surprising if these cells also function as physiological accessory cells but can be understood when one considers the possibilities of membrane antigen processing, co-operation

with mononuclear phagocytes in antigen processing and ontogenetic relationships within the "DC family" (see later).

Contact hypersensitivity. For Langerhans cells and veiled cells, suggestive evidence that these cells act as accessory cells in vivo has come from studies of their behaviour during contact hypersensitivity reactions. In humans hypersensitive to mercuric bichloride, gold chloride, hexachlorophene, mercaptobenzathiazole, nickel sulphate, para-phenylenediamine or pyrethrum and in guinea pigs primed to dinitrochlorobenzene or ferritin topical application of these allergens stimulates a number of Langerhans cell changes (97,158,167-171). Early changes include migration into the dermis and dermal lymphatics, a close apposition to T lymphocytes and an increase in metabolic activity and total cell number. Later, Langerhans cell damage is evident and a decrease in cell number is observed.

Veiled cell changes during contact hypersensitivity (and priming for contact hypersensitivity) have been studied in pigs (172) and mice (162). In these animals skin painting with dinitrofluorobenzene (DNFB) or FITC respectively stimulates a large increase in the output of afferent lymphatic veiled cells and in pigs the percentage of veiled cells in aggregates with T lymphocytes also increases.

All of these Langerhans cell and veiled cell responses during contact hypersensitivity reactions, particularly a juxtaposition to T lymphocytes, point to an in vivo accessory cell role for these cells. Likewise the position of interdigitating and marginal zone DC in the centre of T lymphocyte traffic areas in peripheral lymphoid organs and the close contact of thymic DC with immature T lymphocytes in the thymic medulla could be used to argue in favour of their in vivo accessory cell function. For Langerhans cells and veiled cells in contact hypersensitivity, however, an alternative

interpretation of the apposition to T lymphocytes is that such apposition is a reflection of DTH effector T lymphocyte function consistent with the damage that occurs to Langerhans cells and their reduction in number. Evidence that this is not the sole explanation though is that appositions occur as early as 3-5 hr following allergen challenge, well before active effector T lymphocytes can be expected to be generated. Also, in pigs challenged with DNFB large numbers of blast lymphocytes (probably T lymphocytes) appear in the afferent lymph (172) and similar observations have been made using oxazolone as an allergen in sheep (173), suggesting local activation of T lymphocytes by Langerhans cells and/or veiled cells.

Other evidence of an accessory role for Langerhans cells comes from experiments in which the ability of skin or other sites, naturally or artificially deficient in Langerhans cells, to support priming for contact hypersensitivity has been tested. Examples of naturally deficient sites are the scales of the mouse tail (35) and hamster cheek pouch (38) neither of which support priming for contact hypersensitivity to DNFB either per se (173,174) or when grafted onto the body walls of syngeneic recipients (175). As examples of artificially depleted sites each of ultraviolet (UV) light (173,176), UV light and prednisolone (176) or 4-beta phorbol 12 myristate 13 acetate (PMA; 177) treated mouse body wall, UV treated guinea pig body wall (178) and tape stripped human body wall (179) have been tested. These sites, too, do not support priming for contact hypersensitivity.

Adoptive transfer studies

In the adoptive transfer approach antigen labelled isolated DC are transferred into syngeneic or allogeneic recipients and their ability to act as accessory cells is measured as a capacity to prime for or induce recipient T lymphocyte responses. Two different types of adoptive transfer

experiment have been attempted. In the first type of experiment, DC are labelled with antigens in vitro. In the second type of experiment, DC are labelled in vivo or alternatively are not labelled but have the potential to provide antigenic (or antigen-like) stimuli by virtue of allogeneic cell surface molecules.

Both mouse Langerhans cells and mouse splenic DC have been tested in the first type of experimental approach. Unlike trinitrophenol (TNP) coupled peritoneal macrophages, TNP coupled Langerhans cells, when injected subcutaneously into syngeneic mice, primed animals for a long lived from of contact hypersensitivity upon re-challenge with TNP (180). Likewise, TNP coupled Langerhans cells and TNP coupled splenic DC primed mice for a long lived form of contact hypersensitivity when injected intravenously (180-182). These studies clearly show that mouse Langerhans cells and splenic DC can act as accessory cells when transferred to syngeneic recipients and the inference is that this same function is performed by these cells in normal in vivo physiological situations. It should be stressed, however, that both cell types were artificially introduced to antigen in vitro and this may preclude an ability of these cells to present antigen to T lymphocytes in an immunogenic form.

The second experimental approach has thus provided stronger support for the notion that different DC types function as accessory cells under normal physiological conditions. On Langerhans cells, mouse skin painted with DNFB and grafted onto the body wall of syngeneic recipients (183) sensitised the recipients to contact hypersensitivity upon re-challenge with DNFB. Similar grafts, however, depleted of Langerhans cells by tape stripping (before painting), did not sensitise and neither did grafts transplanted onto allogeneic recipients.

Several different demonstrations have been made for lymph node DC.

Isolated lymph node DC from mice, skin painted with FITC or rhodamine isothiocyanate (RITC), sensitise to FITC or RITC specific contact hypersensitivity respectively, when adoptively transferred to syngeneic recipients (162). Similarly, this has been shown using oxazolone and TNP as antigens and lymph node DC from both normal (184) and nude mice (using congenic euthymic recipients, 185).

For splenic DC, demonstrations in this type of assay have been made in both experimental autoimmune disease and in an ability to overcome an immune response gene (Ir gene) defect. In experimental autoimmune disease, splenic DC from rats with experimental autoimmune encephalomyelitis and splenic DC from mice with experimental autoimmune thyroiditis induced these diseases when adoptively transferred to syngeneic recipients (186,187).

The Ir gene defect is a class II MHC Ir gene defect and occurs in mutant bm12 mice. These mice differ from their strain of origin (C57BL/6 - $H\text{-}2^b$) by three amino acids in the beta chain of the $I\text{-}A^b$ molecule and as a consequence active anti-H-Y male antigen CTL are not generated from in vitro cultures of whole spleen cells from female mice [pre-immunised with phosphate buffered saline (PBS) or whole male spleen cells] and whole male spleen cells (in contrast to the strain of origin where pre-immunisation with whole male spleen cells does allow such a response). If, however, the female mice are pre-immunised with male splenic DC rather than whole male spleen cells, then active anti-H-Y specific CTL can readily be demonstrated in these cultures (albeit in the presence of exogenous IL-2, 188)

Finally, veiled cells have been shown to act as accessory cells in the second type of experimental approach. Rats, injected subcutaneously with syngeneic HSN sarcoma cells, develop large skin tumours. If, however, rats are pre-immunised with intestinal lymph veiled cells from syngeneic donors with Peyers patch HSN tumours, then only a minority develop small skin

tumours upon subcutaneous challenge with HSN sarcoma cells (189).

Transplantation studies

The last in vivo experimental approach, that has suggested a physiological accessory cell role for different DC types, is the transplantation approach which has provided convincing evidence that DC act as "passenger leucocytes", inducing the rejection of transplanted allografts. Often, this has depended upon demonstrations that DC depleted allografts either do not prime for "second set" allograft rejection or themselves enjoy extended survival in the host. Additionally, however, demonstrations that isolated DC types induce the rejection of otherwise long term allografts or accelerate the rejection of normal allografts, when adoptively transferred to hosts, have lent further support to this notion.

Demonstration by depletion. Most studies of allografts depleted of DC have provided evidence of an interstitial DC "passenger leucocyte" role. For example, in mice, full pancreatic islet and thyroid allografts, depleted of interstitial DC by class II MHC antibody plus complement and 33D1 antibody plus complement respectively, are not rejected by the host (and neither do the thyroid allografts prime hosts fro accelerated "second set" rejection, 190-191). In rats, too, depletion interstitial DC from full cardiac allografts by cyclophosphamide treatment and irradiation allows prolonged survival (192).

The depletion method has also been used to show that interstitial DC play a role in the induction of the rejection of MHC matched allografts. In this regard, in the dog, MHC matched kidney allografts survive for extended periods in the host if the donors are first irradiated (to deplete kidney interstitial DC) and reconstituted with host bone marrow (193). That host DC additionally play an inducer accessory role in the rejection of MHC matched

allografts has too been shown in this species. Thus, prolonged survival of MHC matched kidney allografts can also be achieved by prior irradiation of the hosts and reconstitution with host bone marrow depleted of DC precursors with the use of methylprednisolone (142)

Finally, the role of Langerhans cells as "passenger leucocytes" in graft rejection, has been demonstrated by this method (194,195). Fully allogeneic mouse corneal allografts, pre-treated with an irritant to induce Langerhans cells into the corneum, normally prime hosts for second set rejection of body wall allografts. In contrast, untreated mouse corneal allografts, which are devoid of Langerhans cells do not prime for "second set" rejection.

Demonstration by adoptive transfer. Each of veiled cells, splenic DC, interstitial DC and peripheral blood DC have been shown to induce or accelerate the rejection of allografts when adoptively transferred to grafted recipients. In rats, veiled cells of donor origin (but not donor T or B lymphocytes) have been shown to induce the rejection of otherwise long term DC depleted kidney allografts when injected intravenously into hosts at the time of transplant (196).

The role of splenic DC as inducers of long term allograft rejection has been demonstrated in studies of pancreatic islet, skin and thymic allografts. In the case of pancreatic islet allografts, fully allogeneic DC depleted long term grafts are rapidly rejected by mice following intravenous injection of donor splenic DC (197).

A study showing that splenic DC can induce long term skin allograft rejection represents another example of the ability of these cells to overcome an Ir gene defect. In this context, the class II MHC Ir gene defect in mutant bm12 mice (see page 21) also manifests as an inability of female mice to reject male skin grafts. Pre-immunisation of female mice with male

splenic DC (but not whole male spleen cells), however, abolishes this defect and male skin to female skin allografts are subsequently rejected (188).

With regard to the thymic allografts, in mice, long term survival of fully allogeneic foetal thymic lobes, grafted onto adult recipient's renal capsule, can be achieved by pre-treatment of the lobes with deoxyguanosine (dGuO; 198), which depletes the lobes of DC (199) and lymphocytes (200). Mouse T lymphocytes do not express class II MHC (201) and thus it is most likely that depletion of thymic DC is responsible for this effect. Furthermore, in support of this notion is the finding that dGuO treated thymic lobe allografts are rapidly rejected if the hosts are pre-immunised with donor splenic DC (202,203).

Finally, the function of interstitial and peripheral blood DC in this type of assay has been demonstrated in studies of liver and bone marrow allografts respectively. In rat liver allograft studies primary fully allogeneic grafts are rejected rapidly as "second set" grafts if the hosts are intravenously injected (before transplant) with donor isolated liver interstitial DC (204). Similarly, in dogs, it has been shown (both by depletion and adoptive transfer) that DC are the active component of donor peripheral blood mononuclear cells which prime irradiated hosts for the rejection of class I and class II MHC matched donor bone marrow allografts (148).

Thymic dendritic cells and negative selection

Evidence from a variety of different in vivo experimental approaches has suggested that thymic DC may be the principal accessory cells of the immune system which negatively select T lymphocytes. Many of these approaches, however, such as studies of allogeneic bone marrow chimaeras (e.g. 205,206) and studies of the expression of TcR variable region beta (V-beta) gene

expression during T lymphocyte ontogeny (e.g. 207,208) have only shown that bone marrow derived thymic cells (i.e. thymic DC, macrophages or even T lymphocytes themselves) play a role in negative selection or additionally that thymic epithelial cells do not; and in these situations that thymic DC are the main accessory cells involved has been inferred from the fact that in the induction of the immune response thymic DC (under non-physiological conditions) and other members of the "DC family" act as potent accessory cells. Exceptions to this are some studies of thymic allografts and thymic organ cultures (not strictly an in vivo approach but considered so here for convenience) which have provided more direct evidence that thymic DC are the principal accessory cells involved in negative selection.

Thymic allografts. Long term dGuO treated foetal thymic allografts (haplotype a; see page 24) are soon recolonised by host (haplotype b) bone marrow derived cells. Newly developed fully mature T lymphocytes from the grafts, however, are intolerant of a MHC [as detected by both proliferative and CTL responses in the mixed lymphocte reaction (MLR), 198,209,210]. As dGuO depletes from the lobes lymphocytes and thymic DC (but spares thymic epithelial cells and macrophages, 199,200 and E. Jenkinson - personal communication) then this suggests, a priori, that lymphocytes and/or thymic DC play a role in tolerance induction. Further, from other considerations, although mouse T lymphocytes may be involved in negative selection leading to an inability to generate functional CTLs in the MLR it is unlikely that they would also be involved in the type of negative selection that would result in a lack of proliferation in the same response (given the absence of class II MHC from these cells). Thus, in the latter type of negative selection there is evidence that thymic DC alone play a role in this process.

Thymic organ cultures. Recolonisation of explanted dGuO treated mouse thymic lobes has also been achieved in vitro by culture of such lobes with foetal liver or foetal thymic fragments (separated from the dGuO treated lobes by a filter to allow the immigration of stem cells only, 200,211). When the lobes and fragments are in allogeneic combinations (i.e. thymus-haplotype a, fragments-haplotype b), then fully mature T lymphocytes that develop from the lobes, as in the in vivo situation, are not tolerant of a MHC, again pointing to the crucial role of thymic DC in this type of negative selection. Also, however, and again as in the in vivo situation, the T lymphocytes are tolerant of b MHC. In vitro, this tolerance could be explained by the migration into thymic lobes of foetal liver and thymus DC precursors, which then differentiate into DC in situ. In vivo, however, in addition to these recently differentiated DC, mature peripheral DC that had migrated into the thymus and transformed into thymic DC would probably also contribute to this tolerance.

One other recent thymic organ culture study has provided evidence that thymic DC are the principal physiological accessory cells that tolerise T lymphocytes (312). In this regard, T lymphocytes that matured from 21 d old explanted foetal mouse thymi that were cultured for 1 d with highly purified allogeneic mouse splenic DC in vitro 7d previously (i.e. at the time of thymus removal), were specifically tolerant of DC type MHC as detected by an inability to generate CTL in MLRs. Moreover, on a per cell basis, the DC were far more effective than unfractionated spleen cells or thymus cells in inducing this tolerance. The conclusion that thymic are the most important accessory cells which tolerise T lymphocytes, of course, depends upon the assumption that thymic DC and splenic DC are closely related, which, given later discussions is safe.

The "dendritic cell" family in vitro

In vivo studies of the "DC family" have provided much evidence that these cells are capable of acting as accessory cells, but generally direct comparisons with other putative accessory cells have not been made. Studies of isolated DC in vitro have not only confirmed their accessory cell activity but in addition have shown that these cells are superior to other cells in this respect. These observations together with the in vivo observations have provided support for the concept that not only are the different members of the "DC family" significant accessory cells in vivo but are the principal accessory cells of the immune system.

As well as allowing for the testing of DC accessory cell activity, studies of isolated DC in vitro have provided for an elucidation of the molecular mechanisms involved which in turn has permitted speculation as to the basis of the potency of DC. Also, in vitro studies have allowed a more extensive analysis of the cell surface marker characteristics of the "DC family" and the results of such studies have been valuable for a variety of reasons, not least in deciding ontogenetic relationships both within the family itself and in relation to other cell types.

Isolation of different dendritic cell types

A major obstacle in the study of DC in vitro is the lack of a DC specific marker that would allow their isolation from tissues in a simple one step procedure. Although DC specific antibodies have been described (see later) these antibodies have not proved useful in this type of isolation. Consequently, DC are often isolated from tissues in a series of steps each of which successively enrich for these cells. Some of these steps exploit physical properties of DC and others make use of DC marker expression. Owing to both tissue and species differences it is difficult to generalise as to

these methods and thus in the following discussions, using selected examples, different DC types will be considered separately.

Langerhans cells from guinea pigs, mice and humans. Few studies have attempted to isolate Langerhans cells to high purity. One commonly used procedure in mice and guinea pigs, which achieves partial enrichment, however, involves selection of low density epidermal cells by spinning on columns of bovine serum albumin (BSA; 213,214). Another feature of Langerhans cells is their transient adherence to plastic surfaces and thus overnight adherence (to deplete keratinocytes) has also been used in the partial purification of mouse Langerhans cells (215). Sometimes non-adherent cells are treated with Thy1 antibody plus complement (to ensure keratinocyte depletion) and then further enriched for Langerhans cells by panning with LCA antibodies (216,217)

In contrast to other DC types, Langerhans cells have also been enriched in single step procedures which utilise Langerhans cell surface marker expression - in guinea pigs and mice by rosetting with antibody coated ox red blood cells (ORBC; 53,218) and in humans by panning with CD1a antibodies (219,220). With respect to other somatic cells, the markers that are used in these techniques, i.e. FcRIgG and CD1a antigen respectively, are not truly Langerhans cell specific. However, among epidermal cells their expression is relatively Langerhans cell restricted and the strong expression of these molecules upon these cells thus permits their simple enrichment by these methods.

Mouse peripheral lymphoid dendritic cells. Enriched populations of mouse lymph node and splenic DC have too been obtained by selecting low density fractions (162,221) or alternatively by selecting firstly 1-2 hr adherent cells and then overnight non-adherent cells (which primarily depletes

lymphocytes and macrophages respectively, 203,222). Sometimes, the density fractionation and adherence steps have been combined (in that order) to achieve further DC enrichment (223). However, by far the most commonly used procedure in the isolation of these cells, and one which provides for nearly pure populations uses an additional subsequent antibody coated sheep RBC (SRBC) rosetting or re-adherence step to ensure macrophage depletion (224-226). Another method that has been employed in the purification of mouse lymph node and splenic DC has taken advantage of their relative radiation resistance. In this method low density cells are first irradiated before overnight culture on plastic petri dishes. The non-adherent cells are then depleted of dead or dying lymphocytes by density centrifugation and a final FcRIgG rosetting step again ensures macrophage removal (227-229).

Unlike mouse lymph node and splenic DC, mouse Peyers patch DC are completely non-adherent precluding the use of transient adherence in their isolation. These cells, therefore, have been purified by culturing peyers patch cells with irradiated autologous sodium periodate treated splenic T lymphocytes which form clusters with the the DC. Following isolation of the cultured cells by a combination of density and rate zonal centrifugation, dead and dying T lymphocytes are then released from clusters by EDTA treatment and separated from DC by density centrifugation alone (230).

Human peripheral blood dendritic cells. A major difference between human peripheral blood DC and other DC types is that these cells are not initially of low density. Rather, the low density profile develops after 1-2 d in culture. Accordingly, most studies that have attempted to isolate human peripheral blood DC have avoided density fractionation as the initial step and instead have used either transient adherence or SRBC rosetting (which allows T lymphocyte depletion) itself almost invariably followed by transient adherence. Thereafter, different studies have varied widely in the

type and number of additional steps used to further enrich for DC. Some commonly used techniques, however, include selection of low density cells (after 1-2 d culture, 231); depletion of monocytes by re-adherence (147) or carbonyl ion ingestion (149); depletion of monocytes and B lymphocytes (both FcRIgG expressing) by panning on antibody coated plates (232) or by rosetting with antibody coated ORBC (233); and depletion of each of monocytes, B and T lymphocytes using cocktails of antibodies specific for these cells in complement mediated lysis techniques (234) or again panning procedures (235).

Recently, one group have shown that human peripheral blood DC are in fact of an initial high density and based upon this have developed a new isolation technique which yields highly pure populations of DC. In this technique, the high density cells are depleted of T lymphocytes by SRBC rosetting and cultured for 1-2 d. Low density cells are then selected and finally are depleted of residual FcRIgG bearing cells by panning on antibody coated plates (150,236).

Mouse and human thymic dendritic cells. Mouse thymic DC in thymic cell suspensions prepared by manual teasing of thymi are completely non-adherent. Consequently, these cells have been isolated by firstly selecting low density fraction and subsequently depleting contaminating cells with antibodies and complement (237) or combined radiation resistance and FcRIgG rosetting (227). In contrast, mouse thymic DC from collagenase digested thymi are transiently adherent and thus these cells have been purified by the standard technique used for mouse lymph node and splenic DC (213,215,238).

In humans, only thymic DC from manually teased thymi have been isolated. These DC were purified by low density and panning with CD2 antibodies (to remove contaminating T lymphocytes, 239).

Rat dendritic cells. Rat DC are non-adherent and thus again it has not been possible to use transient adherence as a method of enrichment. With regards to Langerhans cells, these have been enriched by low density, radiation resistance and FcRIgG rosetting (240) and similar methods have been used in the purification of liver interstitial (240) and lymph node (241) DC. Of other DC types, i.e. veiled cells (242), lung wall interstitial (243) and splenic (242,244) DC, purification protocols have omitted the irradiation step. In addition, however, rat veiled cells have been purified by low density alone (196); low density followed by rosetting using erythrocytes coated with T lymphocyte, B lymphocyte or monocyte/macrophage antibodies (245); or lack of adherence to nylon wool (246).

Other dendritic cell types. Several isolated reports have described the purification of DC from other tissues. These DC include dog (88), rabbit (87,161) and human (161) veiled cells (all isolated by selection of low density cells); rabbit lymph node DC (247, isolated by low density, irradiation resistance and FcRIgG rosetting); and guinea pig lymph node DC (248, isolated by low density followed by depletion of contaminating cells using specific antibodies attached to magnetic beads). Recently, the isolation of human tonsillar DC has also been documented. However, this will be discussed in more detail in Chapter 2.

Assessment of enrichment of in vitro dendritic cells and their relationship to dendritic cells in vivo

In general the more steps incorporated into an isolation protocol the greater the purity of the DC. For example, some single step Langerhans cell isolation protocols give less than 10% purity (249) whereas the four step procedure, commonly used in the isolation of mouse lymph node and splenic DC yields populations of greater than 90% purity (250-252).

In the assessment of DC purification the identification of the distinctive dendritic morphology has been useful and on occasion has been used as the sole criterion (e.g. 221,245). Alone, however, morphological assessment can be unreliable, given that in most tissues other cell types (e.g. keratinocytes and follicular DC) may also exhibit dendritic morphology. Also, in the case of the lengthier multi-step isolation procedures DC may tend to withdraw their processes and "round up". Frequently, therefore, alternative methods of monitoring DC enrichment have been employed.

The majority of these alternative methods have, too, exploited properties (or lack of properties) of DC that are not DC specific. However, when several different methods are used, with or without morphology, then a reliable estimate of DC purity can be achieved. One method has involved an assessment of the expression of enzymic activities, e.g. expression of ATPase upon guinea pig Langerhans cells (72) and absence of non-specific esterase, acid phosphatase and peroxidase upon mouse lymph node and spleen (253), rabbit lymph node (247), human thymus (239) and human peripheral blood DC (149). Another method has taken advantage of the inability of the majority of DC types to endocytose antigens and has been employed for mouse lymph node and splenic (253,254) and rabbit lymph node (247) DC.

Examination of cell surface immunological marker expression has also been important in the assessment of DC purity. Strong expression of class II MHC has been used to monitor the enrichment of virtually all DC types (e.g. 150,227,247) and expression of FcRIgG (249) and CD1a antigen (219) have been used in the assessment of Langerhans cell enrichment. With regards negative features, i.e. markers absent from DC, usually the expression of several are examined simultaneously to monitor contamination by different cell types. Examples include FcRIgG (149,216); T lymphocyte restricted antigens such as

Thy-1 (230), CD3 (255) and CD4 (150) antigens; B lymphocyte restricted antigens such as Ig (247) and CD20 (255) and CD22 (256) antigens; and monocyte/macrophage restricted antigens such as F4/80 (237), CD11b (235), CD14 (236) and CD35 antigens (256).

Several markers expressed specifically upon DC have now been described and as well as allowing for the assessment of enrichment these have provided clues as to in vitro-in vivo relationships. In the mouse three such markers are the 33D1, NLDC145 and MIDC8 antigens. Original reports suggested that all three of these markers were expressed upon the great majority of isolated splenic DC (227,257). A recent more detailed study, however, has shown that only 10% of isolated splenic DC express the NLDC145 antigen. Furthermore, these cells express the B lymphocyte antigen J11d and the T lymphocyte antigen ly2 but do not express the 33D1 antigen and this in contrast to the remaining 90% of isolated mouse splenic DC which express 33D1 antigen but none of NLDC145, J11d or ly2 antigens (215). This finding is important in the context of the relationship between in vitro and in vivo mouse splenic DC. By immunohistology the NLDC145 antibody reacts specifically with interdigitating DC of the central PALS (258; as does the MIDC8 antibody, 227) whereas the 33D1 antibody reacts specifically with marginal zone DC (151). This, therefore, suggests that the in vitro mouse splenic DC consists primarily of a population of cells analogous to the marginal zone DC plus a minor population of cells that are analogous to the interdigitating DC.

One problem with this interpretation, however, is highlighted by studies of mouse lymph node DC using the same antibodies. Although detailed comparative studies have not yet been performed, independently each of the NLDC145, MIDC8 and 33D1 antibodies have been shown to react with the majority of isolated lymph node DC (227,257). By immunohistology NLDC145 and

MIDC8 react specifically with the paracortical interdigitating DC and this argues for a direct relationship between this cell and the in vitro DC (227,258). However, the 33D1 antibody does not stain any cells in mouse lymph nodes (151) suggesting that changes in 33D1 antigen expression and/or it's sensitivity to detection occur upon isolation; and further that such changes should be borne in mind when considering in vitro versus in vivo splenic DC relationships.

Isolated mouse thymic DC have also been examined for their expression of 33D1 and NLDC145 antigens. DC from manually teased thymi, which are non-adherent, express 33D1 antigen (237). In contrast, DC from collagenase digested thymi, the majority of which are transiently adherent, express the NLDC145, J11d and 1y2 antigens but not 33D1 antigen (215). To explain this, and by analogy to mouse spleen, it has been hypothesised that mouse thymus contains both a 33D1 antigen expressing DC population, which is released from manually teased and collagenase digested thymi, and a 33D1 antigen non-expressing DC population, which is released only from collagenase digested thymi. However, to date only the NLDC145 antibody has been studied by immunohistology of thymus sections, where, like the MIDC8 antibody, it stains most if not all medullary DC (227,258). In contrast, the 33D1 antibody has not been examined by immunohistology and thus the existence of an in vivo 33D1 antigen expressing mouse thymic DC population still remains speculative particularly when one again considers the possibility of changes in 33D1 antigen expression or detection sensitivity during isolation.

Of the remaining mouse DC types, as with lymph node and thymic DC, few if any laboratories have routinely used the 33D1, NLDC145 or MIDC8 antibodies to monitor enrichment. Expression of these antigens by these cells, however, has been studied. In skin sections for example, Langerhans cells express NLDC145 and MIDC8 but not 33D1 antigens (227,258,259); and in

vitro, isolated Langerhans cells, too, display this phenotype (214,215,259), suggesting a direct relationship between the in vivo and in vitro cell (although unlike in vivo and in vitro lymphoid DC this relationship has never seriously been doubted). Veiled cells, express both the NLDC145 and MIDC8 antigens in situ and as isolated cells have also been shown to express the NLDC145 antigen, again arguing for a direct relationship (227,258). Expression of these antigens on indeterminate cells and interstitial DC, however, has been examined only in situ, where the former have been demonstrated to express NLDC145 antigen (258) and the latter 33D1 antigen (in pancreas and thyroid, 191,197) but not NLDC145 or MIDC8 antigens (in liver and kidney, 227,258).

DC specific markers have also been described in other species where again they have only infrequently been used to monitor enrichment but nonetheless have provided information on in vitro-in vivo relationships. In rats, one such marker is the 1F119 antigen, which is expressed upon veiled cells, lymph node and splenic interdigitating and thymic DC in situ and upon isolated cells suggesting direct relationships (260). In guinea pigs, a specific marker is the Msgp5 antigen, which is expressed upon Langerhans cells, indeterminate cells, lymph node and splenic interdigitating DC and thymic DC in situ (248). Also, isolated lymph node DC express this antigen suggesting a direct relationship with lymph node interdigitating DC. Finally, in humans the RFD1 antigen has been shown to be expressed upon both tonsillar interdigitating DC (101) and isolated peripheral blood DC (231).

The "dendritic cell family" as accessory cells in vitro

In the section "Antigen processing/presenting cells and accessory cells" (see page 4), accessory cells were defined as "cells that can induce T lymphocyte responses to MHC plus antigen at the developmental or induction

stages of immunity". This definition was intended to encompass only those T lymphocyte responses that might occur under normal physiological conditions, i.e. in T lymphocyte development, where self MHC plus self antigen are recognised, and in the induction of immunity, where self MHC plus foreign antigen are recognised.

In in vitro studies, however, and indeed in some in vivo studies, the definition of an accessory cell has been extended to cells that can induce T lymphocyte responses to other or similar stimuli, the precise physiological relevance of which, if any, remains unclear. These stimuli include minor histocompatability antigens [both minor lymphocyte stimulating (Mls) and non-Mls - as in the induction of the rejection of MHC matched allografts]; allogeneic MHC [as in the induction of the rejection of MHC mismatched allografts or it's in vitro correlate - the allogeneic MLR (allo MLR)]; self MHC [as in the in vitro autologous MLR (auto MLR)]; and various mitogens such as the plant lectins phytohaemagglutinin (PHA) and concanavalin A (Con A), the oxidising agents sodium periodate and neuraminidase plus galactose oxidase (Na/GaO) and antibodies directed against the CD3 molecule. Often the molecular mechanisms involved in these different responses have not been elucidated in detail. However, with few exceptions, it is widely appreciated that the ability of any one particular cell type to act as an accessory cell in these responses is indicative of the ability of that same cell type to act as an accessory cell as originally defined.

It is in this respect that in the assessment of the ability of different members of the "DC family" to act as accessory cells in vitro, T lymphocyte responses to conventional antigens as well as to these other stimuli have been examined. In these different systems both proliferative and functional (e.g. induction of cytotoxicity and help for B lymphocyte antibody responses) primary and secondary T lymphocyte responses have been

assayed. Neither positive or negative selection, however, have yet been studied in vitro, at least using isolated DC in non-organ culture experimental "set-ups" (see page 26).

Antigen induced T lymphocyte proliferation

Particulate/insoluble antigens. Several different types of particulate/insoluble antigen have been examined. Firstly, with respect to viruses, human peripheral blood DC have been shown to induce primary T lymphocyte proliferative responses to human immunodeficiency virus when the DC were infected with virus in vitro (261). Also, mouse splenic DC infected with influenza virus (either in vitro or in vivo) have been shown to induce primary T lymphocyte proliferative responses, in contrast to resident mouse peritoneal macrophages which did not (262).

Influenza virus infected mouse splenic DC also induce secondary T lymphocyte proliferative responses to virus (262); and similarly, herpes simplex virus (HSV) infected mouse lymph node DC (infected in vivo, 263) and HSV infected human Langerhans cells (freshly isolated and infected in vitro, 264) induce secondary responses. With both types of virus, mononuclear phagocytic cells were also active as accessory cells but induced consistently smaller responses than the DC.

In contrast to viruses, primary bacterial proliferative responses using DC as stimulators have not been clearly defined. Although it has been shown (using cells from healthy donors) that human peripheral blood DC induce T lymphocyte responses to C. Trachomatis (235) and M. Leprae (265), it was not established with certainty whether such responses were indeed primary.

In secondary bacterial responses, mouse lymph node DC are active as inducers of T lymphocyte proliferation to M. Tuberculosis and are more

potent than lymph node or resident peritoneal macrophages (266). Mouse lymph node (267) and splenic (228) DC have also been shown to induce secondary T lymphocyte responses to C. Parvum in different uncloned C. Parvum specific T lymphocyte lines although in these experiments lymph node and activated bone marrow macrophages were more effective accessory cells.

Of the remaining particulate/insoluble antigens, these have been tested only in secondary responses. In this regard, mouse lymph node (267) and splenic (268) DC have been shown to induce proliferation to polymeric flagella protein of S. Typhimurium (POL) and insoluble ovalbumin (OVA) respectively, although less effectively than lymphoid macrophages. Mouse lymph node DC, in the presence of POL, also induce the proliferation of an uncloned POL specific T lymphocyte line, and here the DC are more effective accessory cells than lymph node macrophages (267). With horse RBC (HRBC), mouse splenic DC are more effective accessory cells than resident peritoneal macrophages in the induction of proliferation of an uncloned HRBC specific T lymphocyte line. Individual clones, however, respond to HRBC when presented upon one or the other cell type, not both (269).

Soluble antigens. On primary responses, mouse lymph node DC isolated from animals skin painted (1 d previously) with oxazolone (184), TNP (184,185) or FITC (162,270) induced proliferative response to these agents in previously unsensitised populations of T lymphocytes. Similarly, mouse lymph node DC pulsed with FITC in vitro also induced primary responses (162). In contrast to this, freshly isolated human Langerhans cells failed to stimulate primary proliferative responses to nickel (271) although were active in the induction of secondary responses (see below).

Most members of the "DC family" have been examined in secondary responses. Firstly, freshly isolated mouse Langerhans cells induce secondary responses to keyhole limpet haemocyanin (KLH; using recently sensitised T

lymphocyte blasts, 272), purified protein derivative of M. Tuberculosis (PPD), human serum albumin and OVA (213). Freshly isolated guinea pig Langerhans cells also induce secondary responses to PPD and OVA and to trinitrobenzene sulphonic acid (273,274) and freshly isolated human Langerhans cells have been shown to induce secondary responses to PPD (264,275), soluble HSV antigens (275) and nickel (276). In most of the above studies the Langerhans cells were compared with mononuclear phagocytes and were either as equally effective or more effective as inducers.

Veiled cells can induce secondary T lymphocyte proliferative responses to soluble antigens in vitro and this has been demonstrated using rabbit afferent lymph veiled cells and the antigens KLH (161) and HuIg (87).

A large number of studies have tested the accessory activity of peripheral lymphoid DC. Mouse lymph node DC have been shown to induce secondary responses to PPD (267), OVA (277), KLH (253), oxazolone and TNP (antigen pulsing in vivo, 184) and the same cells also induce proliferation to PPD and OVA in uncloned PPD and OVA specific T lymphocyte lines respectively (267). Again in most of the above studies lymph node DC were compared with mononuclear phagocytes and with only one exception the DC were the more effective accessory cells.

Mouse splenic DC are too active, and where tested, superior to resting or activated macrophages in the induction of secondary proliferation to PPD (253), OVA (223,268) and KLH (253,278). Mouse splenic DC are also more effective than macrophages in the presentation of OVA to an uncloned OVA specific T lymphocyte line (269) and in the presentation of KLH to recently sensitised T lymphocyte blasts (279). With cloned OVA lines, however, as with cloned HRBC lines (see above), individual clones exhibit a strict "all or none" requirement for either DC or macrophages as accessory cells.

Other types of peripheral lymphoid DC that have been examined include

guinea pig lymph node and rat spleen DC. Guinea pig lymph node DC induce secondary responses to OVA, although less effectively than resident peritoneal macrophages (248); and rat splenic DC have been shown to induce secondary responses to myelin basic protein although not to OVA (280).

Demonstrations of interstitial DC accessory activity in secondary responses to soluble antigens have been made using DC from rat tracheal epithelium and lung. Tracheal epithelial DC are more potent than alveolar macrophages and lung DC are more potent than lung macrophages and B lymphocytes in the induction of secondary OVA responses (243).

With regards human peripheral blood DC and soluble antigens, these have been shown to induce secondary proliferative responses to PPD (255,265,281), soluble HSV antigens (235), nickel (276) and tetanus toxoid (255). In the majority of these studies, other cell populations, including whole peripheral blood mononuclear cells and peripheral blood monocytes, were again tested as inducers and were consistently found to be less active.

Finally, one interesting study has examined mouse Langerhans cells and splenic DC as inducers of proliferation in two different cloned sperm whale myoglobin (SWM) specific T lymphocyte lines, using whole SWM as an antigen (217). An important advance in DC biology is the finding that Langerhans cells cultured in vitro in the presence of keratinocytes or certain cytokines, within 3 d, develop into mature peripheral lymphoid DC (see later) and based upon this each of freshly isolated Langerhans cells, Langerhans cells cultured in vitro with keratinocytes for 1 or 3 d and splenic DC were compared in the SWM response. In summary, freshly isolated Langerhans cells induced induce strong proliferative responses and 1 d cultured Langerhans cells induced weak responses to SWM in both clones. In contrast, Langerhans cells cultured for 3 d and splenic DC were inactive as inducers of proliferation.

Peptide antigens. Peptide antigens have only been examined using sensitised T lymphocytes as responders. In this context, freshly isolated, 1 and 3 d cultured mouse Langerhans cells and mouse splenic DC were all equally effective in inducing proliferation to appropriate SWM peptides in two SWM specific T lymphocyte lines (see above; 217).

In other studies mouse lymph node and splenic DC have been shown to induce secondary proliferative responses to the synthetic peptides Glu,Ala,Tyr^{10} (362,391) and poly (Tyr,Glu)-poly(Ala)--poly(Lys) (253); and with both peptides the DC were demonstrated to be superior to macrophages as accessory cells.

Mls antigens. In contrast to other histocompatability antigens Mls antigens play no role in either allograft rejection or the induction of graft versus host disease (282-284; neither do they elicit antibody production, 285). Thus, at least in the induction of immunity, responses to Mls antigens (like auto MLR and mitogen responses) have been described only in vitro. The mechanism of Mls antigen induced responses is controversial. Some have suggested that these super-antigens, which elicit responses in T lymphocytes expressing particular V-beta genes (286,287) are recognised as conventional antigens. Others, however, envisage Mls antigens to be accessory molecules which interact with unique T lymphocyte receptors (distinct from the TcR) thereby allowing some T lymphocyte clones to recognise and respond to non-polymorphic regions of the MHC (288,289).

Concerning DC as accessory cells for Mls responses, to date only mouse splenic DC have been tested. Early studies showed that spleen cells, depleted of B lymphocytes by treatment of mice with anti-IgM antibody from birth, were unable to stimulate primary T lymphocyte proliferative responses to Mls^a (290) and recently this has been confirmed for Mls^d (291), suggesting that only B lymphocytes are active as accessory cells in this

system. Another interpretation of these findings, however, is that under normal conditions other spleen cells (including DC), although not expressing Mls antigen themselves, might also induce responses following absorption of Mls antigens from B lymphocytes in vivo. Indeed, some groups have shown that splenic DC from normal mice induce strong primary and secondary (using T lymphocyte clones as responders) proliferative responses to Mls^a and Mls^d and also that the DC are comparable to B lymphocyte blasts in this respect (292,293). In contrast to this, however, other groups have shown that normal mouse splenic DC (like splenic macrophages but unlike splenic B lymphocyte blasts) are completely inactive in the induction of primary proliferative responses to Mls^a and Mls^d (294,291).

T lymphocyte proliferation in MLRs

Auto MLR. Even in the apparent absence of antigens or other stimuli, in a reaction known as the auto MLR, accessory cells induce proliferative and functional responses in autologous T lymphocytes. To explain this, some investigators have suggested that peripheral T lymphocytes have the capacity to recognise and respond to self MHC (295). Another, more favoured model, however, is that these responses are indeed induced by antigens, either present in the animal at the time of tissue removal and/or introduced into an in vitro culture system in the form of culture media additives or other materials that might be used in cell isolation (296).

In the auto MLR, a variety of different DC types have been tested for their ability to induce primary proliferative responses. Freshly isolated mouse Langerhans cells (214,297); afferent lymphatic veiled cells from dogs (88), rabbits (87) and humans (161); and thoracic duct veiled cells from mesenteric lymphadenectomised rats (298) are all either weak or inactive as accessory cells. On the other hand, 3d cultured mouse Langerhans cells

(cultured in the presence of keratinocytes) are strong inducers of proliferation in this reaction (213).

Mouse lymph node (225) and splenic (299,300), rat lymph node (240) and human peripheral blood (255) DC are also strong inducers of proliferation; and in addition are superior to mononuclear phagocytes, B and T lymphocytes as accessory cells (141,255,267,301).

Several other aspects of mouse splenic DC induced auto MLRs have also been explored. Firstly, these cells induce primary proliferative responses in T lymphocyte populations depleted of ly2 antigen expressing cells but not in T lymphocyte populations depleted of L3T4 antigen expressing cells (250); and this latter observation is in accord with the concept that in the induction of immunity effector T lymphocytes require helper/inducer T lymphocytes to respond.

Secondly, whole mouse splenic DC and mouse splenic DC depleted of the minor ly2 antigen expressing subpopulation are similarly potent inducers of primary proliferative responses (and likewise in the primary allo MLR, 215,302). This is important, for if, as has been hypothesised (see page 33), the ly2 marker distinguishes between interdigitating DC and marginal zone DC analogues, then this study demonstrates that both of these populations are active and equivalent with regards their accessory cell activity in vitro.

Lastly, in contrast to other members of the "DC family", which have not been tested, mouse splenic DC have been shown to induce secondary proliferative responses in the auto MLR using either recently sensitised T lymphocyte blasts or memory T lymphocytes derived from them as responders (303).

Allo MLR. Allogeneic MHC molecules (when expressed upon appropriate antigen antigen processing/presenting cells) induce strong proliferative responses in T lymphocytes - defined in vitro as the allo MLR. Given recent evidence

(304,305), which suggests that the antigen binding grooves of most if not all MHC molecules are occupied with peptides, it would seem likely that such responses are directed against "clothed" (peptide occupied) rather than "naked" (peptide unoccupied) MHC. However, whether or not peptides contribute to allo-recognition is a source of debate (306).

Early studies in the mouse (307), guinea pig (308) and human (220,309) showed that freshly isolated Langerhans cells from these species were active as inducers of proliferation in the primary allo MLR. In later studies in the mouse, however, freshly isolated Langerhans cells were shown to be relatively weak accessory cells when compared to Langerhans cultured for 3 d in the presence of granulocyte-macrophage colony stimulating factor (GMCSF; 216,310), GMCSF plus IL-1 (310), keratinocytes (214,216,217,272,310), keratinocyte conditioned medium (CM; 216) or CM from splenic macrophages stimulated with lipopolysaccharide (LPS ;216).

Veiled cells from the afferent lymphatics of dogs (88), humans (311) and rabbits (161) and from the thoracic ducts of mesenteric lymphadenectomised rats (244) are too active as inducers and interestingly pre-culture of thoracic duct rat veiled cells for 16 hr (before assay) increases their stimulatory capacity (245). Also, as in the mouse splenic DC induced primary auto MLR, when freshly isolated rat thoracic duct veiled cells are used as stimulators of the primary allo MLR purified helper/inducer T lymphocytes but not purified CTL proliferate (91).

Mouse (267) and rat (240) lymph node DC induce primary proliferative responses but by far the most studied peripheral lymphoid DC in this reaction is the mouse splenic DC. Thus, many studies have shown that mouse splenic DC act as accessory cells (e.g. 225,251,312) and many similar studies have shown that these cells are potent accessory cells when compared to a variety of non-DC types, including resting and activated macrophages

and B lymphocytes (252,313,314).

An important observation in the mouse splenic DC induced primary allo MLR, is that in contrast to the mouse splenic DC induced primary auto MLR and the rat veiled cell induced primary allo MLR, both purified helper/inducer T lymphocytes and purified CTL proliferate (250). Further, in this study, by phenotypic and functional criteria, the ability of the CTLs to proliferate could not be explained in terms of a small number of contaminating helper/inducer T lymphocytes. These findings would thus appear to challenge the long standing view that in the induction of immunity CTL have an absolute requirement for helper/inducer T lymphocytes to respond. Rather then, when certain types of DC are used as stimulators, in certain experimental systems, CTL respond without a requirement for help.

In the human, peripheral blood DC are active as inducers of primary proliferative responses (256,315) and again in comparison with other cell types such as peripheral blood mononuclear cells (PBMC), monocytes, B and T lymphocytes these cells are potent accessory cells (147,149,150,255). Human liver interstitial (140) and thymic DC (239) also induce proliferation and the latter cells are more potent than peripheral blood monocytes.

Adherent mouse thymic DC (33D1 antigen negative) also induce primary proliferative responses (213) but non-adherent mouse thymic DC (33D1 antigen positive) have not been directly tested in the primary allo MLR, although may have been tested indirectly. In this regard, low density mouse thymus cells, depleted of cells expressing L3T4, ly2, Ig and class II MHC are devoid of DC and allo MLR inducing capacity. Culture of these cells with IL-1 for 2 d, however, stimulates the differentiation of 33D1 antigen expressing DC from a presumed thymic DC precursor and these recently differentiated thymic DC are strong inducers of proliferation in the primary allo MLR (237).

Fewer DC types have been examined for their ability to induce proliferative responses in the secondary allo MLR. Of those that have been tested freshly isolated guinea pig Langerhans cells are as potent as resident peritoneal macrophages (273) and freshly isolated mouse Langerhans cells comparable to splenic DC (272; contrast with primary response). Also, mouse splenic DC are more potent than resting and activated B lymphocytes and activated macrophages as accessory cells (314).

Lastly, human peripheral blood DC induce proliferative responses in the secondary allo MLR. This has been demonstrated using HLA-DR1 expressing DC and an HLA-DR1 restricted cloned T lymphocyte line. Further, in this system, the DC were more potent accessory cells than HLA-DR1 expressing peripheral blood monocytes (316).

Mitogen induced T lymphocyte proliferation

T lymphocyte responses to mitogens are difficult to categorise as either primary or secondary. On the basis that T lymphocytes are unlikely to have encountered mitogen beforehand, some consider mitogen responses to be primary. Others, however, argue that in any one T lymphocyte population at least a proportion of the clones will have previously undergone division and/or blast transformation in response to antigen (as a result of natural exposure) and that as mitogens, by definition, are polyclonal activators, mitogen responses should be considered as mixed responses with both primary and secondary components. Functionally, for reasons that will be outlined later, it is the latter viewpoint that is perhaps more relevant. For the present, however, the distinction between primary and secondary responses will not be made.

Plant lectins. Little is known of the mechanisms by which the plant lectins, Con A and PHA, induce mitogenesis in T lymphocytes. Both agents,

however, likely achieve their effect by binding to a number of different T lymphocyte surface glycoproteins, including LCA (317) in the case of Con A and CD2 antigen (318), CD3 antigen (319), LCA (320) and the TcR (321) in the case of PHA. In addition, the ability of both agents to induce rapid cell agglutination may also be pertinent to their mechanism of action.

Dealing with Con A first, freshly isolated mouse (214) and guinea pig (274) Langerhans cells and 3 d cultured mouse Langerhans cells (in the presence of keratinocytes, 213) all induce proliferative responses. Freshly isolated and cultured Langerhans cells, however, have not yet been compared as accessory cells.

Veiled cells from the afferent lymphatics of humans (161) and rabbits (87) and from the thoracic ducts of mesenteric lymphadenectomised rats (242) induce proliferative responses to Con A and potency, in comparison with macrophages has been demonstrated. Also, rabbit afferent lymphatic veiled cells have been shown to induce proliferative responses to Con A in allogeneic T lymphocytes (87).

Of peripheral lymphoid DC, guinea pig lymph node DC induce responses but are only as effective as macrophages (248). In contrast, rabbit (247) and rat (242) lymph node and mouse splenic (300) DC are both active and superior to macrophages as accessory cells (237,247,303,322).

Other DC types that have been examined include human peripheral blood DC which are active as inducers and are consistently more effective accessory cells than peripheral blood monocytes (150,261). Also, mouse thymic DC, induce responses, and this has been demonstrated for both adherent and non-adherent populations (237).

In T lymphocyte proliferative responses to PHA, veiled cells, peripheral lymphoid DC and peripheral blood DC have been tested as accessory cells. All of veiled cells from the afferent lymphatics of dogs (88) and

humans (311), rabbit lymph node DC (247) and human peripheral blood DC (323) induce responses and in the last two cases potency in comparison with mononuclear phagocytes has been demonstrated.

In an additional study it has been shown that human peripheral blood DC, in the presence of PHA and cytokines, can induce the clonal expansion of single helper/inducer T lymphocytes and single CTL with high efficiency. Also, in the helper/inducer response peripheral blood monocytes and B lymphocytes were vastly inferior to DC in inducing clonal expansion (236). These findings again call into question the need for helper/inducer T lymphocytes in CTL responses in the induction of immunity and in addition underline the role of the peripheral blood DC as a potent in vitro accessory cell in proliferation induction to mitogens.

Oxidising agents. The mechanisms by which the oxidising agents, sodium periodate and Na/GaO, induce mitogenesis are also largely unknown. Both oxidising treatments, however, lead to the formation of aldehydes upon cell surface glycoproteins and this event has been shown to be crucial in the induction of proliferation, possibly by facilitating accessory cell-T lymphocyte adhesive interactions (for a more detailed discussion see Chapter 3).

Periodate has been used more frequently than Na/GaO as an oxidant in DC studies. On Langerhans cells and periodate oxidative mitogenesis, freshly isolated mouse Langerhans cells are only weak inducers of proliferation whereas mouse Langerhans cultured for 3 d with GMCSF, GMCSF plus IL-1 or keratinocytes are strong inducers of proliferation (214,310).

A large number of studies have tested the ability of peripheral lymphoid DC to induce proliferation. Mouse Peyers patch (230) and splenic (299) DC are both active and the latter have been shown to be potent in comparison with macrophages and resting or activated B lymphocytes

(309,313). In the rat, lymph node (241) and splenic (240) DC induce responses and the former have been shown to be superior to macrophages in this respect (240,322).

Human peripheral blood (147,255) and rat lung (324) and liver (240) interstitial DC also act as accessory cells for proliferation in periodate oxidative mitogenesis and again, in these studies, the DC have been shown to be potent accessory cells in comparison with non-DC types, i.e. mononuclear phagocytes, B and T lymphocytes and fibroblasts.

Finally, in Na/GaO mitogenesis only rat (325) and rabbit (247) lymph node DC have been tested as inducers of proliferation. Both cell types induced responses and the former were shown to be more active accessory cells than resident peritoneal macrophages.

CD3 antibodies. As an integral component of the TcR complex the CD3 molecule plays a central role in conveying to the T lymphocyte the instruction to respond to antigen (326,317). Consequently, CD3 antibodies are thought to induce mitogenesis in T lymphocytes by mimicking triggering through the TcR. CD3 antibodies can induce responses even in the absence of accessory cells and this occurs when they are used in conjunction with phorbol esters (328) or are immobilised, either by attachment to plastic surfaces (329) or by coupling to sepharose beads (330). When soluble CD3 antibodies are used alone as a stimulus, however, there is an absolute requirement for accessory cells (331,332). Further, these accessory cells must express FcRIgG which function to aggregate CD3 antibody molecules (probably indirectly following capping of FcRIgG) and hence TcR complexes in the T lymphocyte membrane (333).

Each of mouse Langerhans cells (fresh and cultured), splenic DC and thymic DC and human peripheral blood DC have been tested as accessory cells in the induction of T lymphocyte proliferation to soluble CD3 antibodies. Of

these DC only freshly isolated mouse Langerhans cells express appreciable quantities of FcRIgG (i.e. > 1 X 10^4 copies/cell - see later) and thus, a priori, one might expect these cells to be the only active accessory cells. In fact, freshly isolated mouse Langerhans cells are extremely weak inducers (213,238) and in this respect are similar to mouse splenic (213) and human peripheral blood (150) DC. In contrast, however, 3 d cultured mouse Langerhans cells (cultured in the presence of keratinocytes) and adherent mouse thymic DC are potent inducers of proliferation (in comparison with macrophages and activated B lymphocytes, 213,238) even though these cells express only very low levels of FcRIgG.

Antigen induced T lymphocyte cytotoxicity

Particulate/insoluble antigens. Studies of the ability of DC to induce cytotoxic responses to particulate/insoluble antigens have focused almost exclusively upon mouse splenic DC and viruses. In primary responses, mouse splenic DC induce cytotoxicity to influenza virus (virus infection in vitro, 262) and HSV (pulsing with high concentrations of heat inactivated virus in vitro, 222), in contrast to resident peritoneal macrophages which do not induce responses to either virus.

Mouse splenic DC also induce secondary cytotoxic responses to influenza virus (where resident peritoneal macrophages are also active but inferior to the DC, 262) and HSV (222) and to Sendai virus (where potency in comparison with whole spleen cells has been reported, 339). In addition, mouse splenic DC from mutant bm14 mice (different from C57BL/6 mice in one amino acid of the H-$2D^b$ molecule) induce secondary cytotoxic responses to Moloney virus and thus overcome a class I MHC Ir gene defect in this strain (334).

Another system in which mouse splenic DC have been tested is in the

response to liposomes bearing allogeneic H-2K molecules (335). Allogeneic H-2K liposomes on their own do not induce anti-H-2K cytotoxic responses in populations of T lymphocytes primed to allogeneic H-2K. In the presence of DC, however, and to a lesser extent B lymphoma cell lines, strong anti-H-2K cytotoxic responses are induced.

Soluble antigens. Freshly isolated mouse Langerhans cells (336) and mouse splenic DC (337) have been shown to induce primary cytotoxic responses to TNP (and with the DC potency in comparison with mononuclear phagocytes has been demonstrated). Mouse lymph node DC induce primary responses to TNP (184) and also to FITC (162) and this has been demonstrated using DC from the lymph nodes of mice that had been skin painted with these agents 1 d previously.

Peptide antigens. In addition to inducing primary cytotoxic responses to whole influenza virus, mouse splenic DC also induce primary cytotoxic responses to the influenza virus nucleoprotein peptides 147-158 and 147-158(Arg 156-) (262).

Non-Mls minor histocompatability antigens. Unlike Mls antigens, non-Mls minor histocompatability antigens are probably recognised by T lymphocytes as conventional antigens are, i.e. as peptides associated with MHC (338,339). One such group of antigens are male H-Y antigens (see pages 21 and 23), which, in vitro, have been studied for their ability to induce secondary cytotoxic responses when presented on mouse splenic DC. In this regard, in both C57BL/6 ($H\text{-}2^b$) and mutant bm12 mice (suffering a class II MHC Ir gene defect in the response to H-Y), male splenic DC are more effective accessory cells than whole male spleen cells as inducers of anti-H-Y cytotoxic responses in whole spleen cell populations from female mice primed in vivo with whole male spleen cells or male splenic DC respectively

(188,340). Also, in mutant bm14 mice (see page 50), which suffer a class I MHC Ir gene defect in the response to H-Y, this defect can be overcome by mere in vitro stimulation with male splenic DC (340-342). The ability of mouse splenic DC to induce secondary anti-H-Y cytotoxic responses in purified populations of CTL, has also been studied in each of the above systems. In C57BL/6 mice purified CTL respond to male splenic DC (but not to whole male spleen cells, 479). Similarly, in mutant bm14 mice, although not in mutant bm12 mice, purified CTL respond (188,340-342).

T lymphocyte cytotoxicity in MLRs

Auto MLR. Hitherto, only one study has examined DC as inducers of T lymphocyte cytotoxicity in the primary auto MLR. In this study, mouse splenic DC were tested and found to be inactive as inducers of cytotoxicity in this reaction (225).

Allo MLR. Despite their relatively weak accessory activity as inducers of proliferation in the primary allo MLR (see page 44) freshly isolated mouse Langerhans cells induce cytotoxicity in this reaction (336). Likewise, mouse splenic DC induce cytotoxicity and this has been shown across both complete H-2 differences (225,314; where potency in comparison with macrophages has been demonstrated, 254) and partial H-2 differences including mice disparate at Ia and H-2D (254), Ia (254), H-2K and H-2D (343), H-2K (254,334), H-2D (254) and I-A (341).

Regarding the ability of mouse splenic DC to induce cytotoxic responses in purified CTL in the primary allo MLR, across an I-A difference mouse splenic DC do not induce cytotoxicity (341). In contrast, across whole H-2 (250,314,342) and H-2K (341,342) differences mouse splenic DC induce strong cytotoxic responses and in these situations, the crucial role of the DC for this effect is again underlined by the fact that whole spleen cells

do not act as accessory cells (341,342). In addition, an important observation that has been made in one H-2K disparate combination, is that pre-treatment of whole spleen cell stimulators with neuraminidase (to remove cell surface sialic acids) then allows these cells, like the DC, to induce cytotoxicity in purified populations of CTL (342).

Mitogen induced T lymphocyte cytotoxicity

Mouse splenic DC have been shown to induce cytotoxicity in Con A mitogen responses (344). In this system, small numbers of DC and Con A (in the presence of CM from PHA and phorbol ester stimulated human PBMC or IL-2 plus a unique CTL differentiation factor) induce active CTL from autologous T lymphocytes which lyse syngeneic targets.

Antigen induced help for B lymphocyte antibody responses

Particulate/insoluble antigens. As inducers of anti-SRBC antibody responses in primary populations of B and T lymphocytes, freshly isolated mouse Langerhans cells are only weak accessory cells (272). Cultured mouse Langerhans cells (in the presence of keratinocytes; 272), mouse splenic DC (272,300,303) and human peripheral blood DC (226), however, induce strong responses and mouse splenic DC have also been shown to induce primary anti-ORBC antibody responses (226). Further, mouse splenic and human peripheral blood DC are more potent accessory cells than mononuclear phagocytes in these respects.

Mouse splenic DC have also been shown to induce anti-SRBC antibody responses in secondary B and T lymphocyte populations and in this response the DC were again superior to mononuclear phagocytes as accessory cells (226).

Soluble antigens. Similar to the induction of primary anti-SRBC antibody

responses, freshly isolated mouse Langerhans cells are only weak inducers of primary anti-TNP antibody responses to the soluble antigen TNP-KLH (272). Mouse Langerhans cells cultured for 3 d (in the presence of keratinocytes) and mouse splenic DC, however, induce strong primary anti-TNP antibody responses to this antigen (303) and mouse splenic DC have been shown to induce primary anti-dinitrophenol (DNP) and anti-fluorescein antibody responses to the soluble antigens DNP-KLH (345) and fluoresceinated sheep gamma globulin (346) respectively.

Help for B lymphocyte antibody responses in MLRs

Finally, mouse splenic DC have also been shown to induce help for B lymphocyte antibody responses in MLRs. Thus, culture supernatants from the mouse splenic DC induced primary auto MLR, in the presence of SRBC or HRBC, stimulate purified populations of autologous B lymphocytes to secrete anti-SRBC or anti-HRBC antibodies respectively (301). Similarly, culture supernatants from the mouse splenic DC induced secondary allo MLR stimulate purified populations of B lymphocytes to secrete anti-SRBC antibodies (314).

The mechanism of dendritic cell accessory function in vivo and in vitro

So far, evidence that different members of the "DC family" are the principal accessory cells of the physiological immune system has been discussed. Little has been discussed, however, as to which types of DC are most likely to perform this physiological role. Obviously, as negative selection occurs predominantly in the thymus then thymic DC are the main DC implicated in this process. Also, if one accepts that the induction of immunity occurs predominantly in peripheral lymphoid organs then it would seem logical to assume that peripheral lymphoid DC are the main DC which act as accessory cells in this stage of the immune response. If this is so, however, then

what of the physiological significance of the findings concerning the accessory cell activity of non-lymphoid DC?

The short answer to this question, is that in physiological terms, non-lymphoid DC probably play only a minor role in the induction of immunity. Despite observations in contact hypersensitivity and demonstrations of accessory activity both in vivo and in vitro (particularly in secondary responses, less so in primary), the absence of these cells from the main sites of T lymphocyte accumulation, likely precludes their role as major physiological accessory cells. This is not, however, to exclude an important antigen processing/presenting and trafficing role for these cells, which may be of relevance to the functioning of the "DC family" as a whole. To elaborate upon this it is first necessary to consider some ontogenetic aspects of the "DC family".

Langerhans cells to veiled cells to lymph node interdigitating dendritic cells

Even before the recent in vitro studies in the mouse (see below), a number of observations suggested that in vivo skin Langerhans cells transform into lymph node interdigitating DC via intermediate afferent lymphatic veiled cells. Firstly, similarities in morphology, ultrastructure (particularly Birbeck granules) and marker characteristics (see pages 7, 10, and 11) indicated a close relationship between the three cell types (58,86,94,347). Secondly, the observation that during contact hypersensitivity reactions Langerhans cells entered dermal afferent lymphatics (see page 18) suggested that Langerhans cells were the precursors of veiled cells; and thirdly, that veiled cells in turn might be the precursors of lymph node interdigitating DC was suggested by the fact that ligation of lymph node afferent lymphatics led to the depletion of interdigitating DC from such lymph nodes (348-349).

Recently, strong confirmatory evidence of this in vivo sequence has been provided by studies of isolated mouse Langerhans cells in vitro. When freshly isolated mouse Langerhans cells are cultured for 3 d in the presence of keratinocytes, they develop into cells that both phenotypically and functionally resemble mouse splenic DC (in particular the interdigitating DC analogues within this population). Phenotypic changes include the development of a more dendritic morphology and the loss of Birbeck granules and enzymic activities such as non-specific esterase (214). Also, there is reduced expression of FcRII-IgG and F4/80 antigen and an increase in the expression of class II MHC (see Table 1.2). Functional changes have been discussed before and include an acquisition of potent accessory activity for primary T lymphocyte proliferative responses in MLRs, for T lymphocyte proliferative responses in periodate oxidative mitogenesis and for primary anti-SRBC and anti-TNP antibody responses (see pages 43, 44, 48, 53 and 54). In addition there is a reduced ability to induce secondary T lymphocyte proliferation to SWM (see page 40).

The mechanism of this keratinocyte mediated differentiation has also been studied (216,310). Because recombinant and purified natural GMCSF (but not a variety of other purified cytokines) induce accessory activity and because GMCSF antibodies (but not other cytokine antibodies) inhibit the ability of keratinocyte CM to induce accessory activity than a major role for GMCSF as a differentiation factor is suggested. GMCSF, however, is not as effective as GMCSF plus IL-1 alpha or keratinocytes (the last two of which are comparably active) in inducing accessory activity for either this response or proliferative responses in periodate oxidative mitogenesis, indicating that GMCSF and IL-1 alpha act in synergy to mediate the differentiation of Langerhans cells into interdigitating DC in vitro.

Taken together with the in vivo findings and the fact that Birbeck

Table 1.2 - Comparison of cell surface markers on isolated mouse Langerhans cells and splenic dendritic cells.[a)]

	Langerhans cells		
Marker	Fresh	3 d cultured	Splenic DC
FcRII-IgG	++	+/-	+/-
F4/80	++	+/-	+/-
L3T4	-	-	-
B220	-	-	-
T200	++	++	++
NLDC145	++	++	+/-[b)]
Thy-1	-	-	-
lyl	-	-	-
Mac-1 alpha chain	++	++	++
class II MHC	+++	++++	+++
33D1	-	-	++[b)]

a) ++++ v. strong, +++ strong, ++ medium, +/- v. weak; based upon 213-216,368,259,302,343,415.

b) 10% NLDC145++/33D1- (probable interdigitating DC analogue); 90% NLDC145-/33D1++ (probable marginal zone DC analogue) -(see page 33).

granules are never found in spleen (55), these in vitro findings are probably representative of a physiological in vivo process in which skin Langerhans cells ultimately transform into lymph node rather than splenic interdigitating DC [see Appendix 1 - (3)]. Presumably therefore, in vitro, mouse Langerhans cells pass through an intermittent veiled cell stage before maturing into interdigitating DC. Although this possibility has not been examined in the mouse, in the rat there is indeed evidence that veiled cells differentiate into interdigitating DC in vitro (245,350). Thus, veiled cells from the thoracic ducts of mesenteric lymphadenectomised rats, after 1-2 d culture in the absence of other cell types or factors, lose their non-specific esterase activity and Feulgen positive cytoplasmic inclusions, maintain their level of expression of class II MHC, and gain expression of the IL-2R and increased accessory activity for proliferative responses in the primary allo MLR (see also page 44).

Lastly, with regards the control of Langerhans cell differentiation in vivo, keratinocytes may release GMCSF and IL-1 constitutively and thus mediate endogenous Langerhans cell differentiation. Alternatively, keratinocyte release of these cytokines and hence differentiation may be antigen dependent; and in favour of this (or at least that antigen accelerates differentiation) are observations in contact hypersensitivity (where Langerhans cell entry into dermal lymphatics is first noticed and where increases in the output of afferent lymphatic veiled cells are seen - see page 18) and the fact that the proportion of afferent lymphatic veiled cells and lymph node interdigitating DC containing Birbeck granules is seen to increase following antigenic stimulation (see pages 11 and 12).

Interrelationships between other members of the "dendritic cell family"

There is less evidence concerning other interrelationships between different

members of the "DC family". On indeterminate cells, one commonly held belief is that these cells are the immediate precursors of Langerhans cells (e.g. 351,352). Certainly, the absence of Birbeck granules and surface ATPase (see pages 9 and 10) would be more suggestive of the indeterminate cell as a precursor of the Langerhans cell rather than a transitionary stage between the Langerhans cell and veiled cell (unless one wishes to propose a "flip-flop" of phenotype).

On splenic marginal zone and interdigitating DC, it would appear that the former are the immediate precursors of the latter. Thus, labelled 33D1 antigen expressing isolated mouse splenic DC (probable marginal zone DC analogues), when injected intravenously into syngeneic mice, by 3 hr are seen to home to the splenic marginal zone, and by 24 hr migrate into the central PALS where they transform into interdigitating DC (353,354). The relationship of these cell types to other members of the "DC family", however, remains unclear. On the basis of logical considerations (see later) and by analogy to the Langerhans cell-veiled cell-lymph node interdigitating DC sequence, one possibility is that splenic marginal zone and hence splenic interdigitating DC are descended from a subset of peripheral blood DC, which, in turn, are derived from interstitial DC. However, adoptive transfer of DC depleted whole mouse splenic cells to syngeneic or semi-allogeneic recipients, depleted of their own DC by lethal irradiation, leads to the reconstitution of their splenic DC compartment and thus splenic DC are unlikely to be derived solely from other DC types (355).

In other possible pathways, which may be important in tolerance induction, some Langerhans cells and some interstitial DC, rather than migrating to draining lymph nodes or spleen, may pass into the circulation, where as peripheral blood DC they travel to the thymus and transform into thymic DC. Indeed, that Langerhans cells might migrate to the thymus via

this route is suggested by the fact that in some peripheral blood (231) and thymic DC (see page 14), Birbeck granules have been identified. Again, however, thymic DC are unlikely to be directly derived solely from other DC types. Thus, in vitro, IL-1 mediated differentiation of DC depleted mouse thymic cells into DC has already been described (see page 45) and in a similar study GMCSF has also been shown to induce such differentiation (356).

Antigen processing/presentation and dendritic cells

Although for DC to induce T lymphocyte responses to viruses in the induction of immunity (where CTL often respond in the absence of helper/inducer T lymphocytes) or to negatively select CTL reactive to intracellular antigens in T lymphocyte development (where such responses may also be helper/inducer T lymphocyte independent) class I MHC processing/presentation alone may be sufficient, for responses to other (extracellular) antigens, class II MHC processing/presentation must be seen as a requirement for accessory activity. However, in situ, lymphoid DC display little or no ultrastructural evidence (i.e. phagosomes, lysosomes and phagolysosomes - see pages 12 and 13) of an ability to process antigens via this pathway. Also in situ, interdigitating DC do not express FcRIgG or complement receptors (see page 13; necessary for the phagocytosis of opsonised antigens); and in vivo, lymph node interdigitating DC do not pinocytose or phagocytose injected substances (see page 17).

Further to this, a number of in vitro observations have served to underline this apparent anomaly as follows:

1) Studies of isolated splenic (221,278,357) and thymic (358) DC have confirmed the absence of phagosomes, lysosomes and phagolysosomes. In addition, isolated splenic DC contain few pinocytic vesicles (221).

2) Lymph node and splenic DC do not express sufficient quantities of FcRIgG or CR1 that would otherwise allow rosetting with antibody coated (186,221,241) or antibody plus C3b coated (221,247) SRBC. Similarly, thymic DC do not form rosettes with antibody coated SRBC (237-239).

3) By immunofluorescence, FcRII-IgG cannot be detected on isolated mouse splenic DC (214,302) and neither FcRII-IgG nor CR3 can be detected on isolated mouse thymic DC (215,237). Also, by radioimmunoassay, it has been shown that mouse splenic and thymic DC express only 1×10^3 and 1×10^4 copies/cell respectively of FcRII-IgG (213).

4) In contrast to various types of isolated macrophage, isolated mouse lymph node and splenic, rat lymph node and human thymic DC do not pinocytose or phagocytose any of soluble antigens [gold, FITC, OVA and horse radish peroxidase (HRP), 221,268], particulate/insoluble antigens (M. Tuberculosis, S. Albus, Zymosan, C. Albicans, polystyrene, latex and colloidal carbon, 221,239,240,266,269,359), opsonised antigens (antibody coated SRBC and C. Albicans, 239,266) or immune complexes (HRP-anti-HRP, 221).

At least for lymph node interdigitating DC, the notion of a Langerhans cell derivation provides a solution to this problem. Langerhans cells are well endowed with phagosomes, lysosomes and phagolysosomes (see page 9); express FcRIgG and CR3 in situ (see page 8) and FcRIgG (77,214,273; including in the mouse, FcRII-IgG at 2×10^4 copies/cell, 213), CR1 (249,360,361) and CR3 (213,214,259) in vitro; and pinocytose and phagocytose a wide variety of intradermally injected substances in vivo (see page 16). Thus, to induce responses to extracellular antigens, lymph node interdigitating DC, although requiring to present antigens on class II MHC, may not themselves require class II MHC antigen processing ability. Rather, in vivo, this latter function could be performed by these cells at an immature stage in their life history i.e. as skin Langerhans cells.

It is upon the basis that Langerhans cells may process antigens for lymph node interdigitating DC, that pathways in which interstitial DC ultimately transform into splenic DC and in which interstitial DC and Langerhans cells ultimately transform into thymic DC (see page 58) may also exist. In these schemes, interstitial DC and Langerhans cells would process antigens for presentation by these other types of lymphoid DC. Of course, for interstitial DC, this demands that these cells, like Langerhans cells, are too able to process antigens via the class II MHC pathway. Direct evidence of this is lacking although it is relevant that some types of interstitial DC do express FcRIgG and complement receptors (see page 15).

Another attraction of these various differentiation pathways is that they would provide an efficient mechanism whereby antigens could be disseminated into lymphoid organs. Apart from an inability to process antigens via the class II MHC pathway, one other problem that lymphoid DC might encounter, is that by virtue of their location, often being some distance from sites where foreign antigen might first be encountered or where self antigen naturally resides, concentrations of antigen in the immediate vicinity of these cells might be too low to permit the induction of T lymphocyte responses. The presence of Langerhans cells and interstitial DC at virtually all of these distal sites and their ability (or proposed ability) to both process extracellular antigens and differentiate into lymphoid DC, however, overcomes this problem, allowing the passage of antigens, into lymphoid organs, in an already cell associated form and thus in relatively high local concentrations. In this view then, the primary physiological function of veiled cells and peripheral blood DC would be as trafficers of antigen from various peripheral locations to lymph nodes, spleen and thymus. Indeed, it would seem unlikely that these cells also played a major role in the processing of extracellular antigens as neither

cell type displays ultrastructural evidence of an ability to degrade extracellular antigens (see pages 11 and 15), neither cell type expresses FcRIgG or complement receptors (see page 11 and 147,149; including FcRIII-IgG, CR1 and CR3 on human peripheral blood DC, 256,281,362) and neither cell type endocytoses a variety of substances including HRP (147), ferritin (92), Zymosan (147), latex (92,147,232,362) or antibody coated SRBC (92,149).

Although there exists a strong logical argument therefore, that non-lymphoid DC process and traffic antigens for presentation by lymphoid DC, it should be recalled, that in vitro , there have been many demonstrations of the ability of isolated lymphoid DC to induce strong T lymphocyte responses to both non-viral particulate/insoluble and complex soluble extracellular antigens (see pages 37-40, 50,51). Also, in those in vitro non-physiological systems where recognition of MHC plus peptide is hypothesised to play a role and where there is evidence that helper/inducer T lymphocytes respond, i.e. MLRs and non-Mls minor histocompatability antigen responses, lymphoid DC are, too, potent accessory cells (see pages 43-46,51). In the allo MLR and non-Mls minor histocompatability antigen responses, it could be argued that the antigens concerned are endogenous self antigens (i.e. self to the strain of origin of the DC) and thus again that lymphoid DC precursors performed the processing. The same would apply in the auto MLR, if this were a response to endogenous but foreign antigens; or alternatively, if it were a response to foreign antigens present in culture media additives or in cell isolation materials, then the continual bathing of the DC in these antigens, for some time before assays are "initiated", could allow their presentation by the small amount of class II MHC antigen processing/presenting machinery that lymphoid DC might possess. Such arguments, however, cannot be applied to lymphoid DC and physiological antigens and hence other mechanisms have been hypothesised to explain this phenomenon.

One possibility is that lymphoid DC can present antigens without processing and this would be in accord with the observation that pretreatment of mouse lymph node DC with chloroquine or ammonia, two lysosomotropic agents which inhibit lysosomal enzyme activity does not affect the ability of these cells to induce secondary proliferative responses to OVA and KLH (268). In contrast to this though, in other studies it has been shown that chloroquine abolishes the ability of mouse lymph node DC to induce secondary proliferative responses to OVA and C. Parvum (267) and of mouse splenic DC to induce secondary proliferative responses to KLH (278) and secondary cytotoxic responses to allogeneic H-2K bearing liposomes (where methylamine, another inhibitor of lysosomal enzyme activity, also blocked, 335) suggesting a requirement for lysosomal processing. Further, mouse lymph node DC do degrade both POL and monomeric flagella protein of S. Typhimurium (267), and similarly, mouse splenic DC degrade KLH (278).

Another possibility is that membrane enzymes play a significant role in the processing of extracellular antigens for presentation by lymphoid DC (266,278). Here antigen processing and association of generated peptides with class II MHC (or class I MHC) would be envisaged to occur at the cell surface only. An alternative theory, however, is that membrane enzymes assist the processing and presentation of antigens by the conventional class II MHC pathway by first achieving limited degradation of antigens to a size and/or form that can then be "handled" by the DC.

Finally, it is worth considering two other mechanisms by which lymphoid DC present antigens on class II MHC in vivo. Firstly, there is the possibility that interdigitating and thymic DC (and Langerhans cells) ingest whole self cells (see page 17).

Secondly, non-DC types might process antigens for presentation by lymphoid DC. The most obvious candidates here are macrophages. Macrophages

are well equipped with phagosomes, lysosomes, and phagolysosomes (363) express FcRIgG and complement receptors (364,365) and are the principal endocytic cells of the immune system (366,367). In addition, their juxtaposition to DC in all lymphoid organs (135,151) would make them well suited for this role. Indeed, in in vitro mouse studies, it has been shown that macrophages enhance the ability of splenic DC to induce secondary T lymphocyte proliferative responses to OVA and insoluble OVA (268), KLH (228,279) and C. Parvum (228) and primary antibody responses to SRBC (226,345,346); and that such enhancement is at least in part mediated by peptides, derived from native antigens and released by macrophages, is suggested by the observation that in KLH and C. Parvum responses pretreatment of macrophages with chloroquine abolishes their enhancing ability (228).

IL-1 and dendritic cells

Second signals. A long standing view, is that in order to respond to antigen, T lymphocytes, in addition to recognising MHC plus peptide, must induce the synthesis of and bind accessory cell derived IL-1 (see page 4). In this view, IL-1 acts as a "second signal", where, following binding to T lymphocyte IL-1R (induced by the "first signal" - MHC plus peptide), it stimulates T lymphocytes to express IL-2 and IL-2R (an alternative model proposes that IL-2R are induced by the "first signal"), which, in a penultimate step then interact to induce responses.

Certainly, in DC induced T lymphocyte responses, there is evidence that IL-2/IL-2R interaction plays a central role. For example, in periodate oxidative mitogenesis, mouse splenic DC have been shown to induce both IL-2 secretion and IL-2R synthesis in proliferating T lymphocytes (301,313). Also, in the mouse splenic DC induced primary auto MLR, the DC induce IL-2

synthesis in T lymphocytes (368) and IL-2 antibodies block the production of T lymphocyte derived helper factors (distinct from IL-2) required for B lymphocyte anti-SRBC or anti-HRBC antibody secretion (301). However, whether or not IL-1 functions as a "second signal" in DC induced responses has been a source of much controversy.

To expand upon this none of rat veiled cells and splenic DC (stimulated with LPS plus silica, 244, or with Con A plus T lymphocytes, 242), mouse splenic DC [stimulated with LPS, interferon-gamma (IFN-gamma), tumour necrosis factor-alpha (TNF-alpha), IL-3, macrophage colony stimulating factor (MCSF) or PMA, 369,370] and human peripheral blood DC (stimulated with LPS, 281,371, or with recently in vitro sensitised alloreactive T lymphocyte blasts, 316) release IL-1. Also, although it has been shown that rat veiled cells and splenic DC express membrane IL-1 activity (constitutively, 244), that human interdigitating DC express membrane IL-1 beta (constitutively - see page 13), and that human peripheral blood DC express membrane IL-1 alpha (in response to PHA and PMA, 372), other types of DC, such as mouse splenic DC do not express this form of IL-1 either (again when stimulated with LPS, IFN-gamma, TNF-alpha, IL-3, MCSF or PMA, 370,371).

This controversy has been heightened by studies that have examined the effect of IL-1 antibodies upon DC induced T lymphocyte proliferation. An early report indicated that IL-1 antibodies could inhibit human peripheral blood DC induced secondary T lymphocyte responses to PPD (281). Recently, however, it has been shown that IL-1 antibodies have no effect upon human peripheral blood DC induced T lymphocyte proliferation in each of the primary and secondary allo MLRs and Con A, PHA and CD3 antibody mediated mitogenesis (316); and similarly, IL-1 antibodies do not affect mouse splenic DC induced proliferation in Con A mitogenesis (237).

Enhancement of accessory function. Apart from the question over it's role as a "second signal", IL-1 has been implicated in DC function in other ways. One of these has already been discussed and concerns the role of IL-1 as a differentiation factor, i.e. for Langerhans (see page 56) and thymic DC (see pages 45 and 59). Another concerns the ability of IL-1 to directly enhance DC accessory activity.

With regard to this, partially purified IL-1 has been shown to boost T lymphocyte proliferative responses in the rat veiled cell induced primary allo MLR (244). Similarly, purified IL-1 alpha boosts mouse splenic and thymic DC induced Con A mitogenesis (237) and the mouse splenic DC induced primary allo MLR (303); and in addition IL-1 alpha boosts mouse splenic DC induced B lymphocyte anti-TNP and anti-SRBC antibody responses (303). Further, in mouse DC induced responses, there is evidence that enhancement by IL-1 occurs solely at the DC level (237,303). Thus, 1) pretreatment of splenic and thymic DC with IL-1 alpha is as effective in enhancing Con A mitogenesis and the allo MLR as adding IL-1 alpha continuously throughout assay; 2) pretreatment of Con A pulsed or allogeneic T lymphocytes with IL-1 alpha has no effect upon proliferation in these respective responses and neither does IL-1 induce proliferation in Con A pulsed T lymphocytes or in recently in vitro sensitised alloreactive T lymphocyte blasts; and 3) enhancement of responses by pretreatment of DC with IL-1, cannot be explained by mere ""carry-over"" of IL-1 into assays and then action at the T lymphocyte level as IL-1 antibodies have no effect upon such enhancement and neither do pretreated DC express IL-1 activity.

The mechanism of this IL-1 enhancement has also been studied (303). IL-1 does not increase the expression of class II MHC upon mouse splenic DC and neither does it increase their viability. Interestingly, however, IL-1 increases the capacity of these cells to cluster T lymphocytes, thus

explaining it's boosting effect (see later).

Presumably, enhancement of DC accessory activity by IL-1 in vitro, is representative of another physiological in vivo process in which macrophages (being a major source of IL-1) regulate DC. Indeed, in in vitro mouse studies, it has been demonstrated that supernatants from latex stimulated peritoneal macrophages boost the ability of splenic DC to induce secondary proliferative responses to KLH and primary proliferative responses in the auto MLR (279); and in addition, these same macrophages (which are inactive as accessory cells on their own) have been shown to boost the splenic DC induced primary allo MLR, even when the macrophages and DC are in allogeneic combinations (373).

Other aspects of the mechanism of dendritic cell accessory function - the basis of lymphoid dendritic cell potency

As discussed previously (see page 6), induction of T lymphocyte responses by accessory cells involves more than simply TcR-MHC plus peptide and cytokine-cytokine receptor interactions - a variety of other T lymphocyte and accessory cell surface molecules have now been directly implicated in T lymphocyte activation. Pin-pointing the reasons for the potency of lymphoid DC accessory activity, therefore, would seem difficult in that any one of a number of accessory cell molecules or any combination thereof may be responsible. Furthermore, to add to this difficulty, still further T lymphocyte and accessory cell molecules have been implicated in T lymphocyte activation by indirect evidence - direct evidence of their role in T lymphocyte-accessory cell interaction has not yet been forthcoming (see however Chapters 4-9). Thus, current ideas about the mechanism of lymphoid DC potency, being based by necessity only upon the knowledge of the involvement of molecules implicated in accessory cell function by direct

evidence, may be only partially correct.

Despite these misgivings, two salient features of lymphoid DC have frequently been forwarded as conferring upon these cells potent accessory activity. The first is the ability of lymphoid DC to cluster with T lymphocytes. The second is the hyper-expression of MHC molecules upon lymphoid DC.

Dendritic cell - T lymphocyte clustering

A characteristic of lymphoid DC induced T lymphocyte responses in vitro is the early formation (within 1-2 hr) of cellular clusters. This has been most studied with mouse splenic DC where it has been observed in each of secondary responses to KLH and OVA (374), the primary auto MLR (214,223,301), the primary (214,250,251,374) and secondary (374) allo MLR, and Con A (375) and periodate oxidative mitogenesis (214,299,312). In all of these examples the clusters are seen to consist of a single central DC surrounded by many T lymphocytes.

Importantly, this early clustering process appears to be essential for the induction of subsequent T lymphocyte proliferative and functional responses. Thus, in the mouse splenic DC induced primary auto MLR (227), primary allo MLR (250,251) and periodate oxidative mitogenesis reaction (299), T lymphocytes in separated clusters (separated after only several hr culture), proliferate comparably to T lymphocytes in unfractionated cultures; and similar observations have been made with regards the production of IL-2 and helper factors for B lymphocyte anti-SRBC and anti-HRBC antibody responses in the auto MLR (301). These findings suggest that in unfractionated cultures T lymphocyte responses occur exclusively within clusters and hence this in turn suggests that T lymphocytes require to cluster with lymphoid DC, for relatively prolonged periods, in order to

proliferate and differentiate.

Exactly why lymphoid DC-T lymphocyte clustering is essential for T lymphocyte responses is unclear at present. The most likely explanation, however, is that clustering allows increased time for TcR-MHC plus peptide (or equivalent ligand) binding and then capping of TcR in the T lymphocyte membrane above a threshold level that is required for T lymphocyte triggering. Similarly, clustering may also allow increased time for other DC-T lymphocyte surface molecular interactions that play a role in the induction of responses. One such set of molecular interactions may be those involved in the delivery and reception of a "second signal" and in this context clustering might also provide for the formation of tight junction bound DC-T lymphocyte intercellular spaces (as have been observed between interdigitating DC and T lymphocytes in peripheral lymphoid organs - see page 12), which would be essential for response induction in that they would allow for both the protection of secreted "second signal" cytokines from degradation by extracellular enzymes and the "build up" of such cytokines within these intracellular spaces in high local concentrations. In addition to this, another possibility, which cannot be excluded, is that clustering itself delivers signals to T lymphocytes that are required for proliferative and functional responses.

Given the requirement for clustering then, one possible basis for the potency of lymphoid DC accessory activity, may be an enhanced capacity to participate in this process. Increased clustering would result in an increase in the number of T lymphocytes that are recruited into cell cycle and hence bigger T lymphocyte responses. Indeed, in support of this idea, ability to act as an accessory cell does correlate with an ability to cluster T lymphocytes. For example, resting mouse splenic B lymphocytes, which are weak accessory cells in the primary auto and allo MLRs and in

periodate oxidative mitogenesis (see pages 43, 44 and 48), cluster few (if any) T lymphocytes in these reactions (301,312,314,374). The same cells, however, do bind recently in vitro sensitised ly2- alloreactive T lymphocyte blasts (314,374) and LPS or anti-Ig activated mouse splenic B lymphocyte blasts do bind T lymphocytes in the primary allo MLR (368); and in both of these cases this coincides with increased accessory activity. Likewise, with regards to mouse macrophages, these are weak accessory cells in primary auto and allo MLRs (see pages 43 and 44) and Con A (see page 47) and periodate oxidative (see page 48) mitogenesis and are also poor binders of T lymphocytes in these reactions (250,251,301,312,374,375). Mouse macrophages, however, do bind recently in vitro sensitised ly2- alloreactive T lymphocyte blasts (374) and again this coincides with increased accessory activity. Further evidence of this nature comes from studies of non-lymphoid DC. Thus, the increase in mouse Langerhans cell accessory activity for primary MLRs and periodate oxidative mitogenesis, following their culture for 3 d with keratinocytes (see pages 42, 44 and 48), is paralleled by an enhanced capacity to cluster T lymphocytes (213,214). Also, human peripheral blood DC, which are strong stimulators of primary auto and allo MLRs (see pages 43 and 45), avidly cluster with T lymphocytes in these responses [in contrast to peripheral blood B lymphocytes and monocytes which are weak accessory cells (see pages 43 and 45) and cluster few (if any) T lymphocytes, 149,315].

Clearly, therefore, understanding the molecular mechanisms of clustering would enable a molecular explanation of the potency of lymphoid DC accessory activity. The most likely candidate adhesion molecules that would be expected to be involved are the well characterised LFA-1 molecule (376,377) and either or both of it's ligands ICAM-1 (378) and ICAM-2 (379). LFA-1/ICAM interactions have been implicated in many different types of

intercellular adhesion involving leucocytes (e.g. 380-384) and that these molecules might also mediate accessory cell-T lymphocyte adhesion is logical for several reasons. Firstly, activation of T lymphocytes by TcR complex cross linking (using CD3 antibodies) is known to stimulate a rapid (albeit short lived) increase in the avidity of their LFA-1 molecules for ICAM-1 (385) and this could explain why in the mouse splenic (251,314) and human peripheral blood DC (315) induced primary allo MLRs the DC preferentially cluster T lymphocytes of the appropriate allogeneic MHC specificity. Secondly, both recently sensitised blast and memory T lymphocytes express higher levels of LFA-1 and ICAM-1 than unsensitised virgin T lymphocytes (386-390). This would provide an additional explanation for the preferential binding of alloreactive T lymphocytes by DC and in addition an explanation for the increased magnitude of secondary over primary T lymphocyte responses. Thirdly, IL-1 has been shown to up-regulate ICAM-1 expression upon fibroblasts (391,392) and endothelial cells (393-395) and in the latter case this results in an increased capacity to bind T lymphocytes. Therefore, the idea that an LFA-1/ICAM-1 interaction plays a role at least in lymphoid DC-T lymphocyte adhesion is attractive for the additional reason that an IL-1 mediated increase in lymphoid DC ICAM-1 expression could provide a basis for the ability of this cytokine to enhance the capacity of these cells to both cluster with and induce T lymphocyte responses (see page 66).

Despite these arguments, however, to date there is conflicting evidence as to whether or not LFA-1/ICAM interactions mediate accessory cell-T lymphocyte clustering. Considering primary and mitogen induced T lymphocyte responses first, in the mouse splenic DC induced primary allo MLR (374) and periodate oxidative mitogenesis reaction (299,312) and in the mouse resident peritoneal macrophage induced oxidative mitogenesis reaction (312), clustering, which is normally examined at $37^{o}C$, has been shown to be

abolished at 4°C. Such temperature dependency is a characteristic of LFA-1/ICAM mediated cell-cell adhesion (384,385,396) thus strongly implicating an LFA-1/ICAM interaction. Furthermore, in the mouse splenic B lymphocyte blast induced primary allo MLR (368) and in the mouse splenic DC and resident peritoneal macrophage induced periodate oxidative mitogenesis reactions (312) an antibody against the beta chain of LFA-1 completely blocks accessory cell-T lymphocyte adhesion. However, in the mouse splenic DC induced primary allo MLR, despite the susceptibility of the clustering process to low temperature, the same LFA-1 beta chain antibody does not block clustering in this response (251,368) although profoundly inhibits proliferation (251,312).

With respect to other putative receptor-ligand interactions, two such, which been shown to mediate the adhesion of transfected cells, are those between CD4 antigen and class II MHC (397,398) and CD8 antigen and class I MHC (399,400). However, in the mouse splenic DC and resident peritoneal macrophage induced periodate oxidative mitogenesis reactions neither L3T4 nor ly2 (the mouse equivalents of CD4 and CD8 antigens respectively) antibodies inhibit clustering (although both types of antibody inhibit proliferation in the DC induced response, 312, consistent with the involvement of these molecules in signal transduction rather than adhesion, 401-403). Also against an adhesive role for a CD4-class II MHC interaction is the fact that class II MHC antibodies (as well as L3T4 antibodies) have no effect upon clustering in the mouse splenic DC induced primary allo MLR (251) and additionally this observation would seem to rule out the role of a TcR-class II MHC interaction in clustering.

Another candidate interaction is that between Mac-1 and unknown ligand which has previously been implicated in neutrophil adhesion (404). Mac-1 antibodies, however, have no effect upon clustering in the mouse splenic DC

and B lymphocyte blast induced primary allo MLRs (368).

Lastly, it is important to consider an interaction between the CD2 antigen and it's widely distributed ligand LFA-3 (405-408). CD2 antigen/LFA-3 interactions have been implicated in a variety of T lymphocyte adhesion phenomena (e.g. 405,408-410) and in addition both of these molecules have been shown to be highly expressed upon blast and memory T lymphocytes relative to virgin T lymphocytes (386-389). To date, however, any role of the CD2 antigen and LFA-3 in accessory cell-T lymphocyte clustering has not been directly examined, although indirectly, if they do participate then they probably play only a minor role, as CD2/LFA-3 mediated intercellular adhesion, is not affected by low temperature (405).

In secondary responses, hitherto, mechanisms of clustering have only been examined using recently in vitro sensitised ly2- blast T lymphocyte, or memory T lymphocytes derived from these blasts, as responders thus allowing the delineation of two different types of accessory cell-T lymphocyte adhesion. The first type is termed antigen independent adhesion in that at 37^{o}C, KLH, OVA or allo MHC specific T lymphocytes will cluster as equally efficiently with mouse splenic DC not expressing their TcR ligand as those that do express their ligand (374). The second type is termed antigen dependent adhesion in that at 4^{o}C these same T lymphocytes cluster only with ligand expressing DC (and to the same degree as at 37^{o}C, 251,374). Mouse splenic B lymphocytes and resident peritoneal macrophages are largely incapable of antigen independent binding at 37^{o}C but both can bind alloreactive T lymphocyte blasts in an antigen dependent fashion at either 4 or 37^{o}C (and again temperature does not affect the extent of binding, 314,374).

In physiological terms binding to ligand negative and positive accessory cells would be representative of the adhesion of antigen non-

specific and specific secondary T lymphocytes respectively. In the former case that binding can be abolished by low temperature once more suggests that an LFA-1/ICAM interaction may be involved. The same LFA-1 beta chain antibody that fails to inhibit clustering in the mouse splenic DC induced primary allo MLR (see page 72), however, also fails to inhibit this type of binding (as do antibodies against class I and class II MHC, L3T4, ly2 and Mac-1, 251). In the latter case, clearly, that at $4^{o}C$ binding is antigen dependent shows, that in contrast to primary responses, a direct TcR-class II MHC interaction can mediate adhesion in secondary responses. Indeed, in support of this, class II MHC and TcR antibodies (but not class I MHC, L3T4, ly2, LFA-1 or Mac-1 antibodies) inhibit the antigen dependent adhesion of alloreactive ly2- T lymphocyte blasts and memory cells to mouse splenic DC at $4^{o}C$ (251).

In summary therefore, although an enhanced capacity to cluster T lymphocytes provides at least part of an explanation for the potent accessory activity of lymphoid DC, controversy over the precise mechanisms involved has precluded a definition of potency in molecular terms. Further to this, although MHC is known to be hyper-expressed upon DC (see below) it remains to be established whether other candidate adhesion molecules, such as LFA-1 and ICAMs, are also hyper-expressed upon these cells. Preliminary studies in the mouse have indicated that LFA-1 is in fact absent from splenic DC (259,312) suggesting that if augmented adhesion via the LFA-1/ICAM pathway contributes to an enhanced ability to cluster T lymphocytes then hyper-expression of ICAM molecules by lymphoid DC must be responsible.

Lastly, it is important to consider other mechanisms by which lymphoid DC might bind increased numbers of T lymphocytes. Firstly, there is the dendritic morphology which would provide a larger surface area for T lymphocyte binding over other accessory cell types. Secondly, there is the

possibility that lymphoid DC are deficient in hypothetical anti-adhesion signals. One such anti-adhesion signal might be cell surface negative charge which could interact with negatively charged groups on the opposing cell surface and thus effect anti-adhesion by mediating "like-like" charge repulsion between cells. Certainly, the MHC molecules of mouse splenic DC are known to be deficient in at least one negatively charged moeity, i.e. sialic acid (342), and that such deficiency is important in conferring upon cells potent accessory activity has already been discussed (see page 53 and, however, below).

Hyper-expression of MHC

The second feature of lymphoid DC, that is often considered to be responsible for their potent accessory activity, is their hyper-expression of MHC molecules. As well as immunohistological findings, in vitro studies of lymphoid DC from several different species have confirmed this hyper-expression (e.g. 133,215,240,247,248,411). Also, in quantitative terms, isolated mouse splenic DC have been shown to express 5-10 X more class II MHC than whole spleen cells (302) and splenic B lymphocytes (368), 2-10 X more class II MHC than splenic, liver and thymic macrophages (359,412) and 12.5, 4.6 and 2.6 X more class I MHC than whole spleen cells, splenic LPS blasts and splenic Con A blasts respectively (342). The same is also true of human peripheral blood DC which have been estimated to express as much as 4 X more class II MHC than peripheral blood monocytes (413) and one interesting possibility that has been suggested by some studies (although not confirmed in others - 231,256) is that this hyper-expression is at the level of HLA-DP and DQ rather than HLA-DR (232,233).

Given the central role of MHC in the antigen presentation step of T lymphocyte activation (confirmed for class II MHC with mouse splenic and

human peripheral blood DC by the fact that class II MHC antibodies inhibit T lymphocyte proliferative and functional responses induced by these cells, e.g. 254,255,262,335) it would seem logical to surmise that hyper-expression of MHC has a bearing upon potent accessory activity. In this context, the level of expression of MHC and hence the concentration of antigen-MHC complexes upon the surface of weaker accessory cell types might be limiting such that some antigen specific T lymphocytes, although clustered to these cells, might not be stimulated to above the threshold that is required for their recruitment into cell cycle. In addition it might be pertinent to consider that the role of MHC in accessory cell function, and therefore the consequences of hyper-expression, might not be restricted to the presentation of antigen. Hence, there is the probability that MHC is directly involved in the clustering of T lymphocytes itself (sensitised T lymphocytes only - see above) and the possibility that MHC serves as an accessory cell receptor which controls their synthesis and release of IL-1 (as suggested for monocytes, 414).

One cell type which has been shown to express as much class II MHC as lymphoid DC (mouse splenic DC in this example), yet has only weak accessory activity in most systems, is the freshly isolated mouse Langerhans cell (214,259). However, this is not necessarily to argue against a role for MHC in determining potency, rather that a number of different factors, including the ability to cluster T lymphocytes, are likely to regulate this. Interestingly, mouse Langerhans cells, when cultured in the presence of keratinocytes, have been shown to increase their expression of class I and class II MHC and this has sparked off a debate as to whether this change, in addition to an increased capacity to cluster T lymphocytes, underlies the concomitant increase in accessory activity (again in most systems). Largely, this debate has centered around the kinetics of the increase in MHC

expression. Some investigators, for example, claim that class II MHC expression peaks to reach a plateau level at 12 hr of culture. At this time point there is no increase in accessory activity and the conclusion is thus that an increase in MHC expression is not important (213,214,216,259). Other investigators, however, claim that class II MHC expression does not peak until 48-72 hr of culture and thus that increases in MHC expression could well contribute to an increase in accessory activity (415). If in fact an increase in MHC expression does not relate to an increase in accessory activity in this system then it is important to point out that again this does not contradict the notion that hyper-expression of MHC has a bearing upon potency. Merely, that when MHC is expressed upon cells at a level that is equal to or above that on freshly isolated Langerhans cells or lymphoid DC then it is not a limiting factor in T lymphocyte activation.

In a more general context, studies of the accessory activity of Langerhans cells in CD3 antibody mitogenesis (see page 49) have provided information as to which of hyper-expression of MHC molecules or an ability to cluster T lymphocytes is the more important in determining potent accessory activity. To reiterate, in CD3 antibody mitogenesis, the number of effective TcR ligands, functionally equivalent to MHC, that any one accessory cell could present to a T lymphocyte, is thought to be directly proportional to the number of copies of FcR it expresses. Thus, the finding that freshly isolated mouse Langerhans cells, which express 2×10^4 copies/cell of FcRII-IgG (antibodies against FcRII-IgG completely block mitogenesis in this system showing that other FcR types are not involved) but only poorly cluster T lymphocytes, are weak accessory cells for mitogenesis whereas 3 d cultured mouse Langerhans cells, which express only 2×10^3 copies/cell of FcRII-IgG but avidly cluster T lymphocytes, are strong accessory cells for mitogenesis strongly argues in favour of

clustering rather than hyper-expression of MHC being the dominant element which controls potency of accessory activity [see Appendix 1 - (4)].

Despite this, hyper-expression of MHC by lymphoid DC most readily explains the ability of these cells to overcome Ir gene defects (see pages 21, 23, 50 and 52). It is difficult to envisage how the clustering of increased numbers of antigen specific T lymphocytes alone can correct a defect, the primaril cause of which (assuming that determinant selection is the operative principle) is probably a low accessory cell surface concentration of antigen-MHC complex (although at the single cell level, "tighter" binding of T lymphocytes to lymphoid DC, with an increased area of membrane contact, could play a role). In contrast, hyper-expression of MHC would be expected to correct such a defect by providing for an increased accessory cell surface concentration of antigen-MHC complex that is supra-threshold for stimulation of at least some antigen specific clones.

Similarly, it is difficult to envisage how the clustering of increased numbers of T lymphocytes by lymphoid DC, or indeed human peripheral blood DC, allows these cells to effect helper T lymphocyte independent CTL activation (see pages 45, 48 and 52). Hyper-expression of class I MHC, however, with increased TcR complex capping in the CTL membrane could result in the generation of quantitatively increased or qualitatively different levels of intracellular second messengers which instruct the CTL to respond in the absence of help; and it is along these lines that an alternative interpretation (apart from increased clustering) of the finding that pre-treatment of whole mouse spleen cells with neuraminidase allows these cells also to activate CTL directly (see pages 53 and 75) is that reduction of the negative charge associated with class I MHC molecules permits greater antigen-class I MHC association (342).

Finally, while still on the issue of direct CTL activation it is

relevant to discuss the seemingly alarming consequences of this phenomenon for self tolerance. Working on the assumption that self tolerance is maintained by the deletion or innactivation of autoreactive regulatory T lymphocytes, escape of CTL from the influences of regulatory T lymphocytes would be expected to result in the development of autoimmunity. However, it is possible that autoreactive effector T lymphocytes are also deleted from or are inactivated in the peripheral T lymphocyte pool and this would be in accord with observation that direct CTL activation, which has been shown to occur in each of the mouse splenic DC induced response to non-Mls minor histocompatability antigens and allo MLR and in the human peripheral blood DC induced PHA mitogenesis reaction, does not occur in the mouse splenic DC induced auto MLR (see page 43).

Origin of dendritic cells

As a last section to this introduction it is interesting to reflect upon further ontogenetic aspects of the "DC family". Likely and tentative interrelationships within the "DC family" have been discussed before (see pages 55 and 57) but a more thorny issue concerns the relationship of these cells to other cell types.

Bone marrow origin. It is now well accepted that all members of the DC family are bone marrow derived. The first demonstrations of this were for Langerhans cells and in the definitive experiments allogeneic bone marrow chimaeras were used. Thus, lethally irradiated mice, receiving allogeneic bone marrow allografts, within 14 d of reconstitution, were found to contain Langerhans cells in their skin of donor origin (416,417). Subsequently, allogeneic bone marrow chimaeras were used to demonstrate a bone marrow origin for each of indeterminate cells (418), veiled cells (92), interdigitating DC (419), isolated mouse splenic DC (355) and thymic DC

(129,420). Also, although this experimental approach has not yet been used to show a bone marrow origin of interstitial and peripheral blood DC, that these cells are bone marrow derived can be inferred from the fact that both express LCA (see page 15 and 147,233) and both are probably closely related to other members of the "DC family" (see page 57).

Marker studies. Aside from bone marrow origin, there is little information available to indicate if DC represent a separate cell lineage and consequently most investigators that have attempted to answer this question have cited marker studies as evidence. Clearly, on this basis, it would seem unlikely that DC are closely related to either T or B lymphocytes for DC express few of the markers that are associated with these cell types (e.g. 47,149,213-215,256,261,302,372). However, whether or not DC are unrelated to mononuclear phagocytes is less certain. Concentrating on isolated mouse splenic DC, Steinman and colleagues are ever eager to point out that the absence of markers such as FcRIgG and CR1 and the lack of endocytic activity (see page 60) show that DC are distinct from mononuclear phagocytes. Also, in support of their argument would be the findings that in vitro, mouse, rat and rabbit lymph node DC do not express FcRIgG (see page 60), guinea pig lymph node DC do not express the MsgM macrophage marker (248), mouse thymic DC do not express CR3 (see page 60) and human peripheral blood DC do not express any of the mononuclear phagocyte markers CD14 antigen, FcRIII-IgG, CR1 and CR3 (see page 32 and 232,261); and in situ, too, human veiled cells and mouse interdigitating DC do not express FcRIgG or complement receptors (see pages 11 and 13). Such an argument, however, appears to ignore the fact that indeterminate cells, Langerhans cells, and some types of interstitial DC (which together could represent the precursors of the entire "DC family" - see pages 55 and 57) do in fact express most mononuclear phagocyte markers that have so far been studied. Thus, for example, mouse

indeterminate cells express FcRIgG in vitro (77,421) and human indeterminate cells express CD14 antigen in situ (see page 10); mouse Langerhans cells express FcRII-IgG, CR3 and F4/80 antigen, both in vitro and in situ (see pages 8,56 and 60) and human Langerhans cells express CD14 and CD33 antigens and FcRIgG and complement receptors in vitro (see page 60 and 422) and CD15c and CD68 antigens in situ (see page 8); and mouse lung interstitial DC express FcRIgG and CR1 (see page 15) and human liver interstitial DC express CD14 and CD68 antigens in situ (see page 15). Moreover, it is also the case that lymphoid DC do indeed express at least some mononuclear phagocyte markers. Thus, CR3 and F4/80 antigen are expressed upon mouse splenic DC in vitro (see page 56); Mac-2 and Mac-3 antigens are expressed upon mouse interdigitating DC and CD14 and CD15 antigens are expressed upon human interdigitating DC in situ (see page 13); F4/80 antigen is expressed upon mouse thymic DC in vitro (215); CR3 and Mac-2 antigens are expressed upon human thymic DC in situ (see page 14); and CD36 antigen is expressed upon human peripheral blood DC in vitro (232).

AIM OF STUDIES

Hitherto, all in vitro demonstrations that isolated peripheral lymphoid DC can either act as accessory cells or, in addition, are more potent accessory cells than a number of other cell types, have been made in experimental laboratory animals. Human peripheral lymphoid DC have neither been isolated nor tested for their accessory activity in vitro. Also, and necessarily following from this, studies that have examined the mechanisms of peripheral lymphoid DC accessory activity have exclusively used peripheral lymphoid DC from experimental laboratory animals (particularly mice).

In view of these facts three main aims of the studies presented in this thesis are as follows:

1) To isolate DC and other cell types (for comparative purposes), to high purity, from human peripheral lymphoid tissue (Chapter 2).

2) To examine the ability and comparative ability (with other cell types) of these DC to act as accessory cells for T lymphocyte proliferation (Chapter 3).

3) To elucidate the molecular mechanisms of human peripheral lymphoid DC accessory activity (Chapters 4-8).

As a convenient source of cells for experiments human tonsils have been used and each of the periodate oxidative mitogenesis reaction, the auto MLR and the allo MLR have been employed to examine accessory activity. For pragmatic reasons, the periodate oxidative mitogenesis reaction has also been most used to elucidate the molecular mechanisms of tonsillar DC accessory activity and for this the availability of large panels of antibodies directed against a variety of different human leucocyte cell surface molecules has been exploited. Thus, to identify molecules that play a role in tonsillar DC induced periodate oxidative mitogenesis the effects of these antibodies upon T lymphocyte proliferation has been tested. Further, to determine whether such implicated molecules function at a DC-T

lymphocyte clustering stage or alternatively a "signal transduction" stage in this reaction, the effects of these antibodies upon the formation of tonsillar DC-T lymphocyte clusters in periodate oxidative mitogenesis has too been examined.

A major difficulty in the assessment of tonsillar DC-T lymphocyte clustering is the inability of DC to adhere stably to a variety of substrata. This lack of adhesion tends to preclude the assessment of clustering in sensitive quantitative assays. A fourth aim of this thesis has therefore been to examine the molecular mechanisms of the clustering of tonsillar T lymphocytes to an adherent putative accessory cell type in such a quantitative assay. This cell type is the human promyelomonocytic cell line, U937, induced to differentiate along the mononuclear phagocyte pathway with PMA (Chapter 9).

CHAPTER 2

ISOLATION AND CHARACTERISATION OF DENDRITIC CELLS AND OTHER CELL TYPES FROM HUMAN TONSILS

The principal aim of the studies in Chapters 3-8 has been to examine the accessory function and mechanism of accessory function of different human tonsillar cell populations, particularly DC. Here, methods employed in the isolation of these cells and of responder T lymphocytes (which are also used in adhesion assays in Chapter 9) are described. For DC, the lack of a useful specific marker or physical property, that would allow their simple isolation, has meant that they have had to be isolated largely by a negative selection technique which has been adapted from those used in the isolation of mouse lymphoid, human thymic and human peripheral blood DC (see pages 28-30). For other cell types, for convenience, these have been isolated by positive selection, as by-products of the DC purification process.

The methods and results of experiments designed to examine the purity of isolated tonsillar cell populations are also described here. In the case of DC, again owing to the lack of a useful specific marker, purity has been assessed both by morphology and by examining preparations for the expression of markers that are known to be expressed upon non-DC cell types. In the case of other tonsillar populations, by contrast, purity has been assessed by marker expression alone, using antibodies that specifically react with these populations.

Lastly, to examine any ontogenetic relationship between tonsillar DC and macrophages, these cells have been chosen for an extended analysis of cell surface phenotype. Also, for DC, such analysis has been performed with a view to providing additional information of relevance to later studies.

Tonsils. Pairs of human tonsils were obtained from patients (aged 1 to 30 yr) undergoing routine non-urgent tonsillectomies. Tonsils were collected into dry, sterile universal containers (Sterlin, Teddington, Middlesex, UK) and were processed within 1-2 hr of extraction. Only healthy tonsils were processed; tonsils showing signs of bacterial infection (judged visually by the presence of yellow/green foci of infection) were discarded.

Preparation and density fractionation of whole tonsillar cell suspensions. Whole cell suspensions were prepared from tonsils by enzymatic digestion followed by mechanical sieving through nylon mesh. Tonsils were first immersed in 70% ethanol for 1-2 s to kill any bacteria associated with the tonsillar surface. Excess ethanol was then rinsed from tonsils by immersion in Hanks balanced salt solution (HBSS; Gibco, Paisley, Strathclyde, UK) and tonsils were transferred to 102 mm diameter glass petri dishes where they were cut into 3-5 mm^3 pieces with the aid of sterile forceps and a scalpel. The digestion medium was a solution of sterile filtered [using 0.2 uM pore size sterile filters (Schleicher and Schuell, Dassel, FDR)] type II collagenase (1 mg/ml; Sigma, Poole, Dorset, UK) in HBSS. This solution was added directly to the tonsils (10 ml for each pair) and digestion was allowed to proceed for 1.5 hr at $37^{o}C$ in a 5% CO_2/humidified incubator (Leec, Nottingham, Nottinghamshire, UK). Following this time, the digest was poured onto a 125 uM pore size nylon mesh (Cadisch, London, UK) stretched across the mouth of a 100 ml glass beaker and lymphomedullary cells were separated from surrounding connective tissue by pushing the tonsils through the mesh with the plunger of a 20 ml syringe (Gillete, Isleworth, Middlesex, UK). The resulting cell suspension, collected in the beaker, was then washed at least four times in RPMI 1640 [1000 rpm (170 X g); Gibco] to remove collagenase and debris before density fractionation.

Isotonic, discontinuous Percoll gradient were used to separate tonsillar cells into high and low density fractions. Washed cells were first resuspended in 100% Percoll [nine volumes of stock Percoll (Pharmacia, Uppsala, Sweden) to one volume of 1.5 M sodium chloride (NaCl); final density - 1.0935 g/ml] such that the final cellular concentration was approximately 5 X 10^7 cells/ml. For each gradient, 1 ml aliquots of this suspension were transferred to 10 ml centrifuge tubes (Nunclon, Kamstrup, Denmark) and gradients were made by sequentially layering 1 ml quantities of 70, 60, 50, 40 and 30% Percoll onto the cell suspension with a 1 ml pipette (Sterlin). 70 to 30% Percoll solutions were made by mixing appropriate volumes of 100% Percoll and 0.15 M NaCl and are of densities 1.0726, 1.0657, 1.0587, 1.0518 and 1.0448 g/ml respectively. To separate cells, gradients were spun at 1900 rpm (600 X g) for 15 minutes (min) at room temperature (RT). Cells from each of the interfaces of the gradients were then harvested and collected into 10 ml centrifuge tubes using pasteur pipettes. Those found at the interfaces between 30 and 40 and 40 and 50% Percoll (low density cells) were pooled as were those found at the interfaces between 50 and 60 and 60 and 70% Percoll (high density cells). Finally, both low and high density populations were washed twice in RPMI 1640 medium to remove any excess Percoll before further fractionation procedures.

Dendritic cell isolation. Low density cells from the Percoll gradients were first treated with L-leucine methyl ester (LeuOMe; Sigma) to deplete macrophages and other cells [e.g. natural killer (NK) cells] that are susceptible to the toxic effects of this agent. Cells were resuspended in sterile filtered 5 mM LeuOMe dissolved in HBSS (final cellular concentration - 1-2 X 10^7/ml) and were incubated in this solution for 50 min at RT. Cells were then washed twice in RPMI 1640 to remove LeuOMe and cellular debris.

To ensure the depletion of macrophages and to remove any other

adherent cells that might have survived LeuOMe treatment cells were cultured overnight on 90 mm diameter plastic petri dishes (Nunclon). For this, cells were resuspended in 5% complete medium [RPMI 1640 supplemented with 5% heat inactivated (56^{o}C for 1 hr) foetal calf serum (FCS; Gibco), 10 mM Hepes buffer (Sigma), 2 mM L-glutamine (Gibco), 50 uM 2-mercaptoethanol (Sigma), 100 ug/ml penicillin/streptomycin (Gibco) and 2.5 ug/ml amphotericin B (Gibco)] and plated onto dishes at a final concentration of 1-2 X 10^{7} cells/ml (10ml/dish). After 16 hr culture at 37^{o}C in a 5% CO_2/humidified incubator non-adherent cells were harvested from dishes in three washes of RPMI 1640 (leaving behind firmly adherent cells). Non-adherent cells were then washed twice in RPMI 1640 and resuspended in 5% complete medium.

The next step was to deplete the non-adherent cells of T lymphocytes by SRBC rosetting followed by density centrifugation through Ficoll-Hypaque. The non-adherent cellular concentration was first adjusted to 2 X 10^{7} cells/ml in 5% complete medium and for each ml, 0.2 ml of SRBC [made by mixing one volume of pelleted stock SRBC (Tissue Culture Services, Slough, Berkshire, UK) with nine volumes of PBS] was added. Non-adherent cells and SRBC were then, incubated together for 5 min at RT, pelleted at 0-5^{o}C and incubated in pellet form for 1 hr at this latter temperature to encourage T lymphocyte-SRBC rosetting. Following this, pellets were resuspended gently in their own supernatants and spun over a column of Ficoll-Hypaque [one hundred and twenty seven parts Ficoll 400 (Pharmacia), two hundred parts sodium diatrizoate (Sigma) and two thousand parts distilled water by weight; final density -1.077g/ml] at 2000 rpm (680 X g) for 20 min at RT. This separated the non-adherent cells into a pelleted SRBC+ rosetted T lymphocyte fraction and an interface SRBC- non-rosetted non-T lymphocyte population. SRBC- cells were harvested from the column with a pasteur pipette, washed twice in RPMI 1640 and resuspended in PBS supplemented with 10% FCS before a

final B lymphocyte depletion step.

B lymphocytes were depleted from SRBC- cell fractions by ORBC rosetting followed by spinning over Ficoll-Hypaque as described for T lymphocyte depletion. SRBC- cells were adjusted to a concentration of 4 X 10^7 cells/ml with PBS/10% FCS and were mixed with an equal volume of a murine CD45RA monoclonal antibody (mAb), 3AC5 [a gift from Dr. J. Ledbetter (Oncogen Corporation, Seattle, Washington, USA), supplied at 2mg/ml purified ascites and prediluted to 4ug/ml in PBS]. After 30 min incubation at 0-5^{o}C (to allow antibody-CD45RA antigen binding without cellular internalisation) SRBC- cells were washed twice in PBS at 0-5^{o}C (to remove unbound 3AC5) and resuspended in PBS/10% FCS at 4 X 10^7 cells/ml. ORBC (AFRC, Cambridge, Cambridgeshire, UK) were first precoupled to affinity purified rabbit anti-mouse immunoglobulin (RAMIg; Dakopatts, High Wycombe, Buckinghamsire, UK) to form complexes with the antibody attached to the ORBC via it's Fc portion. For this, five volumes of pelleted stock ORBC were mixed with eighty volumes of 0.9% NaCl (weight/weight in deionised water), ten volumes of RAMIg and eight volumes of a catalyst, chromium chloride (Sigma; a 0.1% solution in 0.9% NaCl), in a 10 ml centrifuge tube. The tube was then shaken vigorously and the reaction was allowed to proceed for 5 min at RT. After this time, the reaction was stopped by adding excess 0.9% NaCl. The RAMIg-ORBC complexes were then washed twice in PBS (RT) to remove excess antibody and catalyst and were resuspended in two hundred and fifty volumes of PBS/10% FCS. RAMIg-ORBC complexes were mixed with 3AC5 treated SRBC- cells in equal volumes. To encourage the formation of B lymphocyte-ORBC rosettes cells were then co-pelleted at 0-5^{o}C. Immediately after this, cells were resuspended in their supernatants and spun over Ficoll-Hypaque (see above) to separate ORBC+ B lymphocytes (pellet) from ORBC- non-B lymphocytes (interface). Finally, interface cells were harvested from the Ficoll-Hypaque, washed twice in RPMI 1640 and used as source of DC.

Macrophage isolation. Macrophages were prepared from low density tonsillar cells by adherence to plastic. Low density cells were resuspended in 5% complete medium at a final concentration of 1-2 X 10^7 cells/ml and 1 ml aliquots of this were pipetted into the wells of 24 well plastic plates (Nunclon). After 2-3 hr at $37^{o}C$ in a 5% CO_2/humidified incubator non-adherent cells were removed from plates in four washes of RPMI 1640 and adherent cells were recultured overnight (16 hr) in 5% complete medium (1 ml/well). Following overnight culture, cells that had detached from the plastic were discarded in four washes of RPMI 1640 leaving the firmly adherent macrophages.

High density T lymphocyte isolation. High density tonsillar cells from the Percoll gradients were first depleted of adherent cells by overnight culture on plastic petri dishes (conditions of culture as for LeuOMe treated low density cells - see "Dendritic cell isolation"). After overnight culture, non-adherent cells were, harvested from dishes in two washes of RPMI 1640, SRBC rosetted and spun over Ficoll-Hypaque (for methods see "Dendritic cell isolation"). Following spinning, SRBC- non-T lymphocytes and Ficoll-Hypaque were aspirated from tubes and the pelleted SRBC+ T lymphocytes were resuspended in Tris ammonium chloride (Tris NH_4Cl) to lyse SRBC. Tris NH_4Cl was prepared by mixing nine volumes of a 0.83% solution (weight/weight in deionised water) of NH_4Cl (Sigma) with one volume of 2.06% Tris buffer (Sigma; weight/weight in deionised water; pH pre-adjusted to 7.65 with 1 M hydrochloric acid) and incubation in this solution was for 2 min at RT. T lymphocytes were then pelleted and washed once in RPMI 1640.

Low density T and B lymphocyte isolation. Low density T and B lymphocytes were prepared from the T lymphocyte-SRBC and B lymphocyte-ORBC rosettes respectively, obtained during the course of DC isolation. Following the removal of interface cells from the Ficoll-Hypaque columns, remaining

Ficoll-Hypaque was aspirated and pelleted rosettes were treated with Tris NH_4Cl to lyse SRBC or ORBC (see "High density T lymphocyte isolation").

Immunophenotyping of dendritic cells, T and B lymphocytes. Cell surface marker expression on isolated DC, T and B lymphocytes was examined by two different indirect immunofluorescence phenotyping methods. DC and T lymphocytes were phenotyped immediately after their isolation from tonsils. By contrast, B lymphocytes were first incubated overnight on 102 mm plastic petri dishes (1×10^7 cells/ml in 5% complete medium) to allow CD45RA antibody internalisation before immunophenotyping.

In the first method antibody incubations were carried out in 1 ml Sarstedt tubes (Sarstedt, Princeton, New Jersey, USA). 1×10^6 isolated cells were resuspended in 100 ul of a primary anti-human antibody [final dilution either 1:10 for culture supernatants (CS) or 1:100 for ascites fluid (AF) preparations], made up in PBS supplemented with 10% normal horse serum (NHS; Flow Laboratories, Irvine, Strathclyde, UK; included to block FcR mediated and non-specific antibody binding to cells), and then transferred to tubes which were incubated for 1.5 hr at 0-5^{o}C (to prevent antibody internalisation by cells). After this time, cells were washed three times in PBS/10% NHS [1700 rpm (200 X g) at 0-5^{o}C; to remove excess antibody] and resuspended in 100 ul of a 1:20 dilution of FITC conjugated RAMIg (Dakopatts) in PBS/10% NHS. Incubation with this secondary reagent was for 45 min again at 0-5^{o}C. Cells were then washed twice (as before) and resuspended in 25 ul of PBS/10% NHS before mounting on glass slides and examination under an inverted fluorescence microscope (Leitz/Wetzlar, Luton, Hertfordshire, UK).

The second immunofluorescence phenotyping method was performed in 96 well U-bottomed radioimmunoassay plates (Gibco). This method was employed to reduce the cell losses during washing encountered in the first method and

thus allowed a more extensive analysis of cell surface antigens on cells. For this method, isolated cells were resuspended in PBS/10% NHS supplemented with 0.2% (weight/weight) sodium azide (NaN_3; Sigma; included as an alternative method of preventing cellular antibody internalisation) and 100 ul aliquots were dispensed to individual wells on plates. Primary antibodies were then added to wells in 10 ul (for CS, final dilution 1:10) or 1 ul (for AF, final dilution 1:100) quantities and plates were incubated for 1.5 hr at RT. Following this, unbound antibody was removed from cells in two washes of PBS/10% NHS/0.25 NaN_3 [1500 rpm (450 X g) at RT]. In this procedure plates were spun directly and after each wash supernatants were removed from plates in a simple "flicking" procedure. Cells were then resuspended in 100 ul of a 1:20 dilution of FITC-RAMIg made up in PBS/10% NHS/0.2% NaN_3 and were incubated with this reagent for 45 min at RT. Finally, after two washes (as before), cells were resuspended in 50 ul of PBS containing 3.75% (weight/weight) formaldehyde (Sigma) and examined under an inverted fluorescence microscope as indicated in the first method.

Immunophenotyping of macrophages. Cell surface marker expression on macrophages was also assessed by indirect immunofluorescence. However, rather than transfer to alternative culture vessels, adherent macrophages were phenotyped directly in the wells of the 24 well plastic plates in which they had been isolated (immediately after isolation).

Following the removal of overnight non-adherent cells from wells, 200 ul of a primary antibody [either CS (1:10) or AF (1:100) in PBS/10% NHS] was added to the macrophages and plates were incubated for 1.5 hr at 0-5^{o}C. After this time, unbound antibody was aspirated from wells, wells were washed twice in PBS/10% NHS (2 X 400 ul with gentle shaking) and the secondary reagent, FITC-RAMIg (200 ul of a 1:20 dilution in PBS/10% NHS) was added. Incubation with FITC-RAMIg was for 45 min at 0-5^{o}C. Unbound FITC-

RAMIg was then aspirated from wells, wells were washed twice (as before) and 200 ul of PBS/10% NHS was re-added. Cells were viewed directly on plates using an inverted fluorescence microscope.

Morphological assessment of dendritic cell enrichment. For morphological assessment of DC enrichment, cell populations from each of the different stages of the DC purification protocol were added to wells of 24 well plastic plates that had been pre-coated with poly-D-lysine (Sigma; 1 mg/ml in PBS). Pre-coating involved layering 1 ml of poly-D-lysine into wells, leaving for 30 min at RT, aspirating and then washing wells twice with 1 ml of RPMI 1640. Cells were added to wells in varying quantities in 1 ml 5% complete medium. After 30 min at RT, supernatant was aspirated, wells were washed twice with 1 ml of RPMI 1640 and 0.6 ml 5% complete medium was re-added. Cells were then viewed on an inverted phase contrast microscope (Leitz/Wetzlar).

Photography. Fluorescing cells were photographed on a manual setting (1 min exposure) using TMAX black and white 3200 ASA film (Kodak, Hemel Hempstead, Hertfordshire, UK; up-rated to 12800 ASA). Cells illuminated by full spectrum light were photographed on an automatic setting using Panatomic-X black and white 32 ASA film (Kodak; down-rated to 16 ASA).

Antibodies. A list of non-MHC antibodies used in immunophenotyping experiments is shown in Table 2.1. All antibodies are mAb and are of murine origin. With the exception of the antibodies TS2/8 (T. Springer - personal communication), M-T310 (Q. Sattentau - personal communication) and 84H10 (423) all cluster designations are according to the Third (424) and Fourth (425) International Workshops on Leucocyte Differentiation Antigens. In addition, grouped designations are according to the Fourth Workshop. Unless otherwise stated cellular reactivity profiles of all antibodies are

according to both workshops.

A list of MHC antibodies used in immunophenotyping experiments is shown in Table 2.2. Again all antibodies are mAb and are of murine origin. Fine specificities of antibodies are described in detail in references 426-435.

Table 2.1 - Non-MHC antibodies used in immunophenotyping experiments

Antibody	CD grouping/ specificity	Isotype	CS/AF[a)]	Source
M-T910	CD2	G1	AF	Workshop 4
TS2/8	CD2		CS	T. Springer
TS2/18.1.1	CD2	G1	CS	T. Springer
T3	CD3	G1	AF	Worshop 4
UCHT1	CD3	G1	CS	P. Beverley
2AD	CD3	M	AF	Workshop 4
Leu3a	CD4	G1	AF	Q. Sattentau
M-T310	CD4		AF	Q. Sattentau
T4	CD4	G1	AF	Workshop 4
T8	CD8	G1	AF	Workshop 4
UCHT4	CD8	G2a	CS	P. Beverley
FMC56	CD9	G1	AF	Workshop 4
VILA1	CD10	M	AF	Workshop 4
MHM24	CD11a	G1	AF	A. McMichael/ Workshop 4
2F12	CD11a	G1	AF	Workshop 4
LPM19C	CD11b		AF	Workshop 4
MO1	CD11b	M	AF	Workshop 4
MN41	CD11b	G1	AF	Workshop 4
TMG6-5	CD11b	G1	AF	Workshop 4
VIM12	CD11b	G1	AF	Workshop 4
14B6.E2	CD11b	M	AF	Workshop 4
44	CD11b	G1	AF	N. Hogg
5A4.C5	CD11b	G1	AF	Workshop 4
BU15	CD11c	G1	AF	Workshop 3
KB90	CD11c	G1	CS	K. Pulford
SHCL3	CD11c	G2b	AF	Workshop 4
3.9	CD11c	G1	AF	Workshop 3
MoU48	CD13	G2a	AF	Workshop 4
CLB-Mon/I	CD14	G2a	AF	Workshop 4
MEM-15	CD14	G1	AF	Workshop 3/4
UCHM1	CD14	G2a	CS/AF	P. Beverley/ Workshop 4
VIM13	CD14	M	AF	Workshop 4
VIMD5	CD15	M	AF	Workshop 4
VIM10	CD15	M	AF	Workshop 4
8-27-F6	CD15		AF	Workshop 4
BW209/2	CD16	G2a	AF	Workshop 4
CLB-FcRgranI	CD16	G2a	AF	A. von dem Borne/Work-shop-4
GRM1	CD16	G1	AF	Workshop 4
GRF1	CD18	G1	AF	Workshop 4
MHM23	CD18	G1	AF	Workshop 4
B4	CD19	G1	AF	J. Ledbetter
2H7	CD20	G2b	AF	J. Ledbetter
VIBE3	CD24	M	AF	Workshop 4
anti-Tac	CD25		AF	T. Waldmann
7G7/B6	CD25	G1	AF	Workshop 4

Table 2.1 contd.

Antibody	CD grouping/ specificity	Isotype	CS/AF	Source
134-4C2	CD26	M	AF	Workshop 4
KOLT-2	CD28	G1	AF	Workshop 4
Ber-H2	CD30	G1	AF	Workshop 4
Ber-H8	CD30	G1	AF	Workshop 4
Ki-1	CD30	G3	CS/AF	H. Stein/ Workshop 4
SG134	CD31	M	AF	Workshop 3/4
2E1	CDw32	G2a	AF	Workshop 3/4
MY9	CD33	G2b	AF	Workshop 4
MY10	CD34	G1	AF	Workshop 4
E11	CD35	G1	AF	N. Hogg
JML-H13	CD35	G1	AF	Workshop 4
NOE2/6/C6	CD38	G2a	AF	Workshop 4
A1	CD39	G1	AF	Workshop 4
G28-10	CD39	G1	AF	Workshop 4
OKT28	CD39	G1	AF	Workshop 4
B-E10	CD40	G1	AF	Workshop 4
G19-1	CD43	G1	AF	Workshop 4
OTH71C5	CD43	G1	AF	Workshop 4
F10-44-2	CD44	G2a	AF	R. Dalchau/ Workshop 4
F10-89-4	CD45	G2a	AF	Workshop 4
136-4B5	CD45	G1	AF	R. Villela/ Workshop 4
562/10D3	CD45	G3	AF	Workshop 4
3AC5	CD45RA	G2a	AF	J. Ledbetter
UCHL1	CD45RO	G2a	CS/AF	P. Beverley/ Workshop 4
4KB5	CD45R	G1	AF	Workshop 4
HuLym5	CD46	G2a	AF	Workshop 4
BRIC126	CD47	G2b	AF	Workshop 4
K31	CD48	M	AF	Workshop 4
WM68	CD48	G2	AF	Workshop 4
101-1D2	CDw50	G1	AF	Workshop 4
13C2	CD51		AF	Workshop 4
097	CDw52	M	AF	Workshop 4
MEM-53	CD53	G1	AF	Workshop 4
LB-2	CD54	G2a	AF	Workshop 4
RR1/1.1.1.	CD54	G1	AF	Workshop 4
WEHICAM-1	CD54	G2a	AF	Workshop 4
84H10	CD54		AF	Workshop 3
143-30	CD55	G1	AF	Workshop 4
NKH1A	CD56	M	AF	Workshop 4
L183	CD57	M	AF	Workshop 4
TS2/9	CD58	G1	CS/AF	T. Springer/ Workshop 4
EMB-11	CD68	G1	AF	Workshop 4
Ki-M7	CD68	G1	AF	Workshop 4
Y-1/82A	CD68		AF	Workshop 4

Table 2.1 contd.

Antibody	CD grouping/ specificity	Isotype	CS/AF	Source
MLR3	CD69	G1	AF	Workshop 4
HNC142	CDw70	G1	CS/AF	G. Delsol/ Workshop 4
BU55	CD71	G1	AF	Workshop 4
Ki-M8	Myeloid group 11D	G1	AF	Workshop 4
MR11	CDw78	M	AF	T. Kishimoto/ Workshop 4

a) CS - culture supernatant, AF - ascites fluid

Table 2.2 - MHC antibodies used in immunophenotyping experiments

Antibody	Specificity	Allotype restriction	Isotype	CS/AF	Source(reference)
W6/32	HLA-A,B,C	None	G2a	CS	J. Bodmer (426)
DA6.164	HLA-DR	Negative on DR7	G1	CS	V. van Heyningen (427,428)
DA6.231	HLA-DP,DQ,DR	None	G1	CS/AF	V. van Heyningen (427-429)
Hig-78	HLA-DP,DQ,DR	None	G1	CS	K. Guy (430)
L243	HLA-DR	None	G2a	AF	Workshop 4 (431)
Tu22	HLA-DQ,DR	DQ - None DR - ?	G2a	AF	G. Pawelec (432,433)
Tu35	HLA-DP,DR	DP - ? DR - None	G2a	AF	G. Pawelec (432,433)
Tu36	HLA-DR	?	G2b	CS	G. Pawelec (432,433)
Tu39	HLA-DP,DR,DY	None	G2a	AF	G. Pawelec (432-434)
1a3	HLA-DQ	None	G2a	AF	R. Wincester (435)

See footnote to Table 2.1

RESULTS

Cell yields. A list of cell yields of different tonsillar cell populations is shown in Table 2.3. Typically, low density cells were less numerous than high density cells and at least half of the latter rosetted with SRBC. LeuOMe treatment and overnight adherence reduced the low density cell number although, likely, (particularly during the adherence step) much of this reduction was due to non-specific losses through cell death and/or washing (for example, few LeuOMe treated cells remained on plates after overnight adherence). Of low density LeuOMe treated overnight non-adherent cells, the great majority of these rosetted with SRBC and ORBC (approximately equal numbers rosetted with each type of RBC), leaving typically small yields of DC (<0.4% of original starting number). Similarly, the yield of macrophages from low density cells was small (again <0.4% of original starting number).

Morphology of dendritic cell enriched populations. Fig. 2.1 shows the morphology (on poly-D-lysine coated plastic) of cells from different stages of the DC purification process. Compared to unfractionated cells, low density cells are more heterogeneous in both size and shape and some cells exhibiting dendritic type morphology are evident (compare also with high density cells which are an almost homogeneous population of small rounded cells). LeuOMe treatment and overnight culture of low density cells did not significantly increase the frequency of cells with dendritic morphology [in accord with relatively small reductions in cell number during these steps (see Table 2.3) and the probability that such cell losses are largely non-specific]. SRBC and ORBC rosetting, however, did increase the frequency such that the resultant end population consisted almost entirely of a population of cells with dendritic morphology.

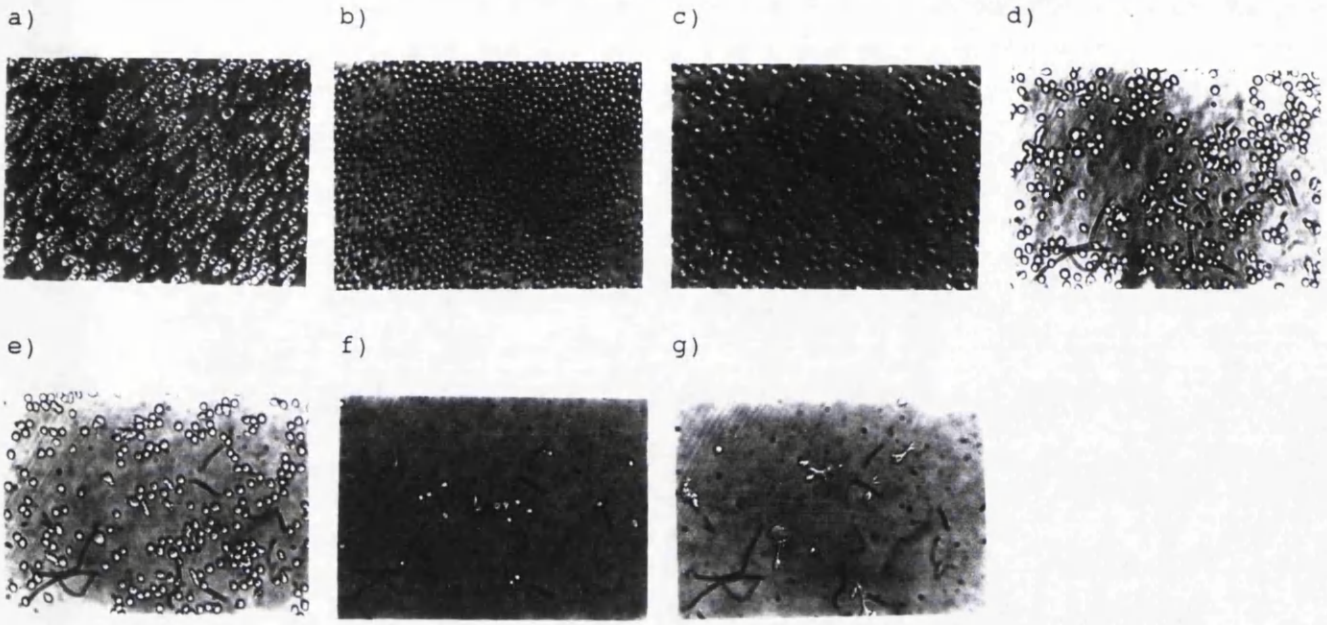
Cell surface phenotype of tonsillar dendritic cells. The results of DC

Table 2.3 - Cell yields from human tonsils.

Cell type	Typical yield per tonsil pair
Unfractionated	8×10^8
Low density	3×10^8 (37.5)[a]
Low density overnight adherent (macrophages)	3×10^6 (<0.4)
Low density LeuOMe treated	2.6×10^8 (32.5)
Low density LeuOMe treated overnight non-adherent	2×10^8 (25.0)
Low density LeuOMe treated overnight non-adherent SRBC+ (low density T)	8×10^7 (10.0)
Low density LeuOMe treated overnight non-adherent SRBC-	1×10^8 (12.5)
Low density LeuOMe treated overnight non-adherent SRBC- ORBC+ (low density B)	8×10^7 (10.0)
Low density LeuOMe treated overnight non-adherent SRBC- ORBC- (DC)	3×10^6 (<0.4)
High density	4×10^8 (50)
High density SRBC+ (high density T)	2×10^8 (25)

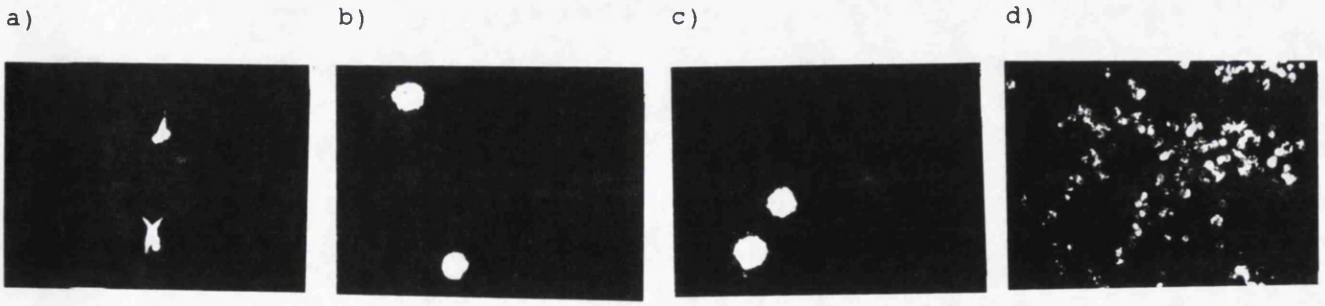
a) Percentage of starting (unfractionated) number.

Fig. 2.1



Phase contrast micrographs of different tonsillar cell populations on poly-D-lysine coated plastic. (a) unfractionated; (b) high density; (c) low density; (d) low density LeuOMe treated; (e) low density LeuOMe treated overnight non-adherent; (f) low density LeuOMe treated overnight non-adherent SRBC-; and (g) low density LeuOMe treated overnight non-adherent SRBC- ORBC-.

Fig. 2.2



Fluorescence micrographs of tonsillar DC, macrophages and high density T lymphocytes. (a) DC and Tu39 (anti-HLA-DP,DR,DY); (b) macrophages and 3.9 (CD11c antibody); (c) macrophages and Tu35 (anti-HLA-DP,DR); (d) high density T lymphocytes and UCHT1 (CD3 antibody).

immunophenotyping experiments, using a broad panel of antibodies, are shown in Table 2.4. Summarising these findings, antibodies which predominantly react with T lymphocytes, i.e. CD2, CD3, CD4, CD8 and CD28 antibodies, either did not stain DC or stained very few cells in these preparations (an exception to this being the CD4 antibody, M-T310, which very weakly stained approximately 20% of cells). Likewise antibodies which predominantly react with B lymphocytes, i.e. CD9, CD10, CD19, CD20, CD24, CD38, CDw78 and one of two CD39 antibodies (OKT28), did not stain DC; although, in contrast to this, another CD39 antibody (A1) weakly stained all DC and a B lymphocyte reactive CD40 antibody also stained all DC, with medium intensity.

With respect to myeloid antibodies, those belonging to CD14, CD16, CDw32 and CD35 clusters again either did not stain DC or stained very few cells in these preparations. A CD13 antibody, however, very weakly reacted with 65% of DC.

Fewer than expected antibodies with reported broad reactivity amongst leucocytes, i.e. non-lineage restricted antibodies, stained DC. Those reactive were generally only weakly so and included CD44 (55%), CD45 (95%), CD45RO (90%), CDw52 (90%), CD53 (90%) and CD58 (45%) antibodies and the CD48 antibody, K31 (90%). CD43, CD45RA, CD46, CD47, CDw50 and CD55 antibodies and the CD48 antibody, WM68 stained few if any DC.

Also of broad reactivity, but considered separately in this thesis, are antibodies against LeuCAM (CD11a, CD11b, CD11c and CD18) and ICAM-1 (CD54) molecules. Of these, CD11a, CD18 and CD54 antibodies reacted strongly with the majority of DC (83, 90-97.5 and 85-95% respectively). CD11b and CD11c antibodies, by contrast, generally stained only a small proportion of cells and with weak intensity.

Most antibodies against activation antigens were unreactive with DC. Unreactive antibodies included those belonging to the CD25, CD26, CD70 and CD71 clusters and the CD30 antibody, Ber-H2. Two activation antigen

Table 2.4 - Cell surface marker expression on human tonsillar dendritic cells[a)]

Antibody	CD grouping/ specificity	Mean percentage positive cells +/- SD[b)]	Intensity[c)]	Number of experiments
M-T910	CD2	0	-	2
TS2/8	CD2	0	-	1
TS2/18.1.1	CD2	0	-	1
T3	CD3	0.7 +/- 1	-	2
UCHT1	CD3	0	-	1
Leu3a	CD4	6	2	1
M-T310	CD4	20	1	1
T4	CD4	0	-	1
T8	CD8	0	-	2
FMC56	CD9	0	-	1
VILA1	CD10	0	-	1
MHM24	CD11a	83 +/- 3.5	4	2
MO1	CD11b	14	2	1
44	CD11b	0	-	2
BU15	CD11c	3	2	1
3.9	CD11c	3	2	1
MoU48	CD13	65	1	1
MEM-15	CD14	6 +/- 1.4	1	2
VIM13	CD14	0	-	1
BW209/2	CD16	0	-	1
GRM1	CD16	6	2	1
GRF1	CD18	90	4	1
MHM23	CD18	97.5 +/- 3.5	4	2
B4	CD19	0	-	2
2H7	CD20	0	-	1
VIBE3	CD24	0	-	1
7G7/B6	CD25	0	-	1
134-2C2	CD26	0.2	1	1
KOLT-2	CD28	0.1 +/- 0.2	3	2
Ber-H2	CD30	0	-	1
Ber-H8	CD30	60	2	1
2E1	CDw32	0	-	2
E11	CD35	5	2	1
JML-H13	CD35	0	-	1
NOE2/6/C6	CD38	0	-	1
A1	CD39	100	2	1
OKT28	CD39	0	-	1
B-E10	CD40	100	3	2
OTH71C5	CD43	0	-	2
F10-44-2	CD44	55 +/- 7.1	2	2
136-4B5	CD45	95 +/- 7.1	2	2
3AC5	CD45RA	5 +/- 7.1	2	2
UCHL1	CD45RO	90 +/- 10	2	3
HuLym5	CD46	0	-	1
BRIC 126	CD47	0	-	1
K31	CD48	90 +/- 0	1	2
WM68	CD48	0	-	2

Table 2.4 contd.

Antibody	CD grouping/ specificity	Mean percentage positive cells +/- SD	Intensity	Number of experiments
101-1D2	CDw50	3	1	1
13C2	CD51	0	-	1
097	CDw52	90 +/- 0	2	2
MEM-53	CD53	90	3	1
LB-2	CD54	85	4	1
84H10	CD54	95	4	1
143-30	CD55	0	-	1
NKH1A	CD56	0	-	2
L183	CD57	2.1 +/- 1.2	2	2
TS2/9	CD58	45 +/- 7	2	2
MLR3	CD69	90	2	1
HNC142	CDw70	0	-	2
BU55	CD71	0	-	1
MR11	CDw78	0	-	2
W6/32	HLA-A,B,C	100 +/- 0	3	2
DA6.164	HLA-DR	100 +/- 0	4	2
DA6.231	HLA-DP,DQ,DR	100 +/- 0	4	2
Hig-78	HLA-DP,DQ,DR	100 +/- 0	5	4
Tu22	HLA-DQ,DR	100 +/- 0	5	3
Tu35	HLA-DP,DR	100 +/- 0	5	3
Tu36	HLA-DR	100 +/- 0	4	2
Tu39	HLA-DP,DR,DY	100 +/- 0	5	3
1a3	HLA-DQ	100 +/- 0	5	2

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a) Assessed by indirect immunofluorescence using the indicated mAb.

b) Figures based upon counts of at least 200 cells. SD - standard deviation. In all experiments RAM-FITC alone gave zero fluorescence.

c) Average intensity was recorded; 1 - v. weak, 2 - weak, 3 - medium, 4 - strong, 5 - v. strong.

antibodies, however, stained DC. These were the CD30 antibody, Ber-H8 (weak staining on 60% of cells), and the CD69 antibody, MLR3 (weak staining on 90% of cells).

All MHC antibodies tested consistently reacted with 100% of DC. With the class I MHC antibody, W6/32, staining was of a medium intensity. With class II MHC antibodies staining was of a strong or very strong intensity and this applied to both antibodies that react with particular class II MHC locus products, i.e. DA6.164 (anti-HLA-DR), Tu22 (anti-HLA-DQ and DR), Tu35 (anti-HLA-DP and DR), Tu36 (anti-HLA-DR) and 1a3 (anti-HLA-DQ), and antibodies which show a more broad specificity amongst class II MHC products, i.e. DA6.231, Hig-78 and Tu39. An example of staining with the Tu39 antibody is shown in Fig. 2.2.

Lastly, a CD51 antibody, which predominantly reacts with platelets, and a CD56 antibody, which has been described as NK cell specific, did not stain DC. In addition, a CD57 antibody, another NK cell reagent, stained only 2% of DC.

Cell surface phenotype of tonsillar macrophages. Table 2.5 shows the results of macrophage immunophenotyping experiments again using a broad panel of antibodies. T lymphocyte reactive CD2, CD3, CD4 and CD8 antibodies and a B lymphocyte reactive CD19 antibody did not stain macrophages. Similar to DC, however, a CD39 B lymphocyte reagent, i.e. the G28-10 antibody, weakly stained 95% of macrophages.

Of myeloid antibodies, those belonging to CD13, CD16 and CDw32 clusters, 3/4 CD14 (CLB-Mon/I, MEM-15 and UCHM1), 1/3 CD15 (VIM10) and 1/3 CD68 (EMB-11) antibodies stained most macrophages, generally with weak intensity. In addition, another CD14 antibody (VIM13) and another CD68 antibody (Ki-M7) stained approximately 50% of cells with weak intensity. CD31, CD33, CD34 and myeloid group 11D antibodies, two other CD15 antibodies

Table 2.5 - Cell surface marker expression on human tonsillar macrophages

Antibody	CD grouping/ specificity	Mean percentage positive cells +/- SD	Intensity	Number of experiments
M-T910	CD2	0	-	1
TS2/18.1.1	CD2	0	-	1
UCHT1	CD3	0	-	1
2AD	CD3	0	-	1
T4	CD4	0	-	1
T8	CD8	0	-	1
UCHT4	CD8	0	-	1
MHM24	CD11a	85 +/- 1.4	3	2
2F12	CD11a	90	4	1
LPM19C	CD11b	0	-	2
MO1	CD11b	90	1	1
MN41	CD11b	94.5 +/- 3.5	2	2
TMG6-5	CD11b	95 +/- 0	2	2
VIM12	CD11b	93	2	1
14B6.E2	CD11b	88 +/- 5.7	2	2
44	CD11b	95	3	1
5A4.C5	CD11b	92.5 +/- 0.7	2	2
KB90	CD11c	93	2	1
SHCL3	CD11c	96	4	1
3.9	CD11c	95	4	1
MoU48	CD13	95	2	1
CLB-Mon/I	CD14	95	3	1
MEM-15	CD14	90	2	1
UCHM1	CD14	95	2	3
VIM13	CD14	52	2	1
VIMD5	CD15	0	-	1
VIM10	CD15	95	1	1
8-27-F6	CD15	0	-	1
CLB-FcRgranI	CD16	95	2	1
MHM23	CD18	95	4	1
M232	CD18	95	4	1
B4	CD19	0	-	1
SG134	CD31	0	-	1
2E1	CDw32	97	2	1
MY9	CD33	10	3	1
MY10	CD34	0	-	1
G28-10	CD39	95	2	1
G19-1	CD43	0	-	1
F10-44-2	CD44	95	3	1
F10-89-4	CD45	95	2	1
562/10D3	CD45	95	1	1
4KB5	CD45R	0	-	1
RR1/1.1.1	CD54	100	4	1
WEHICAM-1	CD54	95	4	1
84H10	CD54	93	4	1
TS2/9	CD58	45	2	1
EMB-11	CD68	95	2	2
Ki-M7	CD68	50	2	1
Y-1/82A	CD68	0	2	1

Table 2.5 contd.

Antibody	CD grouping/ specificity	Mean percentage positive cells +/- SD	Intensity	Number of experiments
Ki-M8	Myeloid group 11D	0	-	1
W6/32	HLA-A,B,C	100 +/- 0	3	1
DA6.164	HLA-DR	0	-	1
DA6.231	HLA-DP,DQ,DR	100 +/- 0	4	1
Hig-78	HLA-DP,DQ,DR	100 +/- 0	4	2
L243	HLA-DR	100 +/- 0	4	1
Tu22	HLA-DQ,DR	100 +/- 0	4	2
Tu35	HLA-DP,DR	100 +/- 0	5	2
Tu36	HLA-DR	100 +/- 0	4	2
Tu39	HLA-DP,DR,DY	100 +/- 0	4	2
1a3	HLA-DQ	100	3	1

See footnotes to Table 2.4.

(VIMD5 and 8-27-F6) and the CD68 antibody, Y-1/82A, however, stained few if any macrophages.

With respect to antibodies with non-lineage restricted specificity, fewer were tested for their ability to stain macrophages than DC. Of those that were examined, however, staining patterns were similar as when DC were used as targets. Thus, CD44, CD45 and CD58 antibodies reacted with macrophages (95% with medium intensity, 95% with weak intensity and 45% with weak intensity respectively) but CD43 and CD45R antibodies did not.

As with DC, CD11a, CD18 and CD54 antibodies reacted with most macrophages (85-90, 95 and 93-100% respectively). Generally, staining was of a strong intensity, excepting the CD11a antibody, MHM24 (medium intensity). In contrast to DC, however, both CD11b (an exception here being the LPMC19C antibody) and CD11c antibodies reacted with most macrophages (90-95 and 93-96% respectively). Staining with CD11b antibodies was generally of a weak intensity whereas staining with CD11c antibodies was of a strong intensity. An example of staining with the CD11c antibody, 3.9, is shown in Fig. 2.2.

Also as with DC, MHC antibodies reacted with 100% of macrophages (an exception this time being one experiment where the DA6.164 HLA-DR antibody failed to react with any macrophages). Again staining with the class I MHC antibody, W6/32, was of a medium intensity whereas staining with class II MHC antibodies (reactive with either particular locus products or of a more broad specificity), when it occured, was generally of a stronger intensity. An example of staining with the Tu35 class II MHC antibody is shown in Fig. 2.2.

Cell surface phenotype of high density tonsillar T lymphocytes. The results of phenotyping experiments using high density tonsillar T lymphocytes as target cells are shown in Table 2.6. T lymphocyte CD2 and CD3 antibodies stained nearly all cells (95-97%) with weak or medium intensity (see Fig.

Table 2.6 - Cell surface marker expression on high density human tonsillar T lymphocytes

Antibody	CD grouping/ specificity	Mean percentage positive cells +/- SD	Intensity	Number of experiments
M-T910	CD2	96	3	1
TS2/18.1.1	CD2	97 +/- 2.8	2	2
UCHT1	CD3	95 +/- 3	2	3
Leu3a	CD4	71.5 +/- 9.2	4	2
UCHT4	CD8	29.5 +/- 3.5	3	2
MHM24	CD11a	95.7 +/- 2.1	4	3
44	CD11b	0	-	1
BU15	CD11c	0.9	2	1
MEM-15	CD14	0	-	2
MHM23	CD18	98 +/- 1	4	3
B4	CD19	4 +/- 2.8	3	2
anti-Tac	CD25	0	-	1
Ki-1	CD30	0	-	1
2E1	CDw32	0	-	2
84H10	CD54	92.5 +/- 0.7	4	2
W6/32	HLA-A,B,C	100	3	2
Hig-78	HLA-DP,DQ,DR	17	4	1

See footnotes to Table 2.4.

2.2 for an example of staining with the CD3 antibody, UCHT1). In addition, a CD4 antibody stained approximately 70% of cells and a CD8 antibody stained approximately 30% of cells with strong and medium intensity respectively.

Of the remaining antibodies that were tested, those belonging to CD19 (B lymphocyte), CD14 and CDw32 (myeloid), CD11b and CD11c (LeuCAM), CD25 and CD30 (activation antigen) clusters reacted with few if any high density T lymphocytes. CD11a, CD18 and CD54 antibodies, however, stained nearly all cells (92.5-98%) with strong intensity. Also, the W6/32 class I MHC antibody reacted with 100% of cells with medium intensity and the Hig-78 class II MHC antibody reacted with 17% of cells with strong intensity.

Cell surface phenotype of low density tonsillar T and B lymphocytes. The cell surface phenotypes of low density tonsillar T and B lymphocytes are shown in Tables 2.7 and 2.8. As with high density T lymphocytes, CD2 and CD3 antibodies stained most low density T lymphocytes (82-85% with weak intensity) and few if any cells were stained by CD14 and CD19 antibodies. In contrast, CD2 and CD3 as well as CD14 antibodies were largely unreactive with low density B lymphocytes whereas a CD19 antibody stained the majority of these cells (86.5% with medium intensity).

Low density tonsillar B lymphocytes were also studied for their expression of class II MHC. All class II MHC antibodies tested (again including antibodies of both restricted and broad specificity amongst different class II MHC locus products) reacted with 100% of B lymphocytes (excepting the Hig-78 antibody which reacted with 90% of cells), generally with strong or very strong intensity.

Table 2.7 - Cell surface marker expression on low density human tonsillar T lymphocytes

Antibody	CD grouping	Mean percentage positive cells +/- SD	Intensity	Number of experiments
TS2/18.1.1	CD2	85	2	1
UCHT1	CD3	82 +/- 2.8	2	2
MEM-15	CD14	0	-	2
B4	CD19	8 +/- 3.6	3	3

See footnotes to Table 2.4

Table 2.8 - Cell surface marker expression on low density human tonsillar B lymphocytes

Antibody	CD grouping/ specificity	Mean percentage positive cells +/- SD	Intensity	Number of experiments
M-T910	CD2	5	2	1
TS2/18.1.1	CD2	7.5 +/- 2.1	2	2
UCHT1	CD3	6.5 +/- 0.7	3	2
MEM-15	CD14	0	-	2
B4	CD19	86.5 +/- 3.5	3	2
DA6.164	HLA-DR	100	3	1
DA6.231	HLA-DP,DQ,DR	100	4	1
Hig-78	HLA-DP,DQ,DR	90 +/- 14.1	4	2
Tu22	HLA-DQ,DR	100	5	1
Tu35	HLA-DP,DR	100	5	1
Tu36	HLA-DR	100	4	1
Tu39	HLA-DP,DR,DY	100	5	1
1a3	HLA-DQ	100	4	1

See footnotes to Table 2.4.

Table 2.9 - Comparison of cell surface markers of tonsillar DC and macrophages

Markers present upon DC and macrophages	Markers present upon macrophages only
CD11a, CD13, CD18, CD39, CD44, CD45, CD54, CD58, class I MHC, class II MHC	CD11b, CD11c, CD14, CD16, CDw32

Markers absent from DC and macrophages
CD2, CD3, CD4[a], CD8, CD19, CD43

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a) One CD4 antibody reacted with 20% of DC but was not tested upon macrophages - see Table 2.4.

DISCUSSION

Rationale of the human tonsillar dendritic cell purification process. In this Chapter, the isolation of human tonsillar DC by a negative selection method, adapted from those used in the isolation of other DC types, is reported. The rationale behind each of the steps used in this technique require further explanation. In the first two steps tonsils were collagenase digested and released low density cells were selected. The purpose of collagenase digestion was to increase DC yield, as has been described for mouse splenic DC (215). Low density cells were selected because most DC types thus far studied are initially of low density (see pages 27-31), excepting human peripheral blood DC, but whose initial high density profile is probably a result of shear forces that these cells are subjected to in the peripheral circulation.

The use of LeuOMe in the next step tonsillar DC isolation is in fact an innovation that has not previously been used in the isolation of other DC types. This agent is directly toxic for mononuclear phagocytes and in the presence of these cells also kills NK cells and some CTL (436-438). It's mechanism of action is unclear but probably LeuOMe freely diffuses into the lysosomes of mononuclear phagocytes whereupon it is converted to the di-peptide condensation product L-leucyl-LeuOMe which then acts as the mediator of cytotoxicity (439,440). Whatever it's mechanism of action, the timing of the LeuOMe treatment step, before adherent cell depletion, to remove each of tonsillar macrophages, NK cells and CTL, was considered crucial. Thus, the adherent step would deplete the majority of macrophages and render NK cells and CTL resistant to the effects of LeuOMe. Also, it has been demonstrated that pre-culture of macrophages, too, renders them LeuOMe resistant (437) and hence the use of LeuOMe after an adherence step, to ensure tonsillar macrophage depletion, would be ineffective.

On the adherence step itself this was primarily used to guarantee macrophage removal but also to deplete other (trace) adherent cell populations, e.g. endothelial cells, epithelial cells and fibroblasts. All DC types so far studied are either transiently or completely non-adherent to plastic (see pages 27-31) and thus DC would not be expected to be depleted after overnight adherence.

In the final two stages of purification low density T and B lymphocytes were separated from DC by SRBC and ORBC (depletion of CD45RA antigen expressing cells) rosetting respectively. In the B lymphocyte depletion step the CD45RA antigen, rather than markers that are more restricted to B lymphocytes, was used to provide a "safety net" for the removal of T lymphocytes. Thus, any T lymphocytes that escaped SRBC rosetting would likely be CD45RA antigen expressing virgin T lymphocytes (441,421) which express low levels of CD2 antigen (see page 73), the molecule that via an interaction with LFA-3 mediates the SRBC rosetting process (410). Of course, the depletion of CD45RA antigen expressing cells in a DC isolation process demands that the DC themselves do not express this marker, which is logical given that lack of CD45RA antigen expression appears to be a feature of more differentiated cells (443,444).

Purity of human tonsillar dendritic cells. DC purification was assessed morphologically and by cell surface phenotyping. Morphologically, most resultant cells (80-90%) were dendritic, supporting successful isolation. In addition, an increase in the frequency of cells with dendritic morphology was observed after density selection, SRBC and ORBC rosetting showing that these steps enrich for DC. That LeuOMe treatment and overnight adherence did not increase the frequency of cells with dendritic morphology can be explained by the fact that the cell populations that would be expected to be depleted in these steps would represent only a small percentage of low

density cells.

Cell surface phenotyping experiments also support the success of the DC isolation protocol by excluding overt contamination by other cell types. Firstly, that T lymphocyte contamination was minimal is shown by that finding that CD2, CD3, CD4, CD8 and CD28 antibodies stain very few or no cells in DC populations. One exception to this is the CD4 antibody M-T310 (which stained 20% of cells) which in light of the above can be interpreted as genuine expression of CD4 antigen by DC.

That B lymphocytes are completely absent is shown by the finding that CD9, CD10, CD19, CD20, CD24, CD38, CD39 and CDw78 antibodies did not stain any cells in DC preparations. One CD39 antibody (A1) and a CD40 antibody, however, stained all cells. Again this can be interpreted as expression of these antigens by DC and is in accord with the observations of another study (see below) and the fact that CD40 antigen expression has been detected upon tonsillar interdigitating DC (see page 13).

Myeloid cells are also insignificant contaminants of DC preparations as judged by the finding that CD11b, CD14, CD16, CDw32 and CD35 antibodies stained at the most very few cells. Staining of 65% of cells in DC preparations with a CD13 antibody is interesting and again is in accord with the findings of a parallel tonsillar DC phenotyping study rather than implying gross myeloid cell contamination.

With regards other cell types, that the DC isolated here are not follicular DC can also be inferred from the lack of staining with CD11b, CD14 and CD35 antibodies and also the lack of staining with CD71 antibodies (445,446). In addition, follicular DC do not express the CD11a, CD18 and CD45 antigens (446), whereas most if not all of the DC isolated here express these molecules. Follicular DC are low density, overnight non-adherent, CD2-CD45RA- cells (446,447) and hence are probably depleted during the LeuOMe treatment step. Another possibility, however, is that follicular DC are

removed by ORBC rosetting by virtue of their close association with B lymphocytes.

DC preparations appear completely free of NK cells. This evidence is provided by the lack of staining with CD56 antibodies, which among leucocytes stain all NK cells in a restricted fashion. Lack of staining with CD56 antibodies also excludes contamination by neural cells and lack of staining with CD55 antibodies excludes contamination by each of endothelial cells, epithelial cells and fibroblasts.

Lastly, as well as the finding that tonsillar DC populations are relatively uncontaminated by non-DC cell types, further evidence that these cells are highly enriched for DC comes from the finding that MHC antigens (particularly class II MHC) are strongly expressed upon all cells.

Isolation and purity of other human tonsillar cell populations. Other tonsillar cell populations were isolated by established positive selection techniques. Little more needs to be expanded upon these methods except that the timing of the low density B lymphocyte isolation step, subsequent and not prior to SRBC rosetting was considered important to reduce contamination of these cells by virgin T lymphocytes.

In contrast to DC, the purity of these other tonsillar cell populations was assessed by phenotyping using specific antibodies. On this theme, that tonsillar macrophages are relatively pure is shown by the finding that 95% of these cells were stained by the macrophage restricted CD68 antibody, EMB-11. In addition, other antibodies known to react with tissue macrophages, but also reactive with some other cell types, e.g. CD11b, CD11c, CD13, CD14, CD16 and CDw32 antibodies, too stained the majority of tonsillar macrophages; and that populations were not contaminated by T or B lymphocytes was suggested by the observation that none of CD2, CD3, CD4, CD8 or CD19 antibodies stained any cells.

Likewise, the high purity of lymphocytes was shown by the finding that most cells in high and low density T lymphocyte populations reacted with a T lymphocyte specific CD3 antibody whereas most cells in low density B lymphocyte populations reacted with a B lymphocyte specific CD19 antibody. Also, most high and low density T lymphocytes were stained by CD2 antibodies (CD4 and CD8 antibodies were too shown to stain high density T lymphocytes with expected frequencies) but few if any cells were stained by CD14 and CD19 antibodies; and most low density B lymphocytes were not stained by CD2, CD3 and CD14 antibodies.

Comparison of cell surface phenotypes of tonsillar dendritic cells and macrophages. Table 2.9 provides a comparison of the cell surface phenotypes of human tonsillar DC and macrophages with respect to those markers that were studied for their expression upon both cell types. Similarities outweigh differences. However, expression or lack of expression of the majority of these markers is either likely to be or is known to be a feature of a number of other cell populations. Perhaps the most convincing argument in favour of a close ontogenetic relationship is the expression of the CD13 antigen by both cell types. Against this though is the expression of CD14 antigen by macrophages but not by DC.

Two other points are of interest. Firstly, no major differences in the expression of CD11a, CD18 and CD54 antigens could be detected between DC and macrophages. Secondly, no difference in the expression of MHC antigens by DC and macrophages (or indeed B lymphocytes) was evident (macrophages that were not stained by the DA6.164 antibody in one experiment may have been from an HLA-DR7 positive individual) with the possible exception of a more intense expression of HLA-DQ by DC which would be in agreement with a previous comparison of human peripheral blood DC and monocytes (see page 75).

Other studies. During the course of these studies Hart and McKenzie also

isolated human tonsillar DC using a technique that lends more heavily upon methods used for the isolation of human peripheral blood DC (448). In this technique tonsillar cells were first T lymphocyte depleted (SRBC rosetting) and then cultured for 1-2 d on plastic before selection of low density cells. Finally, B lymphocytes, myeloid cells and residual T lymphocytes were depleted by fluorescence activated cell sorting (FACS) using a cocktail of CD3, CD20, CD24 and 63D3 antibodies.

Possibly owing to the absence of an enzymatic digestion step, DC yields were slightly lower (0.1%) than the DC yields reported here. The cell surface phenotypes of the two DC types, however, are practically identical. Thus "Hart and McKenzie" type DC express CD13, CD39, CD40, CD45, class I MHC and class II MHC antigens but do not express any of CD2, CD3, CD4, CD8, CD9, CD10, CD11b, CD11c, CD14, CD16, CD19, CD20, CD24, CDw32 or CD35 antigens. The only apparent differences in phenotype are that "Hart and McKenzie" DC reportedly only weakly express CD11a and CD18 antigens and in addition weakly express CD45RA antigen.

Summary

DC, macrophages, low and high density T lymphocytes and low density B lymphocytes have been isolated from human tonsils. DC were isolated by a negative selection method adapted from methods used in the isolation of other DC types. As evidenced by morphology, absence of markers known to be expressed upon other cell types and strong expression of MHC antigens, these DC (which represent only 0.4% of all tonsillar cells) are highly enriched populations. Other cell types were isolated from human tonsils by positive selection methods as by-products of the DC isolation process. The high purity of these cells was shown by their expression of appropriate cell specific markers. An extended analysis of DC and macrophage phenotype revealed both similarities and differences and was not altogether helpful in deciding lineage relationships.

CHAPTER 3

HUMAN TONSILLAR DENDRITIC CELLS AS ACCESSORY CELLS IN VITRO

INTRODUCTION

Amongst the body of evidence that implicates peripheral lymphoid DC as the principal physiological accessory cells involved in the induction of immunity is the demonstration of their potent accessory activity in vitro. All of these demonstrations, however, have been made in experimental laboratory animals. To date, human peripheral lymphoid DC have not been tested as accessory cells in vitro.

To examine this, in this Chapter, isolated human tonsillar DC have been tested for their ability and comparative ability, with tonsillar macrophages and low density T and B lymphocytes, to induce high density T lymphocyte proliferation in three different model systems - periodate oxidative mitogenesis, the auto MLR and the allo MLR. In addition, the kinetics of all three of the DC induced responses have been monitored in order to gain insight into any possible relationships.

MATERIALS AND METHODS

Cells. DC, low and high density T lymphocytes and low density B lymphocytes were isolated from human tonsils as described in Chapter 2. Macrophages were also isolated as described in Chapter 2 except that 102 mm diameter plastic petri dishes rather than the wells of 24 well plastic plates were used as the adherence substrate. Following the removal of overnight non-adherent cells, macrophages were harvested from dishes by gentle scraping using the plunger of a 10 ml syringe (Gillete). Cells were then washed twice in RPMI 1640 before irradiation.

Irradiation of accessory cells. To prevent their proliferation in oxidative mitogenesis assays and MLRs, putative accessory cells (DC, macrophages, low density T and B lymphocytes) were first irradiated. For this purpose cells were resuspended in RPMI 1640 and irradiated at 3000 rads over a 5 min period [250 KV Maximar source (General Electric Company, Fremont, California, USA)]. Cells were then washed once in RPMI 1640 and used in assays.

Oxidation of high density T lymphocytes. For oxidative mitogenesis assays, high density responder T lymphocytes were first oxidised with sodium periodate ($NaIO_4$; Sigma). For this, T lymphocytes were resuspended in a solution of periodate (0.2, 2 or 20 mM made up in PBS and sterile filtered before use) such that the final cellular concentration was 5×10^6/ml. Cells were then incubated in this solution for 30 min at 0-5^{0}C before two washes in HBSS (containing glucose to "mop up" excess periodate) and incorporation into assays.

Proliferation assays. Oxidative mitogenesis assays, auto and allo MLRs were performed in 96 well plates (Nunclon) in 10% human AB serum [complete medium

containing 10% heat inactivated (56^{o}C for 1 hr) human AB serum (North London Blood Transfusion Centre, Middlesex, UK) rather than FCS]. In each of these assays 2 X 10^{5} high density responder T lymphocytes (added to wells in 100 ul volumes) were cultured either alone (i.e. with 100 ul medium) or with varying numbers of irradiated accessory cells (see above; also added to wells in 100 ul volumes). Additional wells contained irradiated accessory cells only (i.e. with 100 ul medium). For oxidative mitogenesis, responder T lymphocytes were first treated with periodate (see above) and were autologous to the accessory cells. For auto and allo MLRs responder T lymphocytes were left unmodified and were autologous and allogeneic to accessory cells respectively. Incubation of cultures was for varying lengths of time in a 5% CO_2/37^{o}C humidified incubator. To measure proliferative responses wells were pulsed with 30 ul (0.75 uCi) of [5-^{125}I]-2-deoxyuridine (^{125}IdUrd; Amersham International, Amersham, Buckinghamshire, UK; supplied at 1 mCi/ml and pre-diluted to 25 uCi/ml in RPMI 1640) and cultures were incubated for a further 6 or 16 hr in a 5% CO_2/37^{o}C humidified incubator. Cells were harvested from wells onto glass fibre filters [Whatman, Maidstone, Kent, UK; using a Skatron cell harvester (Flow Laboratories)] and incorporation of radiolabel into cells was measured by counting the radioactivity associated with the filters in a NE 1600 gamma counter (Nuclear Enterprises, Reading, Berkshire, UK).

Statistics. Results are expressed as mean counts per minute (CPM) +/- 1 SD of triplicate cultures.

RESULTS

The function of tonsillar dendritic cells as accessory cells for oxidative mitogenesis. Fig. 3.1 depicts the results of an experiment that was designed to examine whether or not tonsillar DC could act as accessory cells for T lymphocyte proliferation in oxidative mitogenesis. In this experiment, constant numbers of irradiated DC and periodate modified (either 0.2, 2 or 20 mM) autologous high density T lymphocytes were cultured either alone or in combination as indicated. Culture was for varying times and for each time point proliferation was measured by ^{125}IdUrd incorporation during the last 6 hr. As shown, at each time point examined, only small quantities of ^{125}IdUrd were incorporated by isolated DC (a-c), isolated periodate treated T lymphocytes (all concentrations, a-c) and DC plus 20 mM periodate treated T lymphocytes (c). However, DC induced significant proliferative responses in 0.2 and 2 mM periodate treated T lymphocytes (a,b). In these cases, proliferative responses were: first detected 2 d after culture initiation (around which time they also peaked), persisted for at least 7 d and were greatest using 2 mM periodate.

Fig. 3.2 shows the effect of increasing numbers of DC upon oxidative mitogenesis under conditions that are optimal for this response (defined in Fig. 3.1, i.e. 2 mM periodate in a 2-3 d assay). As shown proliferation increased with increasing numbers of DC added.

Comparison of the dendritic cell induced oxidative mitogenesis reaction with the dendritic cell induced auto and allo MLRs. Fig. 3.3 shows the results of four different experiments which compare the 2-3 d T lymphocyte proliferative responses that occur in the DC induced oxidative mitogenesis reaction and auto MLR (which uses unmodified autologous T lymphocytes as responders). In each experiment DC induced T lymphocyte proliferation in

both types of response [excepting the auto MLR in (a)]. However, although the ratio of the magnitude of the oxidative mitogenesis reaction to the auto MLR varied from experiment to experiment [i.e. from 1.3 using 1 X 10^5 DC per well in (b) to 28 in (a)], in all experiments the oxidative mitogenesis response was greater than that of the auto MLR when measured at this time.

In a similar type of experiment (Fig. 3.4) the 2-3 d T lymphocyte proliferative response in the DC induced oxidative mitogenesis reaction was compared with that in the DC induced allo MLR (which uses unmodified allogeneic T lymphocytes as responders). Again DC induced T lymphocyte proliferation in both types of response and as with the auto MLR, the magnitude of the allo MLR was smaller than that of the oxidative mitogenesis reaction when measured at 2-3 d.

The DC induced auto and allo MLRs were investigated further by examining the kinetics of these reactions. Figs. 3.5 (a,b) and 3.6 show that peak proliferative responses in these reactions (auto and allo MLR respectively) occured at 4 d in contrast to the DC induced oxidative mitogenesis reaction which peaked around 2-3 d [Fig. 3.1 (a,b)]. However, in experiments where the 2-3 d proliferative response in the DC induced oxidative mitogenesis reaction was compared with the 4 d proliferative response in the DC induced auto and allo MLR, the oxidative mitogenesis reaction was always greater in magnitude [Figs. 3.7 (a,b) and 3.8 respectively].

The relative potency of tonsillar dendritic cells, macrophages and low density T and B lymphocytes as accessory cells for the oxidative mitogenesis reaction, the auto MLR and the allo MLR. Fig. 3.9 shows the results of ten different experiments in which tonsillar DC were compared with tonsillar macrophages, low density T lymphocytes and low density B lymphocytes as inducers of proliferation in the 2-3 d oxidative mitogenesis reaction. All

four populations could act as accessory cells for oxidative mitogenesis. Consistently, however, DC induced the greatest T lymphocyte proliferative responses.

The results of experiments in which tonsillar DC, macrophages and low density B lymphocytes were compared as inducers of proliferation in the 96 hr auto and allo MLRs are shown in Figs. 3.10 and 3.11. Only DC could act as accessory cells for the auto MLR; macrophages and B lymphocytes did not induce significant T lymphocyte proliferative responses above that of background in this reaction (Fig. 3.10). In contrast to this, all three of these populations acted as accessory cells for the allo MLR. However, DC were still the most potent inducers of T lymphocyte proliferation (Fig. 3.11).

Fig. 3.1(a)

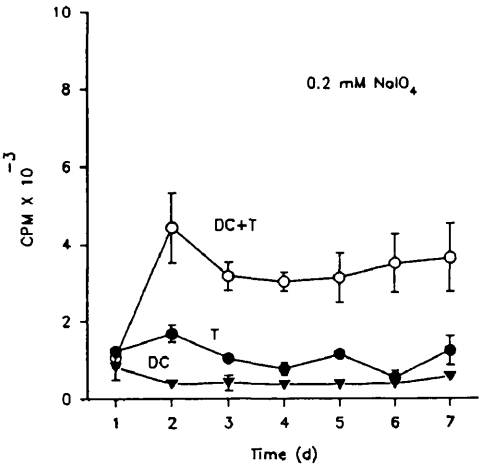


Fig. 3.1(b)

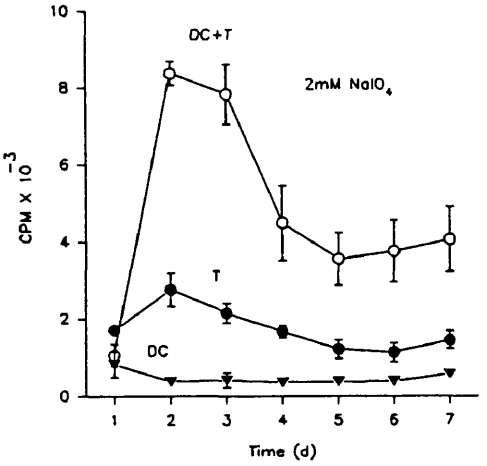


Fig. 3.1(c)

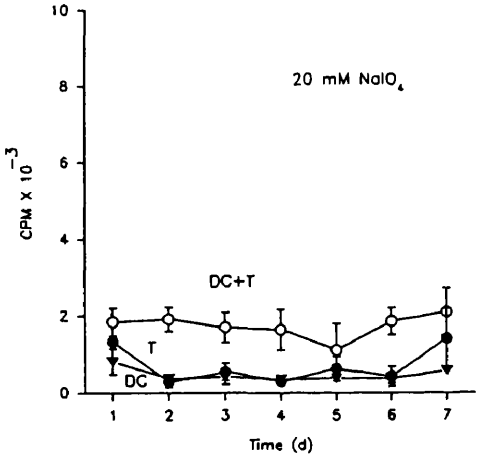


Fig. 3.2

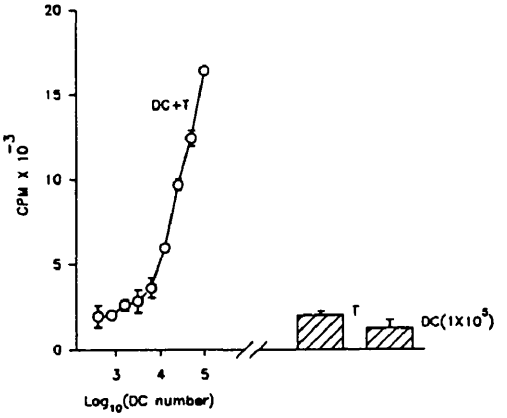


Fig. 3.3(a)

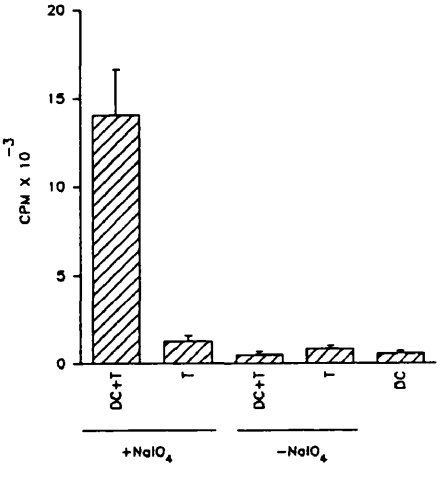
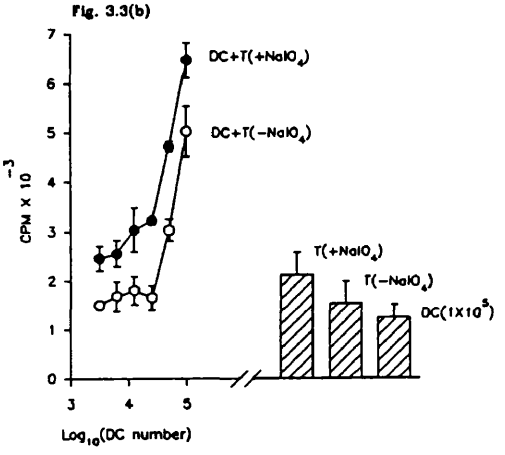


Fig. 3.3(b)



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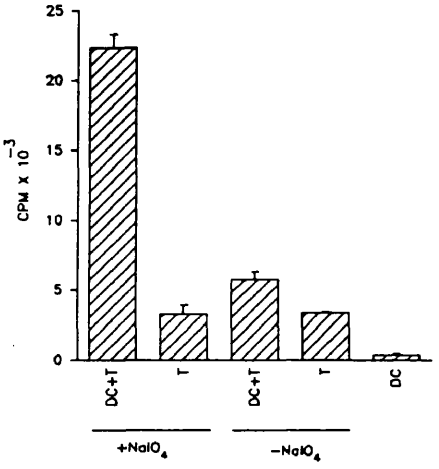


Fig. 3.3(d)

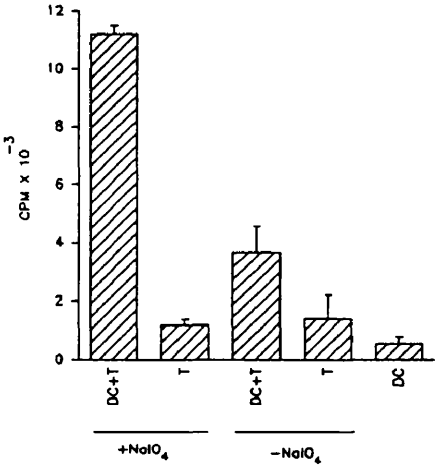


Fig. 3.4

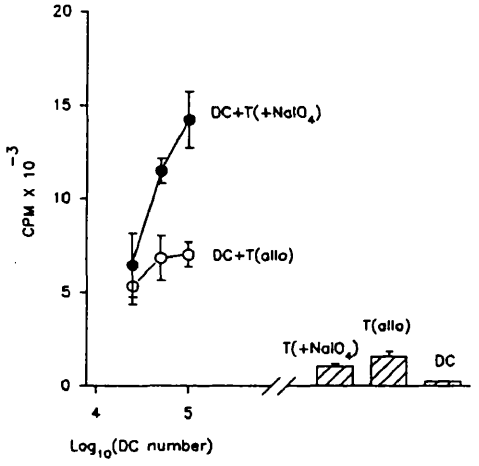
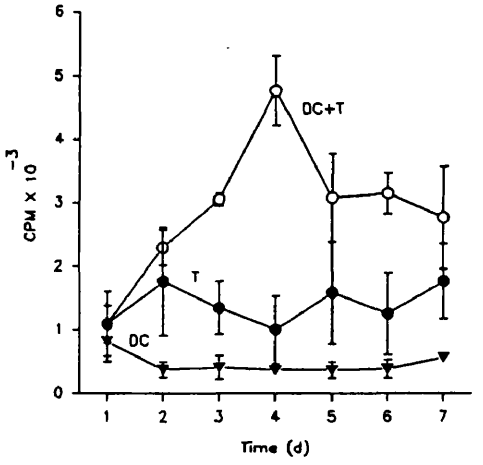


Fig. 3.5(a)



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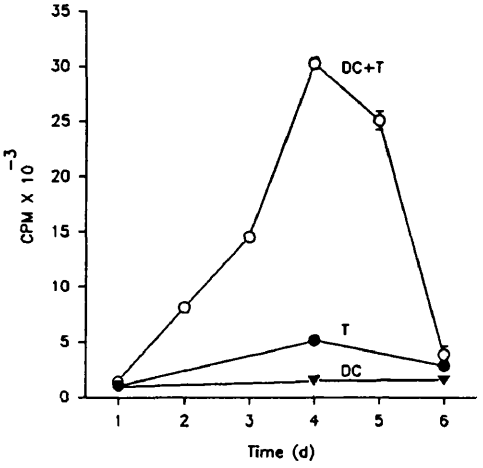


Fig. 3.6

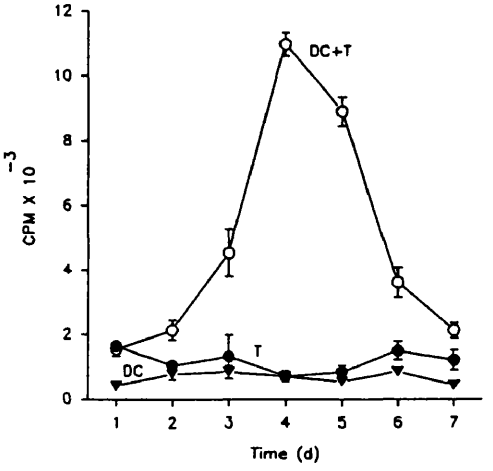
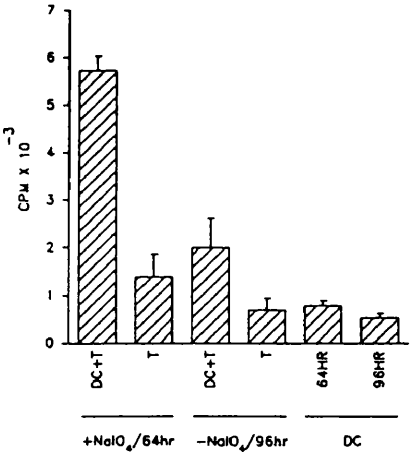


Fig. 3.7(a)



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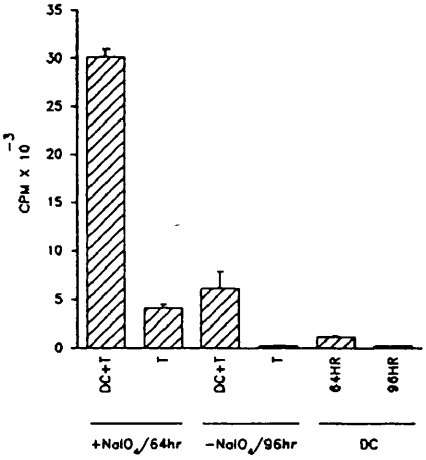


Fig. 3.8

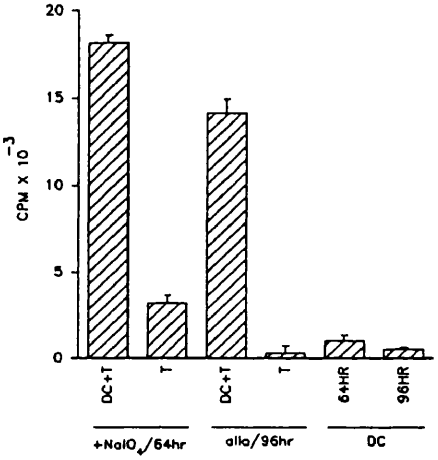
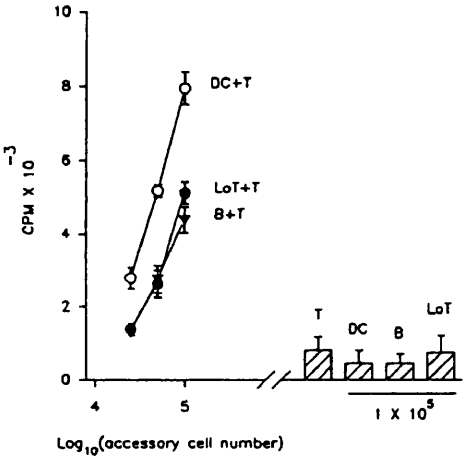


Fig. 3.9(a)



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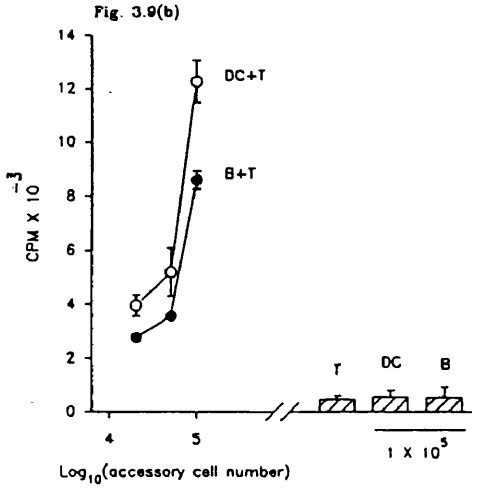
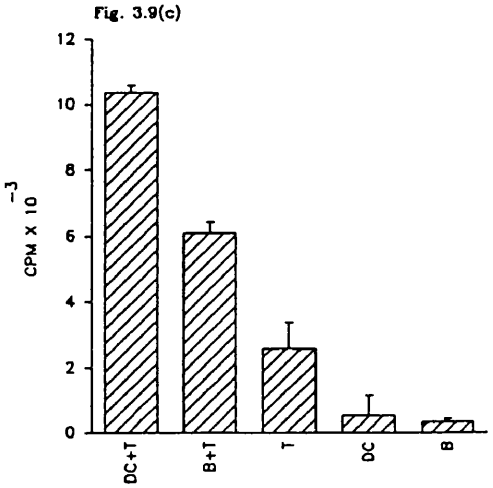


Fig. 3.9(c)



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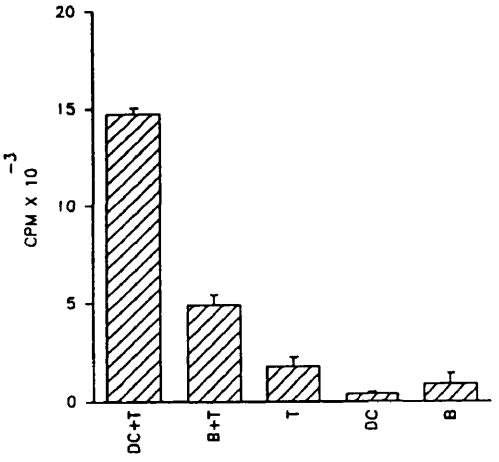

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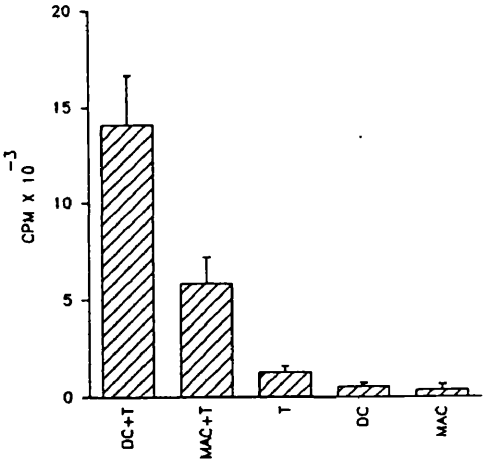

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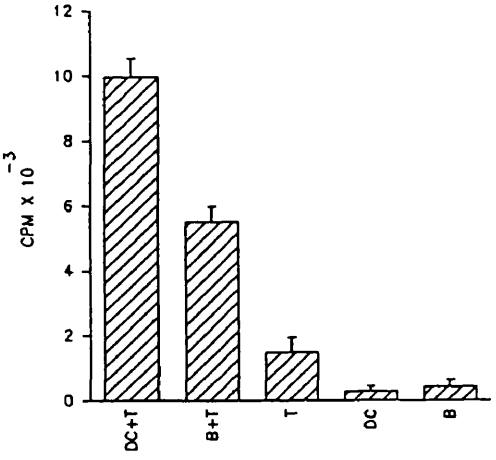

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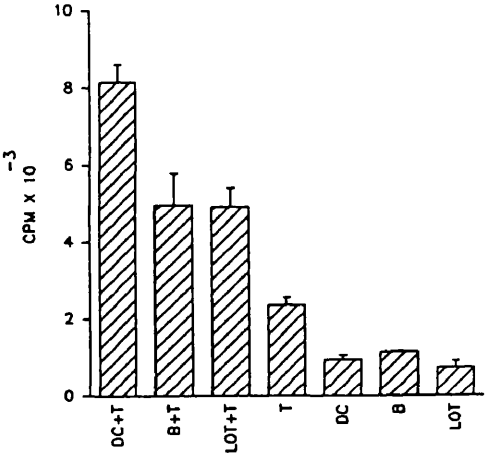


Fig. 3.9(h)

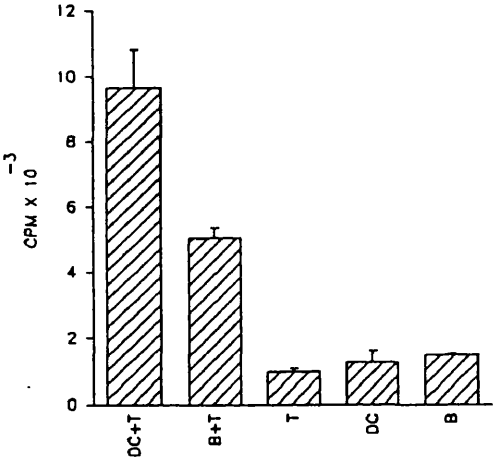
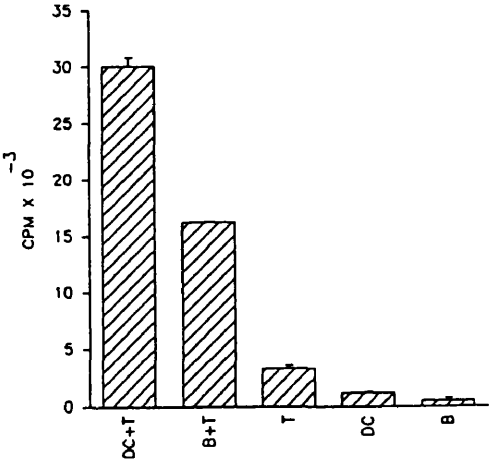


Fig. 3.9(i)



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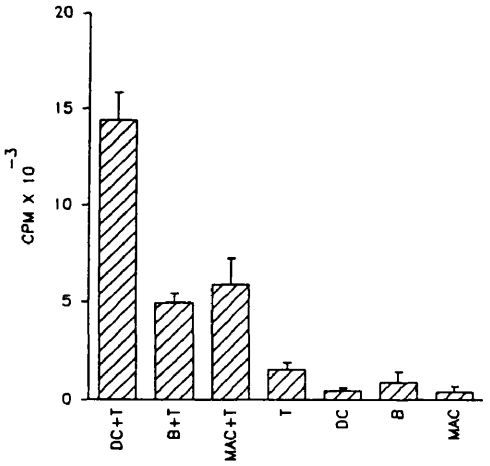


Fig. 3.10

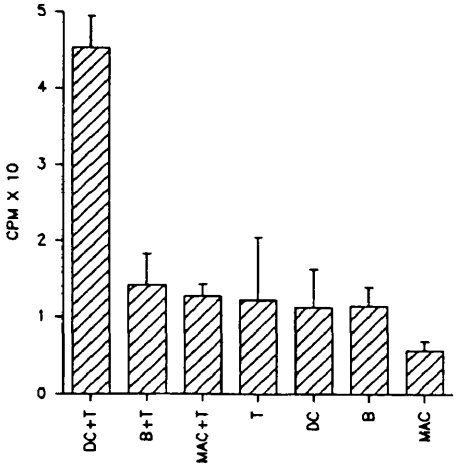


Fig. 3.11

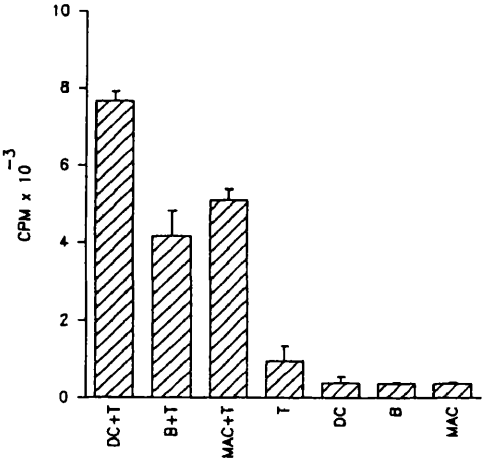


Fig. 3.12

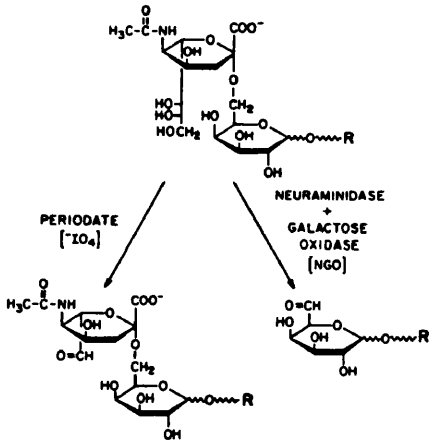


Figure Legends

See Materials and Methods plus:

Fig. 3.1(a-c). DC induced oxidative mitogenesis. DC number - 4 X 10^4/well, pulse length - 6 hr.

Fig. 3.2. DC induced oxidative mitogenesis. $NaIO_4$ concentration - 2 mM, assay duration - 64 hr, pulse length - 16 hr.

Fig. 3.3(a). DC induced oxidative mitogenesis and auto MLR. $NaIO_4$ concentration - 2 mM, DC number - 1 X 10^5/well, assay duration - 64 hr, pulse length - 6 hr.

Fig. 3.3(b). DC induced oxidative mitogenesis and auto MLR. $NaIO_4$ concentration - 2 mM, assay duration - 64 hr, pulse length - 16 hr.

Fig. 3.3(c). DC induced oxidative mitogenesis and auto MLR. $NaIO_4$ concentration - 2 mM, DC number - 5 X 10^4/well, assay duration - 64 hr, pulse length - 16 hr.

Fig. 3.3(d). DC induced oxidative mitogenesis and auto MLR. $NaIO_4$ concentration - 2 mM, DC number - 1 X 10^5/well, pulse length - 16 hr.

Fig.3.4. DC induced oxidative mitogenesis and allo MLR. $NaIO_4$ concentration - 2 mM, assay duration - 64 hr, pulse length - 16 hr.

Fig. 3.5(a). DC induced auto MLR. DC number - 5 X 10^4/well, pulse length - 6 hr.

Fig. 3.5(b). DC induced auto MLR. DC number - 1 X 10^5/well, pulse length - 6 hr.

Fig. 3.6. DC induced allo MLR. DC number - 1 X 10^5/well, pulse length - 16 hr.

Fig. 3.7(a). DC induced oxidative mitogenesis and auto MLR. $NaIO_4$ concentration - 2 mM, DC number - 6 X 10^4/well, pulse length - 16 hr.

Fig. 3.7(b). DC induced oxidative mitogenesis and auto MLR. $NaIO_4$ concentration - 2 mM, DC number - 1 X 10^5/well, pulse length - 16 hr.

Fig. 3.8. DC induced oxidative mitogenesis and allo MLR. $NaIO_4$ concentration - 2 mM, DC number - 8 X 10^4/well, pulse length - 16 hr.

Fig. 3.9(a,b). Accessory cell induced oxidative mitogenesis. $NaIO_4$ concentration - 2 mM, assay duration - 64 hr, pulse length - 16 hr.

Fig. 3.9 (c,d,f,i,j). Accessory cell induced oxidative mitogenesis. $NaIO_4$ concentration - 2 mM, accessory cell number - 1 X 10^5/well, assay duration - 64 hr, pulse length - 16 hr.

Fig. 3.9(e). Accessory cell induced oxidative mitogenesis. $NaIO_4$ concentration - 2 mM, accessory cell number - 8 X 10^4/well, assay duration - 64 hr, pulse length - 6 hr.

Fig. 3.9(g). Accessory cell induced oxidative mitogenesis. $NaIO_4$ concentration - 2 mM, accessory cell number - 8 X 10^4/well, assay duration - 64 hr, pulse length - 16 hr.

Fig. 3.9(h). Accessory cell induced oxidative mitogenesis. $NaIO_4$ concentration - 2 mM, accessory cell number - 5 X 10^4/well, assay duration - 64 hr, pulse length - 16 hr.

Fig. 3.10. Accessory cell induced auto MLR. Accessory cell number - 1 X 10^5/well, assay duration - 96 hr, pulse length - 16 hr.

Fig. 3.11. Accessory cell induced allo MLR. Accessory cell number - 1 X 10^5/well, assay duration - 96 hr, pulse length - 6 hr.

Fig. 3.12. Modification of terminal sialic acid or penultimate galactose on glycosylated molecules by treatment with periodate or neuraminidase plus galactose oxidase. R denotes the remainder of the cell surface glycoprotein or glycolipid molecule.

DISCUSSION

Accessory cell requirements for oxidative mitogenesis. Blast transformation and replication in lymphocytes following their oxidation with periodate or Na/GaO (oxidative mitogenesis) was first described using unpurified populations of lymphocytes from each of rat (449), mouse (450), rabbit (451), calf (452) and guinea pig (453) lymph nodes, mouse spleen (454), mouse thymus (455) and human peripheral blood (456). Some early studies examined the cellular requirements for oxidative mitogenesis and reached two important conclusions. Firstly, because T lymphocyte depleted mouse spleen cells did not proliferate, T lymphocytes were considered the main responding cells (455,457,458). Secondly, because purified lymphocytes from mouse spleen, rabbit lymph node and human peripheral blood, depleted of glass adherent cells or of class II MHC expressing cells also did not proliferate and because responsiveness could be restored by re-addition of these depleted populations to oxidised lymphocytes a second glass adherent class II MHC expressing non-lymphocyte population or accessory cell population was considered to play an essential role in the induction of oxidative mitogenesis (453,459,460-463). At first many considered the mononuclear phagocyte to be the principal accessory cell. However, in those species in which this type of experiment was performed, DC are also glass adherent (albeit transiently) and, of course, express class II MHC. Following the development of techniques for the purification of DC the idea that these cells were the main accessory cells was in fact confirmed. Thus, isolated DC from each of mouse Peyers patch and spleen, rat lymph node, spleen, lung and liver, rabbit lymph node and human peripheral blood were shown to be potent accessory cells for oxidative mitogenesis when compared with mononuclear phagocytes and other putative accessory cell types isolated from the same or other tissues (see page 48).

Human peripheral lymphoid dendritic cells as accessory cells. If one accepts that peripheral lymphoid organs are the main sites in which the induction of the immune response takes place, then of all of the different members of the "DC family", demonstrations that isolated peripheral lymphoid DC are potent in vitro accessory cells are perhaps most critical in support of the notion that DC are the principal physiological in vivo accessory cells involved in the induction of immunity. As outlined above, in in vitro T lymphocyte responses such as oxidative mitogenesis, as well as in most other in vitro model systems, peripheral lymphoid DC from experimental laboratory animals do act as potent accessory cells. However, in humans, although a comparable role has been demonstrated for some non-lymphoid DC types, most notably peripheral blood DC, the function of peripheral lymphoid DC as accessory cells has not previously been investigated.

Using tonsillar DC as an example of human peripheral lymphoid DC the main findings of this Chapter now clarify this issue. Firstly, tonsillar DC were shown to act as accessory cells, not only in the oxidative mitogenesis reaction but also in the auto and allo MLRs. Secondly, in each of these responses, tonsillar DC were potent accessory cells when compared with tonsillar macrophages and low density T and B lymphocytes. Together, these findings add weight to the concept that in humans, as in experimental laboratory animals, peripheral lymphoid DC are significant if not the major in vivo physiological accessory cells involved in the induction of the immunity.

The Chemistry of periodate oxidative mitogenesis. Previously, it has been determined that low temperature (0-5^{o}C), low periodate concentration (1-4 mM) and short periodate incubation times (15-30 min) are the optimal conditions favouring maximal proliferation in periodate oxidative mitogenesis (456,464,465). In this study, too, under conditions of constant

low temperature and short incubation time, pre-incubation of the T lymphocytes with low concentrations of periodate, i.e. 2 mM, gave the greatest proliferative responses. Within these limits the action of periodate on cell surfaces is confined to the oxidation of vicinal hydroxyl groups leading to the formation of cell surface aldehydes (466) and that these aldehydes play an important role in the induction of responses is confirmed by the finding that all of the aldehyde group reactive reagents sodium borohydride (452,467), hydroxylamine (468) and cysteine methyl ester (469), dimedone (470), semicarbazide (468) and thiocarbohydrazide (470) inhibit mitogenesis if reacted with lymphocytes immediately following their oxidation.

Aldehyde reactive reagents have also been used to determine which cell surface molecules mediate periodate induced oxidative mitogenesis. In this regard, reaction of oxidised cells with tritiated sodium borohydride enables the identification of aldehyde containing cell surface molecules as a series of different molecular weight bands. If, however, the cells are treated with the proteolytic enzymes papain, trypsin, chymotrypsin or thermolysin (after labelling), all of which inhibit oxidative mitogenesis, then a common feature is the removal of four high molecular weight glycoprotein bands of 175, 170, 160 and 155 KD and thus any or all of these molecules most likely mediate periodate oxidative mitogenesis (471,472).

Upon these glycoproteins the most favoured functionally relevant target site for periodate oxidation is sialic acid. This contention is supported by the fact that pre-treatment of cells with neuraminidase (before periodate treatment) blocks subsequent mitogenesis (451,468) and also that, in contrast to galactose, mannose, N-acetyl glucosamine, N-acetyl galactosamine, fucose and amino acids, periodate treatment of cells alters the electrophoretic mobility of this sugar (452). Under conditions that are

optimal for oxidative mitogenesis the action of periodate upon sialic acid is shown in Fig. 3.12.

The role of Schiff bases in periodate oxidative mitogenesis: Relationship of the dendritic cell induced oxidative mitogenesis reaction to the dendritic cell induced auto and allo MLRs. The precise role played by aldehydes in periodate oxidative mitogenesis is unknown. However, given that proliferative responses occur if either the T lymphocytes or accessory cells or both are oxidised (460,467), aldehydes probably play a non-specific role in this reaction. Along these lines, a much favoured suggestion is that newly formed cell surface aldehydes rapidly react with cell surface amino groups to form accessory cell-T lymphocyte intercellular Schiff base bridges which are instrumental in the induction of responses (473). Evidence in favour of this comes from the fact that after several hours culture of oxidised cells aldehyde group reactive reagents such as thiocarbohydrazide and dimedone no longer block proliferation (470).

An obvious way in which these Schiff base intercellular bridges could function in oxidative mitogenesis is by allowing for both the adhesion of increased numbers of antigen specific (specific for whichever antigens are recognised in the auto MLR - i.e. endogenous but foreign host antigens and/or foreign antigens present in culture media additives - see page 42) T lymphocytes to accessory cells and a "tightening" of such specific T lymphocyte-accessory cell adhesion, manifest as an increased area of T lymphocyte-accessory cell contact. In fact, a long recognised feature of periodate is it's ability to promote cell-cell adhesion (473). This would result in an accelerated and augmented auto MLR which would be in accord with both the kinetics of the tonsillar DC induced oxidative mitogenesis reaction and auto MLR (as well as allo MLR - another likely antigen specific response - see page 43) reported here and the kinetics of the oxidative

mitogenesis reaction and auto MLR in unpurified populations of lymphocytes reported elsewhere (464,473,474).

In addition to this, however, Schiff base bridges might also "tighten" the adhesion between antigen non-specific T lymphocytes and accessory cells. If this were to occur then these T lymphocytes may also be recruited into cell cycle, following the interaction of their TcR with MHC plus irrelevant antigen (normally, such an interaction would not lead to response owing to both it's extremely low affinity and a limiting degree of accessory cell-T lymphocyte contact). Indeed, this might explain the apparent polyclonal nature of proliferative responses in oxidative mitogenesis for although it has been shown that in oxidative mitogenesis fewer T lymphocytes are recruited into cell cycle than in PHA or Con A mitogenesis (449,473) it is widely accepted that this reaction is more than just an antigen specific response.

If in fact, as postulated, Schiff bases do play an important role in oxidative mitogenesis then there would appear to be a requirement for their lability for full proliferative responses. Thus, aldehyde group reactive reagents such as sodium borohydride and hydroxylamine, both of which reduce Schiff bases and render them more stable (475), inhibit periodate oxidative mitogenesis even when reacted with cells 24 hr after the oxidation step (470).

Summary

To summarise, in this Chapter, isolated human tonsillar DC have been shown to act as accessory cells for T lymphocyte proliferation in each of the periodate oxidative mitogenesis reaction, the auto MLR and allo MLR. Further, in comparison with other tonsillar accessory cell types, potency of accessory cell activity in all of these responses was demonstrable. The kinetics of the three DC induced responses was also examined. Peak T lymphocyte proliferative responses in the DC induced oxidative mitogenesis reaction occurred earlier and were greater than peak proliferative responses in the DC induced auto and allo MLRs. This pattern is consistent with the speculative view that the oxidative mitogenesis reaction is at least in part a Schiff base mediated accelerated and augmented auto MLR.

PREFACE TO CHAPTERS 4-8

As outlined in "Aim of studies", the main purpose of investigations described in Chapters 4-8 has been to elucidate the molecular mechanisms of human tonsillar DC induced T lymphocyte proliferation. For reasons of simplicity of methodology, greater magnitude of response (over MLRs for example) and short time course of the assay (see Chapter 3) the tonsillar DC induced periodate oxidative mitogenesis model of T lymphocyte proliferation has been used for this purpose.

The principal tools of investigation that have been used are antibodies. In this respect, in each Chapter, panels of antibodies have been screened for their effects upon two different aspects of the response. Firstly, upon proliferation itself, thus enabling the identification of molecules that play a role in this reaction. Secondly, upon tonsillar DC-T lymphocyte clustering, thus enabling the categorisation of implicated molecules into those that are involved in this clustering and those that are involved in signal transduction.

In addition to this, at selected points, the effects upon proliferation of adding antibodies at different times to the tonsillar DC induced oxidative mitogenesis reaction has been examined. These experiments have been performed with a view to determining at which time point in the cellular interaction various molecules perform their role. Further, to determine whether or not there are any qualitative differences between the tonsillar DC induced oxidative mitogenesis reaction, the tonsillar DC induced induced auto and allo MLRs and the tonsillar macrophage, and B lymphocyte induced oxidative mitogenesis reactions (with respect to the types of molecules involved), some antibodies have also been tested for their effects upon proliferation in these other reactions.

CHAPTER 4

EFFECT OF MHC ANTIBODIES UPON TONSILLAR DENDRITIC CELL-T LYMPHOCYTE INTERACTION

On theoretical grounds, MHC molecules might be expected to perform multiple different roles in DC induced T lymphocyte responses. For example, there is the presentation of antigen, which in a signal transduction context might be involved in the delivery of both "first and second signals", and in a clustering context, might mediate DC-T lymphocyte adhesion either directly or indirectly via increases in LFA-1 avidity.

Indeed, class II MHC antibodies profoundly inhibit mouse splenic and human peripheral blood DC induced T lymphocyte responses, thus confirming the central role of class II MHC (see page 76). However, in the mouse splenic DC induced primary allo MLR, class II MHC antibodies have no effect upon DC-T lymphocyte clustering, suggesting a signal transduction role for class II MHC (see page 72); and this is in contrast to the secondary allo MLR induced by these cells where antibody inhibition as well as other studies have suggested that class II MHC performs at least a clustering role (see page 73).

In view of these findings, in this Chapter, it has been particularly interesting to examine the effects of MHC antibodies (class I and class II MHC) upon both proliferation and clustering in tonsillar DC induced oxidative mitogenesis.

MATERIALS AND METHODS

Cells. DC, high density T lymphocytes and low density B lymphocytes were isolated from human tonsils as described in Chapter 2.

Proliferation assays. Oxidative mitogenesis assays (2 mM periodate was employed throughout), auto and allo MLRs were performed as indicated in Chapter 3 using varying numbers per well of irradiated DC or low density B lymphocytes as accessory cells and 2 X 10^5 per well of high density T lymphocytes as responder cells. In addition, MHC antibodies were added to accessory cell plus responder T lymphocyte wells (either at culture initiation or at various time points thereafter) in varying concentrations in 20 ul quantities. Culture in oxidative mitogenesis assays was for a total of 64 hr and in MLRs for a total of 96 hr. In all proliferation assays ^{125}IdUrd was added to wells 16 hr before culture termination.

Assessment of cellular clustering. The extent of cellular clustering (with regards size and number of clusters) that occurred when DC were cultured with unmodified or 2mM periodate modified autologous high density T lymphocytes [in the presence (added at culture initiation) and absence of MHC antibodies], or when either of these T lymphocyte populations were cultured alone, was assessed visually on an inverted phase contrast microscope at 4 and 12 hr following culture initiation. Often, these cultures were the same cultures used for the assessment of proliferation in oxidative mitogenesis assays and auto MLRs. Sometimes, however, separate cultures were set up, the conditions of which were exactly the same as for proliferation cultures only proliferation was not subsequently examined.

Photography. Clusters were photographed on an automatic setting using Panatomic X black and white ASA film (down rated to 16 ASA).

Antibodies. A list and details of the MHC antibodies used in proliferation and cluster assays is shown in Table 2.2. All were sterile filtered and if required were dialysed (four to six changes of PBS over 3-4 days for antibodies containing NaN_3) and/or deaggregated (7000 X g/30 min for antibody solutions with visible precipitates) before use.

Statistics. See Chapter 3

In addition, use is made of the expression percentage inhibition of mean proliferation (%IMP) caused by an antibody, where %IMP is as follows:

$$\frac{\text{(mean DC+T CPM) - (mean DC+T+antibody CPM)}}{\text{(mean DC+T CPM) - (mean T alone CPM)}} \times 100$$

Also, where indicated inhibitory or enhancing effects of antibodies were analysed statistically by comparing DC+T triplicates in a one sided two sample t-test. Assumptions of this test are that data are drawn randomly from normally distributed populations with equal variances. Random selection of data and normal distribution of populations were assumed. Equality of variances was tested in a one sided F-test. Where evidence was obtained that population variances were unequal an alternative one sided two sample t-test was employed which uses the test statistic t, where

$$t = \frac{x(1) - x(2)}{\sqrt{\frac{s^2(1)}{n(1)-1} + \frac{s^2(2)}{n(2)-1}}}$$

which under the null hypothesis is distributed as overleaf

$$t \left[\frac{s^2(1)}{n(1)-1}\right]^2 \left[\frac{1}{n(1)+1}\right] + \left[\frac{s^2(2)}{n(2)-1}\right]^2 \left[\frac{1}{n(2)+1}\right]^{-2} \left[\left[\frac{s^2(1)}{n(1)-1}\right] + \left[\frac{s^2(2)}{n(2)-1}\right]\right]^2$$

In all tests the significance level was taken as $p<0.05$.

RESULTS

Effect of a class I MHC antibody upon dendritic cell induced oxidative mitogenesis. Fig. 4.1 shows the effect of the class I MHC antibody, W6/32, upon T lymphocyte proliferation in DC induced oxidative mitogenesis. When incorporated into this reaction from the outset W6/32 strongly inhibited the proliferative response.

The inhibitory activity of W6/32 was investigated further by titrating this antibody into the reaction. Fig. 4.2 depicts the results of two different experiments which show that inhibition of T lymphocyte proliferation by W6/32 was dose dependent.

Effect of class II MHC antibodies upon dendritic cell induced oxidative mitogenesis. The effect of class II MHC antibodies upon T lymphocyte proliferation in DC induced oxidative mitogenesis was initially examined using the Hig-78 antibody. When incorporated into cultures from the outset Hig-78 inhibited T lymphocyte proliferation (Fig. 4.3), an effect which was dependent upon the concentration of added antibody as shown in three different experiments (Fig. 4.4).

In further experiments, other class II MHC antibodies in addition to Hig-78, were screened for their effects upon DC induced oxidative mitogenesis. Fig. 4.5 shows the results of two different experiments in which the effect of Hig-78 was compared with that of DA6.231 and DA6.164 antibodies. In both experiments Hig-78 inhibited proliferation. However, neither DA6.231 nor DA6.164 significantly affected the response.

Figs. 4.6 and 4.7 show the results of two more experiments in which extended panels of antibodies were used. In the first experiment (Fig. 4.6) antibodies Hig-78, Tu22, Tu35, Tu36, Tu39 and L243 inhibited proliferation in contrast to antibodies DA6.231, DA6.164 and 1a3 which did not affect

proliferation, thus confirming (Figs. 4.3-4.5) and extending previous observations. In the second experiment (Fig. 4.7) a similar panel of antibodies used in first experiment was titrated into the assay. Again Tu22, Tu35, Tu36 and Tu39 inhibited proliferation and these effects were dose dependent. However, DA6.231, DA6.164 and 1a3 did not affect proliferation at any of the tested antibody concentrations.

Effect of class I and class II MHC antibodies upon the dendritic cell induced auto and allo MLRs. To examine whether or not MHC antibodies could inhibit DC induced T lymphocyte proliferation in other model systems, the class I MHC antibody, W6/32, and the class II MHC antibody, Hig-78, were incorporated into the DC induced auto and allo MLRs. Fig. 4.8 shows that both of these antibodies inhibited the DC induced auto MLR. Similarly, both antibodies inhibited proliferation in the DC induced allo MLR and in this latter reaction this effect was shown to be dependent upon the concentration of added antibody (Fig. 4.9).

Effect of class I and class II MHC antibodies upon the low density B lymphocyte induced oxidative mitogenesis reaction. The effect of MHC antibodies upon the low density B lymphocyte induced oxidative mitogenesis reaction was also tested. Fig. 4.10 shows that the class I MHC antibody, W6/32, inhibited T lymphocyte proliferation in the B lymphocyte induced oxidative mitogenesis reaction in a dose dependent fashion and Fig. 4.11 shows that the class II MHC antibody, Hig-78, was also inhibitory in this response. Fig. 4.12 shows the results of a single experiment in which the effect of an extended panel of class II MHC antibodies upon the low density B lymphocyte induced oxidative mitogenesis reaction (a) was compared with the effect of this same panel of antibodies upon the DC induced oxidative mitogenesis reaction (b). For both types of reaction the pattern of

inhibition was the same i.e. antibodies Hig-78, Tu22, Tu35, Tu36, Tu39 and 1a3 significantly inhibited proliferation in contrast to antibodies DA6.231 and DA6.164 which did not affect proliferation.

Effect of class II MHC antibodies upon the dendritic cell and B lymphocyte induced oxidative mitogenesis reactions when added at different times. Figs. 4.13 and 4.14 show the results of two different experiments in which the class II MHC antibodies, Tu39 and Hig-78, were incorporated into the DC and B lymphocyte induced oxidative mitogenesis reactions at different times. In the DC induced reaction (Fig. 4.13), Tu39 inhibited proliferation at all time points of antibody addition (from 0 to 48 hr following culture initiation) although significantly less inhibition of proliferation was observed at the 48 hr time point in comparison with earlier time points. Likewise, in the B lymphocyte induced reaction (Fig. 4.14) both Tu39 and Hig-78 antibodies inhibited proliferation when added at all time points up to and including 48 hr and in this response the degree of inhibition was similar irrespective of the time of antibody addition.

Effect of class I and class II MHC antibodies upon dendritic cell-T lymphocyte clustering in dendritic cell induced oxidative mitogenesis. To test the effects of MHC antibodies upon the DC-T lymphocyte clustering process that occurs in the DC induced oxidative mitogenesis reaction, antibodies were added to cultures of DC plus periodate treated autologous T lymphocytes at culture initiation and their effects upon clustering were assessed visually. Fig. 4.15 shows the results of an initial experiment in which the effects of the class I MHC antibody, W6/32, and the class II MHC antibody, Hig-78, were examined. Panels (a) and (b) show the morphology of DC-T lymphocyte clusters in the absence of antibodies and of T lymphocytes alone (in which little endogenous clustering occurs) respectively at 4 hr.

Panels (c) and (d) show the morphology of DC-T lymphocyte clusters in the presence of W6/32 (c) and Hig-78 (d) at this same time point. Neither of these antibodies affected the size of DC-T lymphocyte clusters at 4 hr. The effect of these antibodies was also examined 12 hr after culture initiation. The results are depicted in panels (e-g) which show that neither of these antibodies affected the DC-T lymphocyte clustering process at 12 hr.

The effect of class II MHC antibodies upon DC-periodate treated T lymphocyte clustering was explored in more detail using an extended panel of antibodies. Fig. 4.16 shows that none of the antibodies Hig-78, DA6.231, DA6.164, Tu22, Tu35, Tu36 or Tu39 affected DC-T lymphocyte clustering when assessed at 12 hr. Fig 4.17 shows the results of another experiment in which an extended panel was examined and for which the 64 hr proliferative response was also assayed (Fig. 4.6). After 12 hr culture in this response none of the antibodies Hig-78, DA6.231, DA6.164, Tu22, Tu35, Tu36, Tu39, 1a3 or L243 affected DC-T lymphocyte clustering. Many similar experiments using class II MHC antibodies have been performed. In each of these experiments class II MHC antibodies did not affect DC-T lymphocyte clustering (not shown).

For comparative purposes, the effect of the W6/32 and Hig-78 antibodies upon the DC-T lymphocyte clustering process that occurs in the DC induced auto MLR was also examined. For this, antibodies were incorporated into cultures of DC and unmodified T lymphocytes and their effects upon clustering were again assessed visually. Fig. 4.18 shows that neither W6/32 nor Hig-78 affected DC-T lymphocyte clustering in the auto MLR when assessed at both 4 and 12 hr.

Fig. 4.1

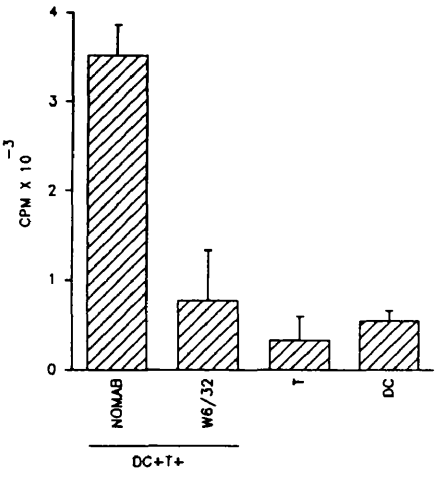


Fig. 4.2(a)

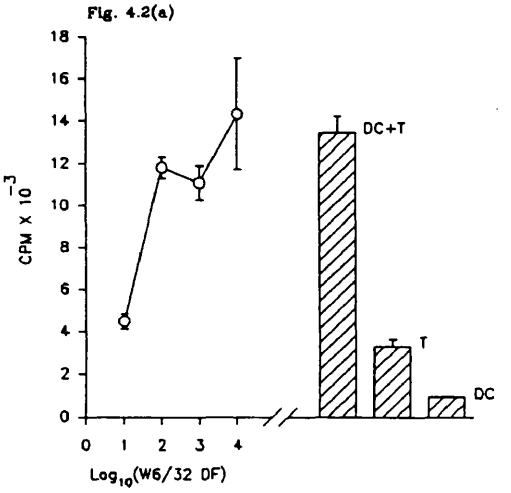


Fig. 4.2(b)

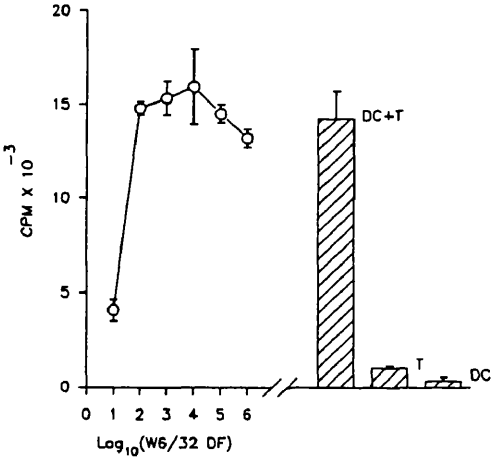


Fig. 4.3

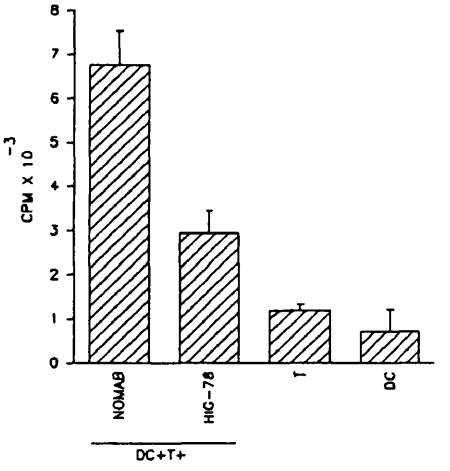


Fig. 4.4(a)

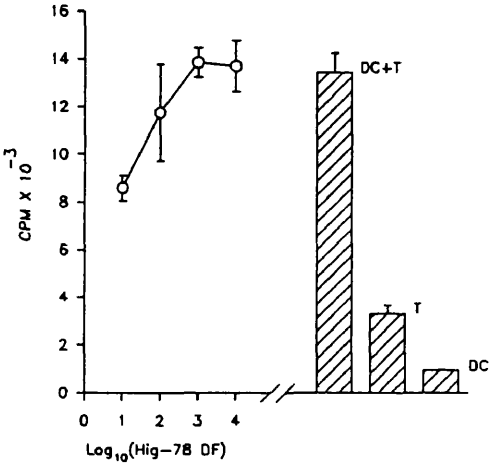


Fig. 4.4(b)

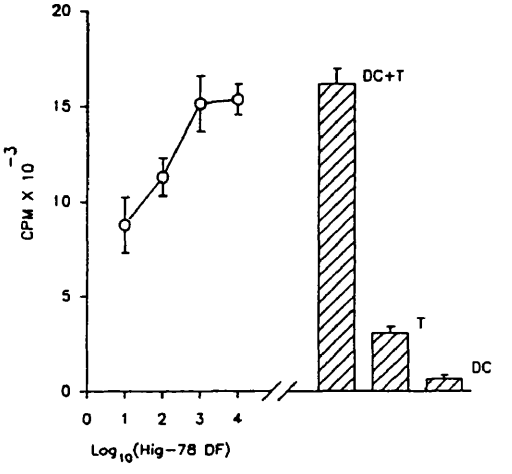
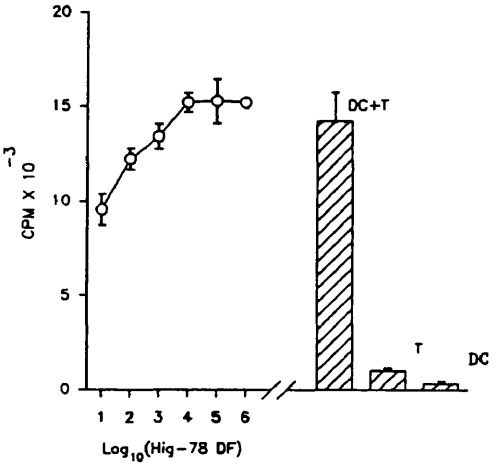


Fig. 4.4(c)



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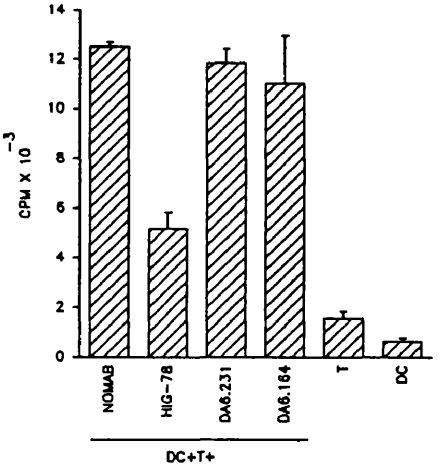

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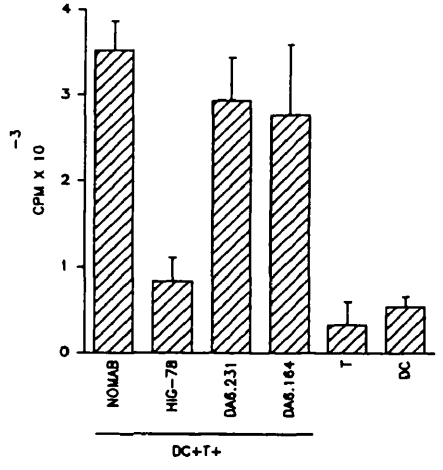

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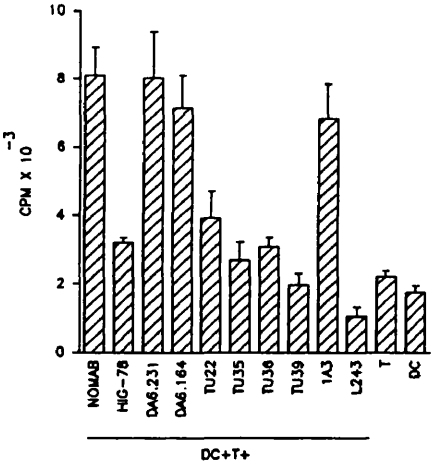

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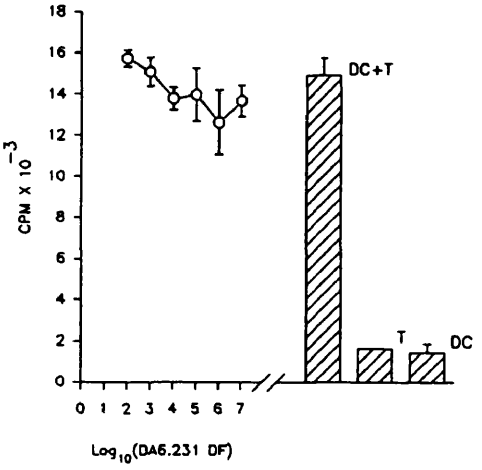

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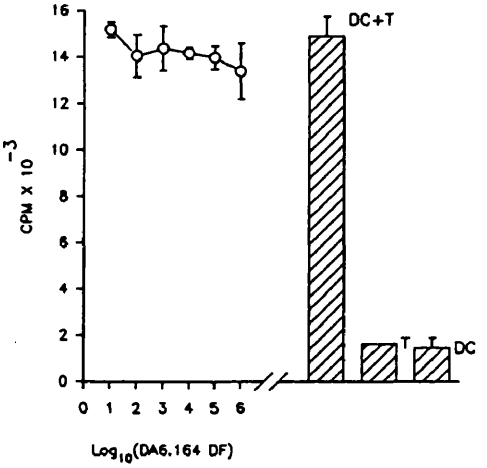

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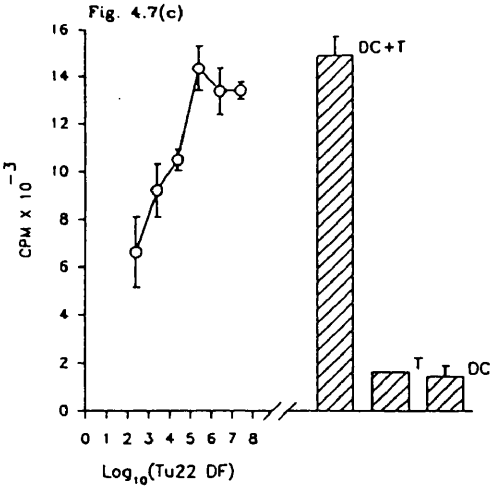

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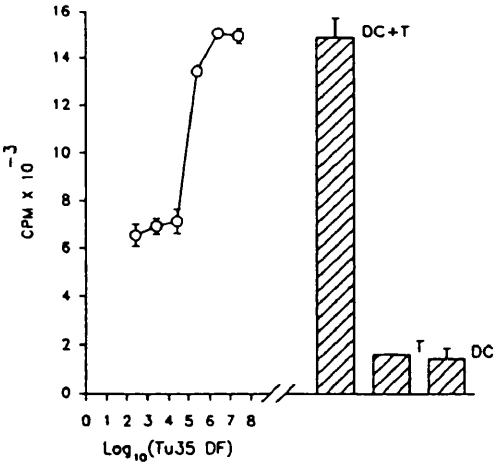

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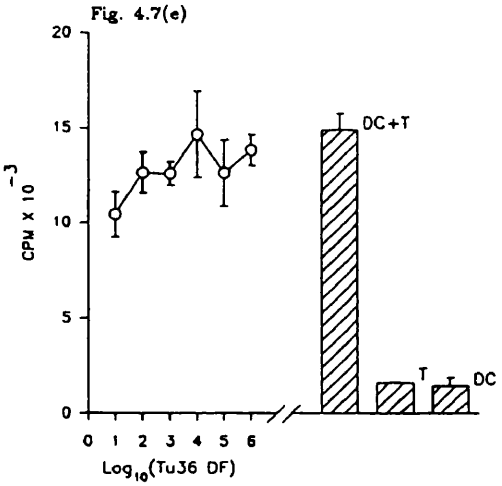

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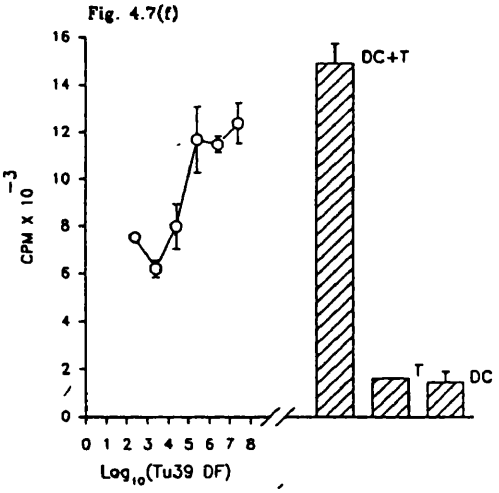

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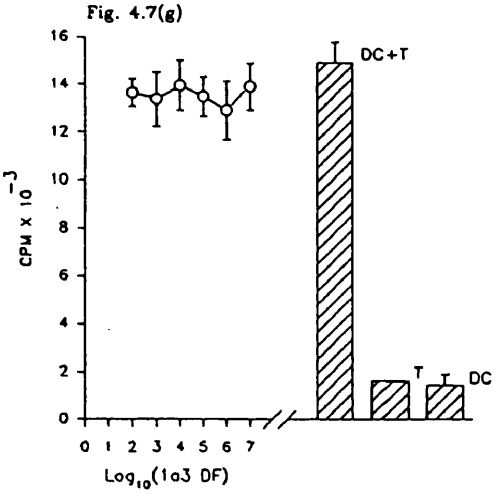
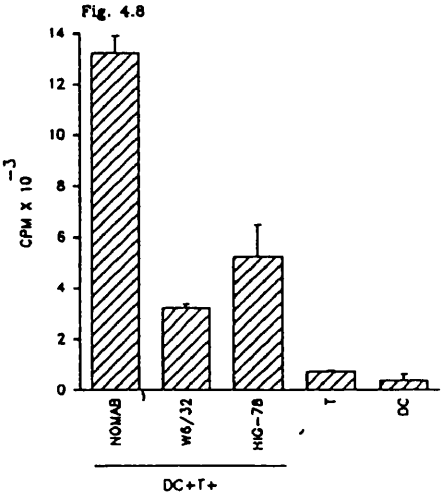


Fig. 4.8



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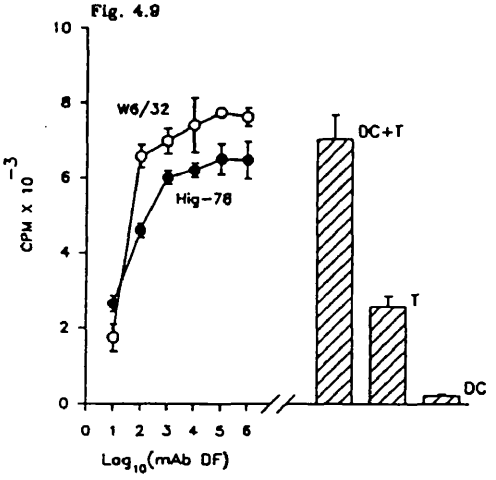


Fig. 4.10

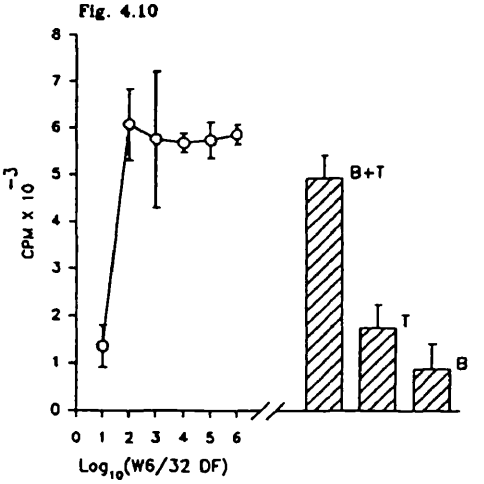
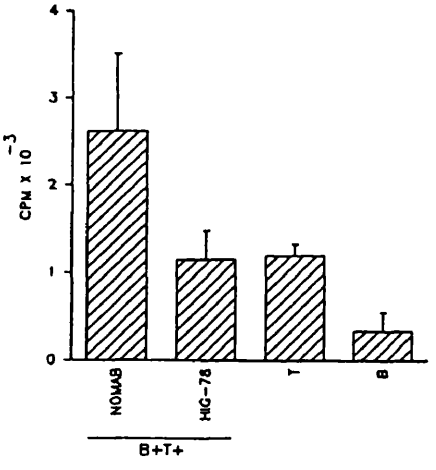


Fig. 4.11



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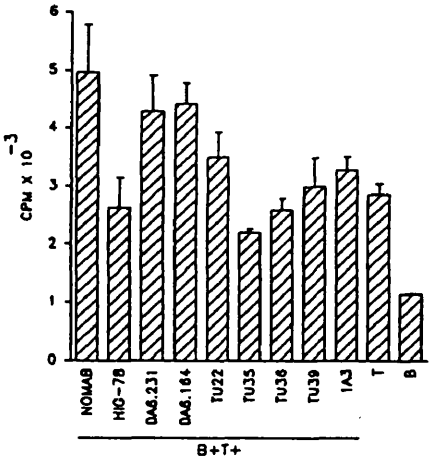

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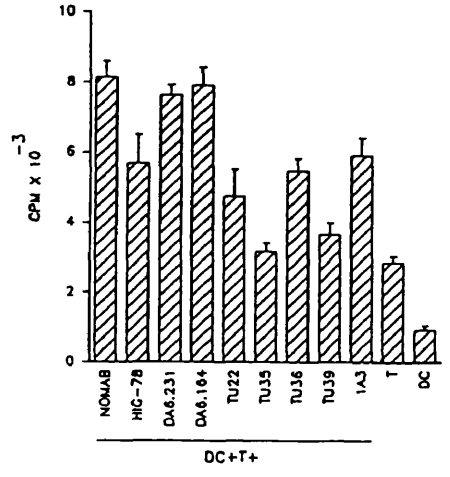

Fig. 4.13

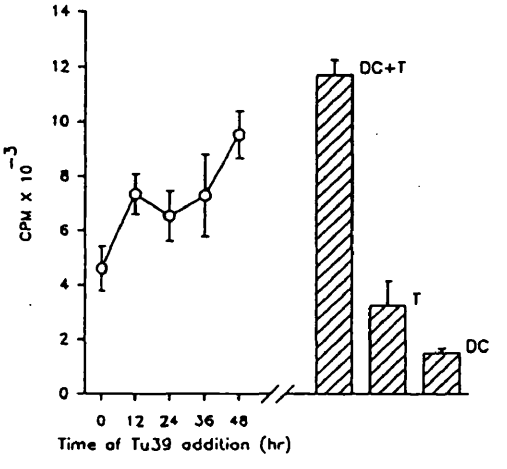

Fig. 4.14

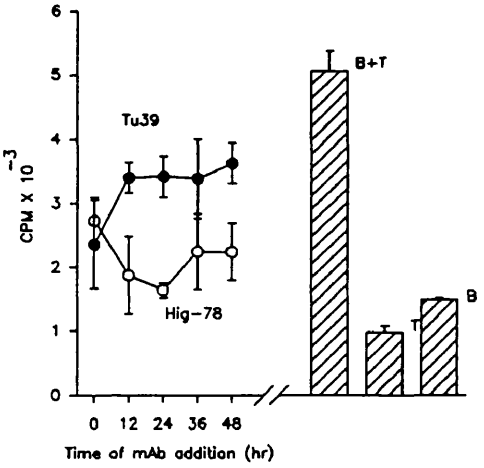
6
5
4
3
2
1
0
CPM X 10^-3
Tu39
Hig-78
B+T
T
B
0 12 24 36 48
Time of mAb addition (hr)

Fig. 4.15

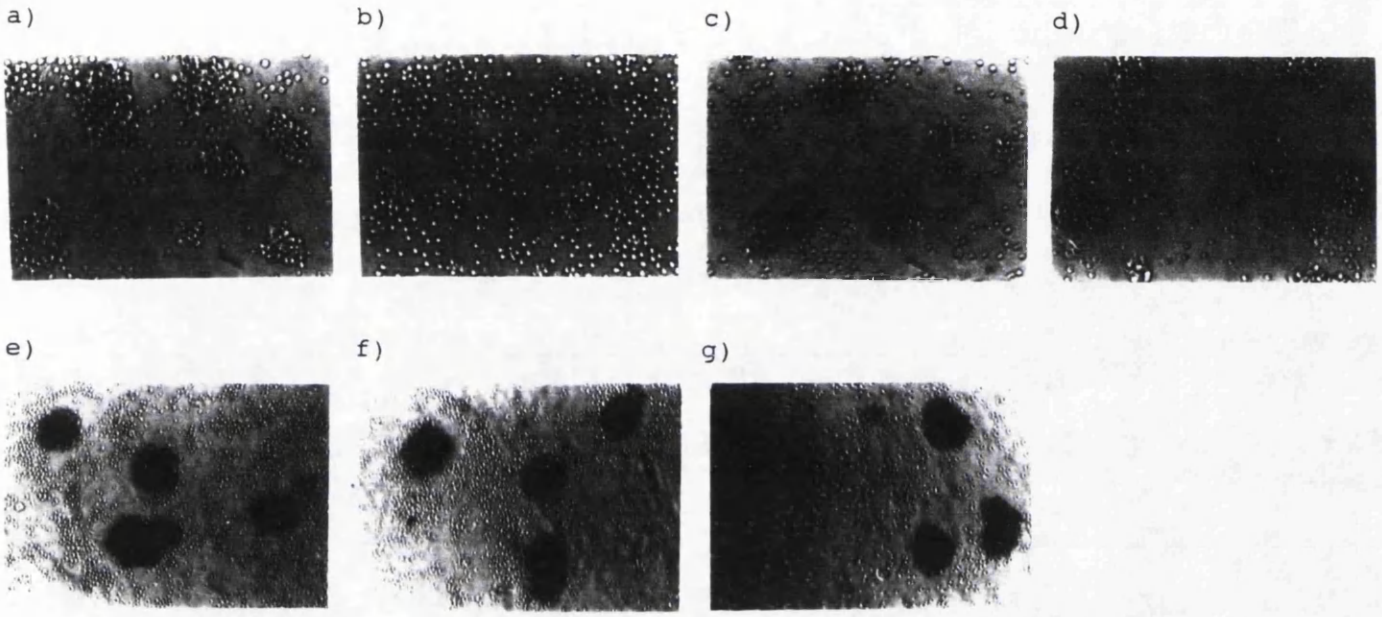

Fig. 4.16

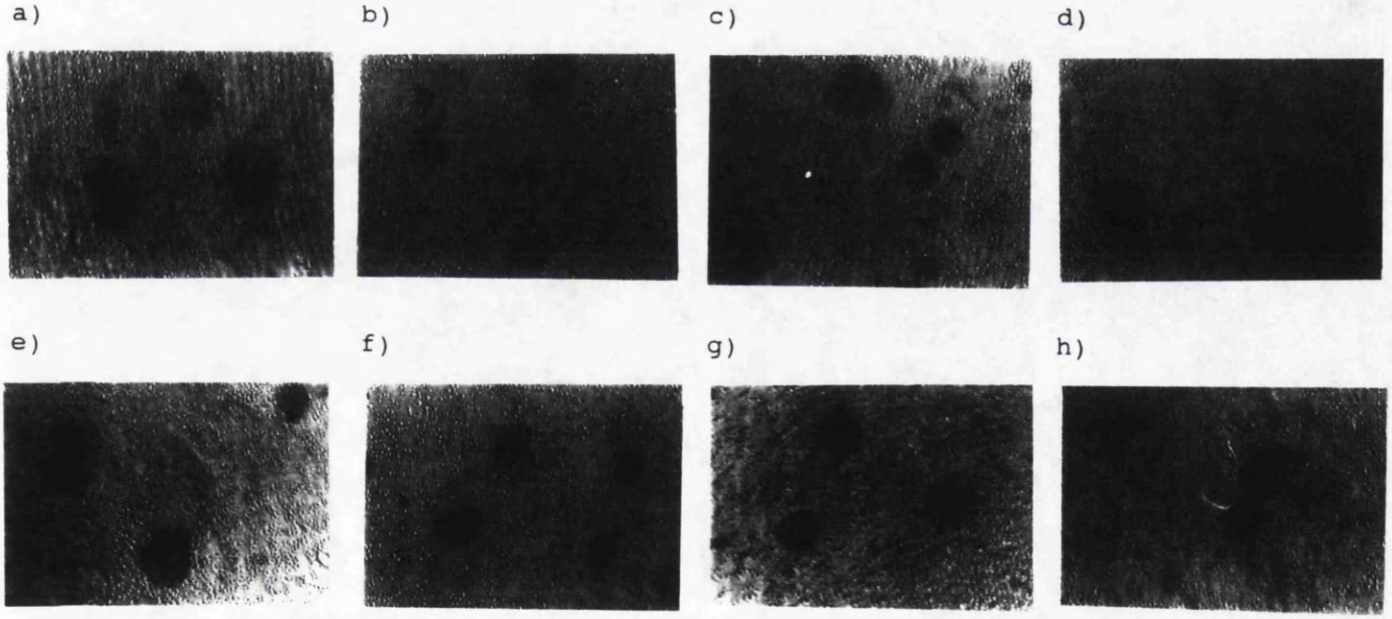

Fig. 4.17

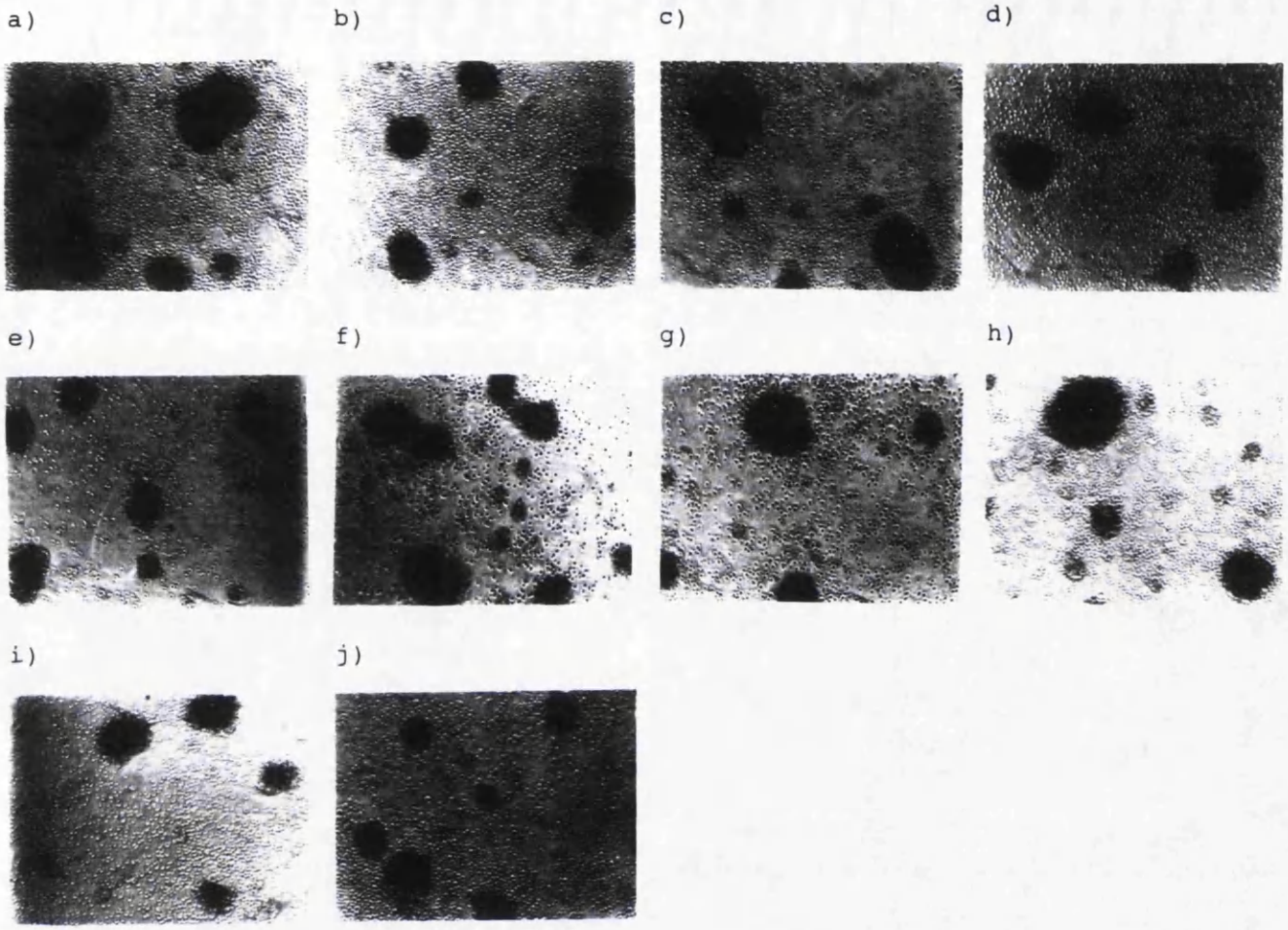

Fig. 4.18

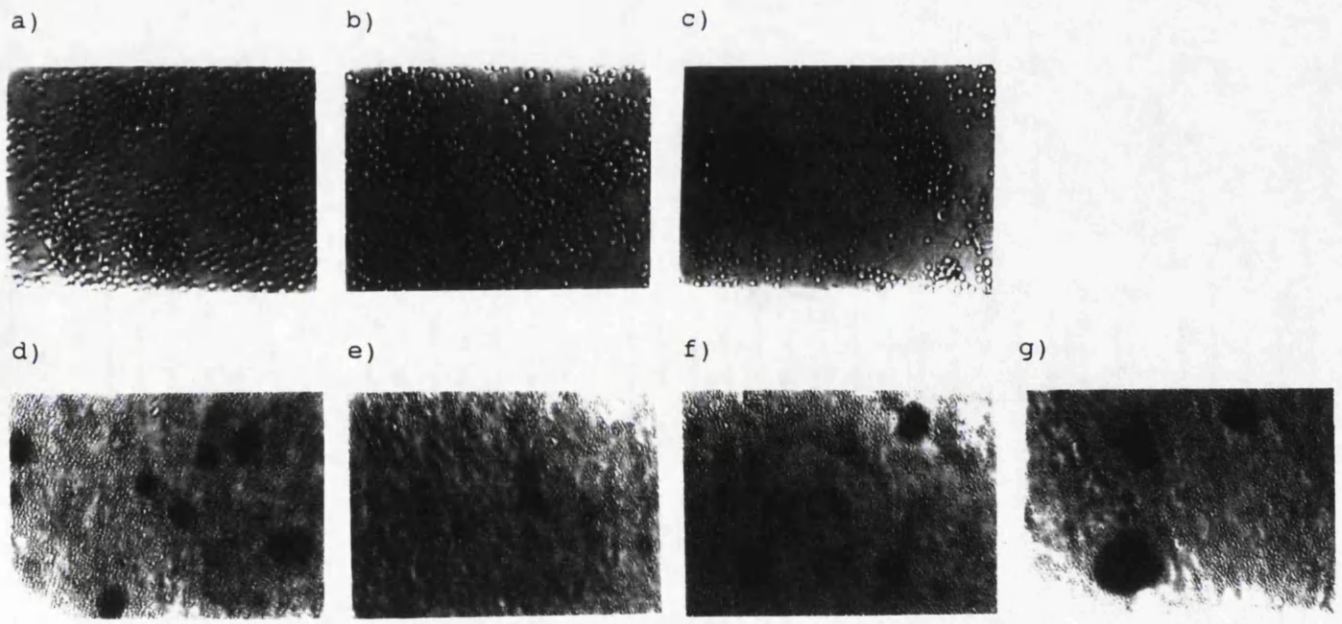

Figure Legends

See Materials and Methods plus:

Fig. 4.1. DC induced oxidative mitogenesis. DC number - 5 X 10^4/well, W6/32 dilution factor (DF) - 10.

Fig. 4.2(a). DC induced oxidative mitogenesis. DC number - 5 X 10^4/well.

Fig. 4.2(b). DC induced oxidative mitogenesis. DC number - 1 X 10^5/well.

Fig. 4.3. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, Hig-78 DF - 10.

Fig. 4.4(a,b). DC induced oxidative mitogenesis. DC number - 5 X 10^4/well.

Fig. 4.4(c). DC induced oxidative mitogenesis. DC number - 1 X 10^5/well.

Fig. 4.5(a). DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, all mAb DF - 10 (DA6.231 - CS), Hig-78 inhibition is statistically significant (SS), DA6.231 and DA6.164 inhibition is not SS.

Fig. 4.5(b). DC induced oxidative mitogenesis. DC number - 5 X 10^4/well, all mAb DF - 10 (DA6.231 - CS), Hig-78 inhibition is SS, DA6.231 and DA6.164 inhibition is not SS.

Fig. 4.6. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well; Hig-78, DA6.164 and Tu36 DF - 10, DA6.231 (AF) and 1a3 DF - 10^2, Tu22, Tu35 and Tu39 DF - 2.5 X 10^2; L243 DF - 4 X 10^2; Hig-78, Tu22, Tu35, Tu36, Tu39 and L243 inhibitions are SS; DA6.231 and DA6.164 and 1a3 inhibitions are not SS.

Fig. 4.7(a-g). DC induced oxidative mitogenesis. DC number - 1 X 10^5/well; DA6.231 - AF; Tu22, Tu35, Tu36 and Tu39 inhibitions, at higher or all antibody concentrations, are SS; DA6.231, DA6.164 and 1a3 inhibitions (or enhancements), at all antibody concentrations, are not SS.

Fig. 4.8. DC induced auto MLR. DC number - 1 X 10^5/well, W6/32 and Hig-78 DF - 10.

Fig. 4.9. DC induced allo MLR. DC number - 1 X 10^5/well.

Fig. 4.10. B lymphocyte induced oxidative mitogenesis. B lymphocyte number - 1 X 10^5/well.

Fig. 4.11. B lymphocyte induced oxidative mitogenesis. B lymphocyte number - 1 X 10^5/well, Hig-78 DF 10.

Fig. 4.12(a,b). DC and B lymphocyte induced oxidative mitogenesis. DC and B lymphocyte number - 1 X 10^5/well; Hig-78, DA6.164 and Tu36 DF - 10; DA6.231 (AF) and 1a3 DF - 10^2, Tu22, Tu35 and Tu39 DF - 2.5 X 10^2; Hig-78, Tu22, Tu35, Tu36, Tu39 and 1a3 inhibitions are SS; DA6.231 and DA6.164 inhibitions are not SS

Fig. 4.13. DC induced oxidative mitogenesis. DC number - 5 X 10^4/well, Tu39 DF - 3.75 X 10^2.

Fig. 4.14. B lymphocyte induced oxidative mitogenesis. B lymphocyte number - 5 X 10^4/well, Hig-78 DF - 10, Tu39 DF - 3.75 X 10^2.

Figs. 4.15-4.18 - DC-T lymphocyte clustering

Fig. 4.15. Oxidative mitogenesis. (a-d) photographed at 4 hr, (e-f) photographed at 12 hr, (a,e) - DC+T, (b) - T alone, (e,f) - DC+T+W6/32, (d,g) - DC+T+Hig-78.

Fig. 4.16. Oxidative mitogenesis. All photographed at 12 hr, all DC+T plus (a) NomAb, (b) Hig-78, (c) DA6.231, (d) DA6.164, (e) Tu22, (f) Tu35, (g) Tu36, (h) Tu39.

Fig. 4.17. Oxidative mitogenesis. All photographed at 12 hr, all DC+T plus (a) NomAb, (b) Hig-78, (c) DA6.231, (d) DA6.164, (e) Tu22, (f) Tu35, (g) Tu36, (h) Tu39, (i) 1a3, (j) L243.

Fig. 4.18. Auto MLR. (a-c) photographed at 4 hr, (d-g) photographed at 12 hr, (a,d) DC+T, (e) T alone, (b,f) DC+T+W6/32, (c,g) DC+T+Hig-78.

Discussion

MHC molecules as signal transducers in tonsillar dendritic cell induced oxidative mitogenesis. One of the main findings in this Chapter is that class I and class II MHC antibodies inhibit T lymphocyte proliferation in the tonsillar DC induced oxidative mitogenesis reaction. Likewise, MHC antibodies inhibit proliferation in the tonsillar DC induced auto and allo MLRs and in the low density tonsillar B lymphocyte induced oxidative mitogenesis reaction. These results suggest that in tonsillar DC induced oxidative mitogenesis, as well as in these other types of response, MHC molecules play an important role in the induction of proliferation.

Another major finding of this Chapter is that in the tonsillar DC induced oxidative mitogenesis reaction neither class I or class II MHC antibodies have any effect upon the formation of DC-T lymphocyte clusters. This suggests that the role of MHC molecules in this response is confined to that of signal transduction.

The role of class I MHC. One problem with the antibody technique of assigning different roles to different molecules in a cell-cell interaction such as tonsillar DC induced oxidative mitogenesis is that of the epitopic specificity of antibodies. Although it can be argued that phenomena such as steric hindrance, induction of conformational changes and cell surface down regulation would allow an assessment of the involvement of several or all epitopes at once of a target molecule, for some antibodies this might not apply. A case in hand is the W6/32 antibody which has been used to examine the role of class I MHC in these studies. As evidenced by it's ability to inhibit class I MHC/CD8 antigen binding in adhesion assays using transfected cells and in cytotoxicity assays using CD8 antigen dependent alloreactive CTL clones (399,476) the W6/32 antibody is known to bind (or at least

functionally block) a region of class I MHC involved in an interaction with the CD8 antigen and therefore one candidate signalling role of class I MHC in tonsillar DC induced oxidative mitogenesis involves such an interaction. However, as shown by an inability of W6/32 to inhibit the cytolytic function of CD8 antigen independent alloreactive CTL clones (476) this antibody is known not to block an interaction of class I MHC with the TcR and, hence, the conclusion that class I MHC functions only in signal transduction in tonsillar DC induced oxidative mitogenesis might be premature.

This reservation not withstanding, in it's signal transduction role via an interaction with CD8 antigen, class I MHC might participate in the delivery of "first signals" to CTL by serving to activate and cross-link the CD8 antigen to the TcR complex on these cells. Thus, CD3 and CD8 antibodies co-immobilised to the same plastic surface, are far more effective inducers of purified CTL proliferation than immobilised CD3 antibodies alone (477,478). Further, in order for CD8 antigen dependent alloreactive CTL to lyse targets simultaneous binding of the CTL's TcR and CD8 antigen to the same target cell class I MHC molecule must occur (476). Indeed, in agreement with observations here, it is now almost well accepted that at physiological levels of class I MHC and CD8 antigen expression the role of a class I MHC-CD8 antigen interaction is exclusively in signal transduction and contributes little to accessory cell-CTL or target cell-CTL adhesion (403,479).

Two other findings in this thesis, however, cast doubt upon this model of the involvement of class I MHC in tonsillar DC induced oxidative mitogenesis. Firstly, CD8 antibodies do not affect T lymphocyte proliferation in this reaction (see Chapter 6). Secondly, in this reaction and in the other responses studied in this Chapter, W6/32 caused at least 75% IMP suggesting additional functions for the W6/32 epitope. One such

function could relate to the finding that the W6/32 antibody induces the release of a soluble suppressor factor from monocytes which inhibits T lymphocyte proliferation in PHA mitogenesis (480). Thus, class I MHC might also function as an accessory cell surface receptor in a pathway in which CD8 antigen acts as a ligand and whose role is to down regulate T lymphocyte activation via release of this suppressor factor. In this hypothesis, W6/32 may mimic this role of CD8 antigen resulting in the inhibition of proliferation of CD4 antigen expressing T lymphocytes as well as of CD8 antigen expressing CTL.

Of course, class I MHC might perform an additional function at the T lymphocyte rather than the accessory cell level. For example, class I MHC has been shown to associate with the CD25 antigen and the TcR upon the surface of T lymphocytes (481-483). These interactions might be functionally important and could be blocked by W6/32.

The role of class II MHC. Less is known of the ability of any of the class II MHC antibodies used here to block functionally relevant sites upon class II MHC and further there is uncertainty concerning the exact specificity of some of these antibodies (see Table 2.2). Thus, as with class I MHC, the conclusion that class II MHC is involved only in signal transduction is a tentative one and the precise signal transduction role performed by this molecule(s) difficult to decide.

Despite this, an obvious way in which class II MHC could function in signal transduction is in the presentation of antigen, i.e. delivery of "first signals", to CD4 antigen expressing T lymphocytes. In addition, an implicated class II MHC MHC-TcR interaction might be involved in the release of "second signals" such as IL-1 from DC. These functions would be expected to be associated with polymorphic epitopes of class II MHC and as a most of the inhibitory class II MHC antibodies used here are thought to detect

monomorphic epitopes then it is predicted that blockade of these functions by these antibodies, if this were to occur, is indirect.

Also in the context of the presentation of antigen, class II MHC might serve to cross-link the CD4 antigen to the TcR complex upon CD4 antigen expressing T lymphocytes, thereby augmenting "first signals" delivered to these cells. The evidence for this pathway is similar to the evidence for such a functional interaction between class I MHC and the CD8 antigen, i.e. in CD3 antibody mitogenesis cross-linking the CD4 antigen to the CD3 antigen is far more effective in inducing the proliferation of CD4 antigen positive T lymphocytes than engaging the CD3 antigen alone (484,485). In this regard the Hig-78 antibody might block this function of class II MHC directly (this amtibody and CD4 antigen are thought to bind a monomorphic epitope present upon each of HLA-DP, DQ and DR, 397,398,430,486) whereas if the other inhibitory class MHC antibodies blocked this function this would have to be by an indirect mechanism (i.e. none of these antibodies react with determinants common to HLA-DP, DQ and DR, 431-435). If in fact any of the inhibitory class II MHC antibodies do block a class II MHC-CD4 antigen interaction in this response then it is again important to point out that the fact that none of the inhibitory class II MHC antibodies affect the formation of DC-T lymphocyte clusters is in accord with the concept that, as with a class I MHC-CD8 antigen interaction, at physiological levels of class II MHC and CD4 antigen expression these two molecules mediate little or no intercellular adhesion (403,479).

Most of the inhibitory class II MHC antibodies did not affect complete inhibition of proliferation. Two exceptions to this, however, are the antibodies Tu39 and L243 which in the same experiment (Fig. 4.6) caused greater than 100% IMP - an observation which raises a number of related issues. Firstly, in the context of the response of CTL, one explanation for

this is that in tonsillar DC induced oxidative mitogenesis CTL proliferation is helper/inducer T lymphocyte dependent. Certainly, for the tonsillar B lymphocyte induced oxidative mitogenesis reaction (where class II MHC antibodies also caused complete blockade) this explanation would seem valid but for tonsillar DC induced responses (including the tonsillar DC induced auto and allo MLR) the same explanation is unlikely, given that a feature of peripheral lymphoid DC is their ability to activate CTL directly (see pages 45, 48, 52). Another explanation for complete inhibition in tonsillar DC induced oxidative mitogenesis, therefore, is that as hinted above CD8 antigen expressing CTL do not proliferate (see also Chapter 6); and one reason for this might be that this response, as a modified auto MLR, in turn a response to extracellular antigens, only involves the presentation of antigens upon class II MHC (see Chapter 3). However, while applicable to the oxidative mitogenesis reaction and auto MLR, this reasoning would seem inappropriate for the allo MLR. Thus, a still further explanation for complete inhibition is that class II MHC performs an as yet undefined function that is involved in the induction of proliferation of both CD4 and CD8 antigen expressing T lymphocytes. As with class I MHC, in this alternative role, class II MHC could be implicated at either or both of the accessory cell and T lymphocyte level.

Secondly, because L243 is HLA-DR specific this might suggest that tonsillar DC induced oxidative mitogenesis is HLA-DR restricted. HLA-DR restriction could result from the determinant selection of whichever antigens mediate an auto MLR component of this reaction, or additionally, if one accepts that oxidative mitogenesis also involves a response of antigen non-specific T lymphocytes to MHC plus irrelevant antigen (see Chapter 3), then HLA-DR restriction could reflect a preferential reactivity of such lymphocytes with this molecule. Whatever, HLA-DR restriction could provide

at least part of an explanation for the only partial inhibition caused by the Hig-78, Tu22 and Tu35 antibodies, i.e. in addition to HLA-DR, these antibodies react with other class II MHC products which might act to "mop up" antibody molecules such that complete blockade of HLA-DR is not achieved.

It is more difficult to explain why the Tu36 antibody, which has been described as HLA-DR specific (432,433), never completely blocked proliferation and why the Tu39 antibody, which reacts with each of HLA-DP, DR and DY (432-434), completely blocked proliferation in one experiment but not in three others. In the former case it could be argued that the assignation of HLA-DR specificity is a tentative one. Also, in the latter case, with complete inhibition, it could be argued that the antibody was in a particularly concentrated form, able to overcome any "mopping up" effect of non-HLA-DR class II MHC molecules. With Tu39 and partial blockade, however, this raises a third issue which concerns and ties in with the inhibitory effects, or lack of inhibitory effects of the 1a3, DA6.164 and DA6.231 antibodies.

In this regard, HLA-DR restriction may not be a constant feature of each and every response. In some responses other class II MHC molecules might also serve as restriction elements. If for example, HLA-DQ were also to act as a restriction element then Tu39 would not effect complete inhibition owing to it's lack of reactivity with this molecule. Likewise, involvement of HLA-DQ in some responses but not in others might explain why the 1a3 antibody, which is HLA-DQ specific (435), moderately inhibited proliferation in one experiment (Fig. 4.12) and had no effect upon proliferation in two others. Such an hypothesis, however, cannot account for the consistent inability of the DA6.164 (HLA-DR specific excepting HLA-DR7, 427,428) and DA6.231 (reactive with HLA-DP, DQ and DR, 427-429) antibodies

to affect proliferation in any experiment and presumably therefore these two antibodies bind class II MHC epitopes that are simply not involved in cell-cell interactions in tonsillar DC induced oxidative mitogenesis.

One final aspect of these studies, is the effect of adding class II MHC antibodies at different times to the tonsillar DC and B lymphocyte induced oxidative mitogenesis reactions. In this respect, that class II MHC antibodies inhibit both responses irrespective of their time of addition to culture suggests that in it's signal transduction role class II MHC functions at a late stage. An alternative view, however, is that an interaction of class II MHC with the TcR and/or CD4 antigen may well be an early event and that inhibition at all time points reflects the fact that such interactions might be equilibriated in turn probably relating to their low affinities (see also Chapter 6).

Another feature of the time course experiments was that in the DC induced response class II MHC antibodies caused less inhibition at the 48 hr time point of antibody addition than at earlier time points whereas in the B lymphocyte induced response the level of inhibition was constant. The most obvious explanation for this observation is that the rate of T lymphocyte proliferation induction is greater in the former response than in the latter. Thus, by 48 hr, in the DC induced response, but not in the B lymphocyte induced response, some T lymphocytes will have become committed to cell division.

Summary

In this Chapter, class I and class II MHC antigens have been shown to function in signal transduction in tonsillar DC induced oxidative mitogenesis. The signal transduction role of class I MHC could involve an interaction with the CD8 antigen leading to the activation of CTL. However, this conclusion is tentative and, further, it is likely that class I MHC performs an additional signal transduction role. The signal transduction role of class II MHC most likely involves an interaction with the TcR and CD4 antigen leading to the activation of helper T lymphocytes. Evidence is presented that in tonsillar DC and B lymphocyte induced oxidative mitogenesis an interaction between class II MHC and the TcR and/or CD4 antigen is of relatively low affinity and occurs at an early stage in these responses.

CHAPTER 5

EFFECT OF LEUCAM AND ICAM-1 ANTIBODIES UPON TONSILLAR DENDRITIC CELL-T LYMPHOCYTE INTERACTION

INTRODUCTION

The ability of lymphoid DC to cluster increased numbers of T lymphocytes over a variety of other accessory cell types is now widely regarded as forming at least one basis for the potent accessory activity of these cells. Understanding the molecular mechanisms of lymphoid DC-T lymphocyte adhesion, therefore, would seem critical in defining this potency in molecular terms.

For a variety of a priori reasons the idea that lymphoid DC-T lymphocyte clustering is mediated by an interaction of the leucocyte cell adhesion molecule (LeuCAM) LFA-1 with either or both of it's ligands ICAM-1 and ICAM-2 is most logical (see page 70). Empirically, however, this possibility has not been explored in detail and moreover of the available experimental evidence there are anomalous findings (see page 71).

In this Chapter, therefore, the role of LFA-1 and ICAM-1 in human tonsillar DC induced oxidative mitogenesis has been examined using large panels of CD11a (LFA-1 alpha chain), CD18 (LFA-1 beta chain) and CD54 antibodies respectively. In addition, the roles of the two other LeuCAM members, Mac-1 and p150,95, have been assessed using antibodies that react with their alpha chains, i.e. CD11b and CD11c antigens respectively.

MATERIALS AND METHODS

Cells. DC, high density T lymphocytes and low density B lymphocytes were isolated from human tonsils as described in Chapter 2.

Proliferation assays. Oxidative mitogenesis assays were performed as indicated in Chapter 3 using varying numbers per well of irradiated DC and low density B lymphocytes as accessory cells and 2×10^5 per well of 2 mM periodate modified autologous high density T lymphocytes as responder cells. LeuCAM and ICAM-1 antibodies were also added to accessory cell plus T lymphocyte wells (at culture initiation or at various time points thereafter) in varying concentrations in 20 ul quantities. All assays were pulsed with ^{125}IdUrd at 48 hr and terminated at 64 hr.

Assessment of cellular clustering. Clustering of DC and 2 mM periodate modified autologous high density T lymphocytes, in the presence (added at time zero) and absence of LeuCAM and ICAM-1 antibodies was assessed visually and photographed at 4 and 12 hr following culture initiation (see Chapter 4). Again, cultures were often the same as those used for the assessment of proliferation in oxidative mitogenesis assays although separate cultures (in which proliferation was not subsequently assessed) were also set up.

Antibodies. Details of MHM24 and 2F12 (CD11a), Mo1 and 44 (CD11b), KB90 (CD11c), GRF1 and MHM23 (CD18), and WEHICAM-1 and 84H10 (CD54) antibodies are given in Table 2.1. A list of other LeuCAM and ICAM-1 mAb used in proliferation and clustering assays is shown in Table 5.1. Cluster designations are according to the nomenclature committees of the Third and Fourth International Workshops on Leucocyte Differentiation Antigens. All antibodies were sterile filtered, dialysed and deaggregated as appropriate (see Chapter 4).

Statistics. See Chapters 3 and 4 plus some results are expressed as percentage change of mean proliferation (% CMP), where % CMP is as follows:

$$\frac{\text{(DC+T+antibody CPM)-(DC+T CPM)}}{\text{(DC+T CPM) - (T alone CPM)}} \times 100$$

Table 5.1 - Additional LeuCAM and ICAM-1 antibodies

Antibody	CD grouping	Species of origin[a]	Isotype	CS/AF[b]	Source
BU17	CD11a	M	G1	AF	Workshop 4
CC51D7	CD11a	M	G1	AF	Workshop 4
CRIS-3	CD11a	M	G2b	AF	Workshop 4
F110.22	CD11a	M	G1	AF	Workshop 4
GRS3	CD11a	M	G1	AF	Workshop 4
HI111	CD11a	M	G1	AF	Workshop 4
ITM3-2	CD11a	M	G2a	AF	Workshop 4
MEM-25	CD11a	M	G1	AF	Workshop 4
MEM-30	CD11a	M	G1	AF	Workshop 4
MEM-83	CD11a	M	G1	AF	Workshop 4
MEM-95	CD11a	M	G1	AF	Workshop 4
TMD3-1	CD11a	M	G1	AF	Workshop 4
VIPIIIB1	CD11a	M	G1	AF	Workshop 4
YTH81.5	CD11a	R	G2a	AF	Workshop 4
122-2A5	CD11a	M	G1	AF	Workshop 4
1524	CD11a	M	G1	AF	Workshop 4
25.3.1	CD11a	M	G1	AF	Workshop 4
459	CD11a	M	G1	AF	Workshop 4
KB23	CD11c	M		CS	K. Pulford
3KB43	CD11c	M		CS	K. Pulford
CLB-54	CD18	M	G1	AF	Workshop 4
IC11	CD18	M	G1	AF	Workshop 4
M232	CD18	M	G1	AF	Workshop 4
YFC118.3	CD18	R	G2b	AF	Workshop 4
YFC51.1	CD18	R	G2b	AF	Workshop 4
11H6	CD18	M	G2a	AF	Workshop 4
MY13	CD54	M	G1	AF	Workshop 4
OKT27	CD54	M	G1	AF	Workshop 4
7F7	CD54	M	G2a	AF	Workshop 4

a) M - mouse, R - rat.

b) CS - culture supernatant, AF - ascites fluid.

Effect of CD11a antibodies upon dendritic cell induced oxidative mitogenesis. Fig. 5.1 shows the results of an initial experiment in which a CD11a antibody (MHM24) was tested for it's effect upon DC induced oxidative mitogenesis. MHM24 inhibited T lymphocyte proliferation in this response in a dose dependent fashion when incorporated into cultures from the outset.

In subsequent experiments, other CD11a antibodies in addition to MHM24 were screened for their effects upon DC induced oxidative mitogenesis. The results, expressed as percentage change in proliferation for each antibody, are summarised in Fig. 5.2 which shows that out of a total of twenty CD11a antibodies tested, thirteen antibodies inhibited proliferation (MEM-25, MEM-95, 25.3.1, MHM24, 1524, 2F12, F110.22, TMD3-1, ITM3-2, CC51D7, VIPIIIB1, GRS3 and HI111), five antibodies enhanced proliferation (CRIS-3, 122-2A5, BU17, MEM-83 and 459) and two antibodies (MEM-30 and YTH81.5) did not significantly affect proliferation.

Effect of CD11b and CD11c antibodies upon dendritic cell induced oxidative mitogenesis. Figs. 5.3 and 5.4 show the results of two different experiments in which CD11b antibodies were tested for their effects upon T lymphocyte proliferation in DC induced oxidative mitogenesis. Neither the Mol (Fig. 5.3) nor the 44 (Fig. 5.4) CD11b antibodies affected proliferation in this response when added to cultures at time zero.

Fig. 5.5 shows the result of a single experiment in which the effect of the 44 (a) antibody upon DC induced oxidative mitogenesis was compared with the effect of three different CD11c antibodies [KB23 (b), 3KB43 (c) and KB90 (d)]. None of these antibodies affected the proliferative response when tested in a range of concentrations.

Effect of CD18 antibodies upon dendritic cell induced oxidative mitogenesis. The effect of CD18 antibodies upon DC induced oxidative mitogenesis was first explored using the MHM23 antibody. When incorporated into cultures from the outset MHM23 inhibited T lymphocyte proliferation in this response (Figs. 5.6) an effect which was dependent upon the concentration of added antibody (Fig. 5.7).

In further experiments, MHM23 and seven other CD18 antibodies were tested for their effects upon DC induced oxidative mitogenesis. The results are summarised in Fig. 5.8 which shows that seven of these antibodies inhibited proliferation (M232, IC11, MHM23, CLB-54, YFC51.1, YFC 118.3 and GRF1) and one antibody enhanced proliferation (11H6).

Effect of CD54 antibodies upon dendritic cell induced oxidative mitogenesis. Five different CD54 antibodies were tested for their effects upon DC induced oxidative mitogenesis. Figs. 5.9-5.11 show that three of these antibodies [84H10 (Fig. 5.9), 7F7 (Fig. 5.10) and WEHICAM-1 (Fig. 5.11)] inhibited the T lymphocyte proliferative response when added to cultures at time zero. Further, the inhibitory effect of 84H10 was shown to be dose dependent. In contrast, Figs. 5.12 and 5.13 show that two CD54 antibodies [OKT27 (Fig. 5.12) and My13 (Fig. 5.13)] failed to affect T lymphocyte proliferation in DC induced oxidative mitogenesis when added to cultures at this same time point.

Effect of CD11a and CD18 antibodies upon the dendritic cell induced oxidative mitogenesis reaction when added at different times. The effect of adding CD11a and CD18 antibodies at different times to the DC induced oxidative mitogenesis reaction was investigated using the proliferation inhibiting antibodies MHM24 and MHM23 respectively. Fig. 5.14 depicts the results of two different experiments which show that the MHM24 antibody

inhibited proliferation only if added to cultures within and including the first 12 hr following culture initiation. When incorporated into cultures at time points after this, MHM24 did not significantly affect T lymphocyte proliferation in this response. Similarly, Fig. 5.15 shows that the inhibitory effect of the MHM23 antibody was dependent upon including this antibody into cultures within and including the first 12 hr following culture initiation. Again, MHM23 did not significantly affect T lymphocyte proliferation in DC induced oxidative mitogenesis when added to cultures at later time points.

For comparison, the effect of adding a CD11b antibody (Mol) at different times to the DC induced oxidative mitogenesis reaction was also investigated. Fig. 5.16 shows that the Mol antibody failed to affect T lymphocyte proliferation in this response irrespective of the time of addition of this antibody to the cultures.

Effect of CD11a, CD11b, CD11c and CD18 antibodies upon B lymphocyte induced oxidative mitogenesis. The effect of CD11a, CD11b, CD11c and CD18 antibodies upon B lymphocyte induced oxidative mitogenesis was also examined. Fig. 5.17 shows that the CD11a antibody, MHM24, inhibited proliferation in this response in a dose dependent fashion when incorporated into cultures from the outset. However, when incorporated into cultures at this same time, neither a CD11b antibody (Mol) nor a CD11c antibody (KB90) affected T lymphocyte proliferation (Fig. 5.18).

Fig. 5.19 shows the results of an experiment in which the effects of adding the MHM24 antibody (a) and the MHM23 CD18 antibody (b) at different times to the B lymphocyte induced oxidative mitogenesis reaction was tested. As with the DC induced reaction, MHM24 and MHM23 only inhibited B lymphocyte induced oxidative mitogenesis when incorporated into cultures at early time points (either culture initiation for MHM24 or culture initiation

and 12 hr for MHM23). At later time points no inhibitory effects were observed.

Effect of CD11a, CD18 and CD54 antibodies upon dendritic cell-T lymphocyte clustering in dendritic cell induced oxidative mitogenesis. Fig. 5.20 depicts the results of an experiment in which a CD11a antibody (MHM24), a CD18 antibody (MHM23) and a CD54 antibody (84H10), each of which inhibited T lymphocyte proliferation in DC induced oxidative mitogenesis, were compared for their effects upon DC- T lymphocyte clustering in this same reaction. As shown, all three antibodies inhibited DC-T lymphocyte clustering when incorporated into cultures at time zero.

All of the other CD11a, CD18 and CD54 antibodies that were screened for their effects upon DC induced oxidative mitogenesis were also tested for their effects upon DC-T lymphocyte clustering in this response.

The effects of CD11a antibodies are depicted in Figs. 5.21 and 5.22. Fig. 5.21 shows that the MEM-25, MEM-95 and 25.3.1 antibodies inhibited DC-T lymphocyte clustering, in contrast to the CRIS-3, 122-2A5, BU17, MEM-30, MEM-83, 459 and YTH81.5 antibodies which did not affect clustering. Fig. 5.22 shows that the F110.2 and GRS3 antibodies also inhibited clustering, in contrast to the ITM3-2 antibody which had no effect. Of the remaining CD11a antibodies 1524, 2F12, TMD-3, CC51D7 and HI111 inhibited clustering but VIPIIIB1 had noe effect (not shown).

Figs. 5.23 and 5.24 show the effects of the CD18 antibodies upon DC-T lymphocyte clustering. Each of the antibodies M232, IC11, CLB-54 and YFC51.1 [Fig. 5.23 (a,c-f)] inhibited clustering as did the antibodies YFC118.3 and GRF1 (Fig. 5.24). However, the 11H6 CD18 antibody did not affect DC-T lymphocyte clustering [Fig. 5.23 (a,b)].

The effects of the other CD54 antibodies upon DC-T lymphocyte clustering are shown in Figs. 5.25-5.27. Only the WEHICAM-1 antibody

inhibited clustering [Fig. 5.25 (a,c)]. None of the antibodies 7F7 [Fig. 5.25 (a,b)], OKT27 (Fig. 5.26) or My13 (Fig. 5.27) affected DC-T lymphocyte clustering.

Tables 5.2-5.4 summarise the effects of CD11a, CD18 and CD54 antibodies upon both T lymphocyte proliferation and clustering in DC induced oxidative mitogenesis.

With respect to the CD11a antibodies (Table 5.2), the majority of those that inhibited proliferation also inhibited clustering (MEM-25, MEM-95, 25.3.1, MHM24, 1524, 2F12, F110.22, TMD3-1, CC51D7, GRS3 and HI111). However, all of the antibodies that enhanced proliferation (CRIS-3, 122-2A5, BU17, MEM-83 and 459), two of the antibodies that inhibited proliferation (ITM3-2 and VIPIIIB1) and both of the antibodies that did not affect proliferation (MEM-30 and YTH81.5) did not affect DC-T lymphocyte clustering.

Of the eight CD18 antibodies that were tested for their effects upon both aspects of the response (Table 5.3) all of the proliferation inhibiting antibodies (M232, IC11, MHM23, CLB-54, YFC51.1, YFC118.3 and GRF1) inhibited clustering. In contrast, the 11H6 antibody, which enhanced proliferation, had no effect on clustering.

CD54 antibodies fell into three different groups according to their effects upon proliferation and clustering (Table 5.4). The first group, consisting of antibodies 84H10 and WEHICAM-1, inhibited both proliferation and clustering. The second group, of which the 7F7 antibody was the sole member, inhibited proliferation but had no affect on clustering and lastly the third group, represented by the antibodies OKT27 and My13, did not affect either event in the cell-cell interaction.

Finally, the CD11b antibodies (Mol and 44) and the CD11c antibodies (KB23, 3KB43 and KB90), all of which had no effect upon T lymphocyte

Table 5.2 - Grouping of CD11a antibodies according to their effects upon proliferation and clustering in dendritic cell induced oxidative mitogenesis

Group 1 - Inhibitors of proliferation and clustering	Group 2 - Enhancers of proliferation no effect on clustering
MEM-25, MEM-95, 25.3.1, MHM24, 1524, 2F12, F110.22, TMD3-1, CC51D7, GRS3, HI111	CRIS-3, 122-2A5, BU17, MEM-83, 459
Group 3 - Inhibitors of proliferation no effect on clustering	**Group 4 - No effect on proliferation and clustering**
ITM3-2, VIPIIIB1	MEM-30 and YTH81.5

Table 5.3 - Grouping of CD18 antibodies according to their effects upon proliferation and clustering in dendritic cell induced oxidative mitogenesis

Group 1 - Inhibitors of proliferation and clustering	Group 2 - Enhancers of proliferation no effect on clustering
M232, IC11, MHM23, CLB-54, YFC51.1, YFC118.3, GRF1	11H6

Table 5.4 - Grouping of CD54 antibodies according to their effects upon proliferation and clustering in dendritic cell induced oxidative mitogenesis

Group 1 - Inhibitors of proliferation and clustering	Group 2 - Inhibitors of proliferation no effect on clustering
84H10, WEHICAM-1	7F7
Group 3 - No effect on proliferation and clustering	
OKT27, MY13	

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proliferation in DC induced oxidative mitogenesis, were also tested for their effects upon DC-T lymphocyte clustering in this response. None of the antibodies affected clustering (not shown).

Fig. 5.1

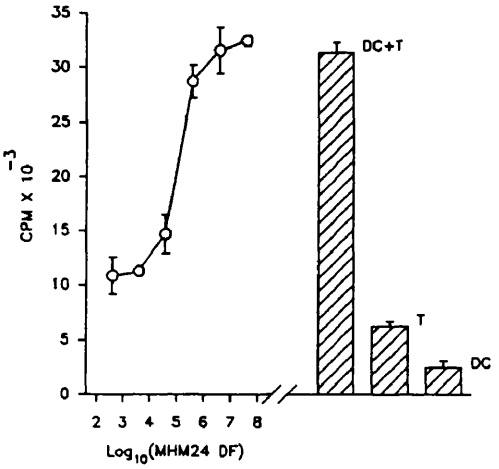


Fig. 5.2

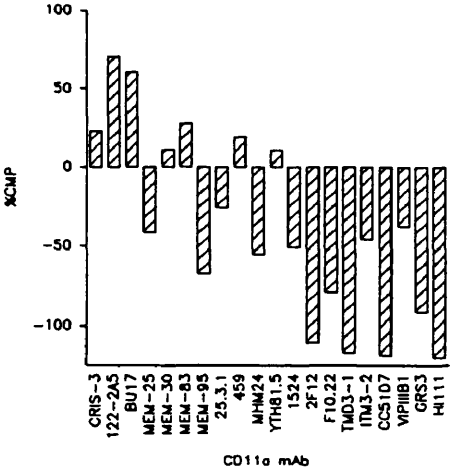


Fig. 5.3

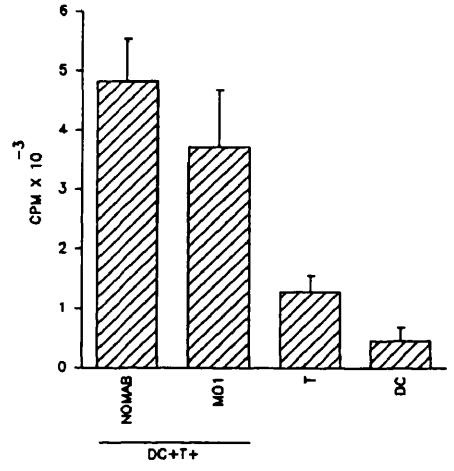


Fig. 5.4

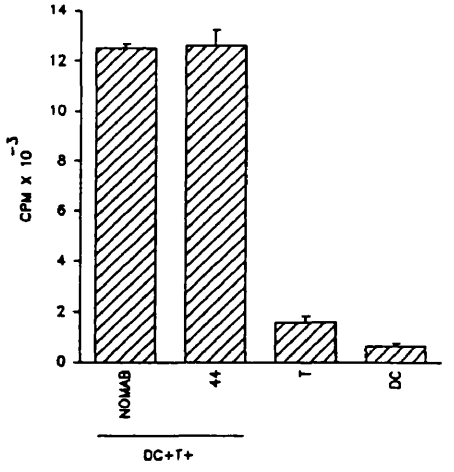


Fig. 5.5(a)

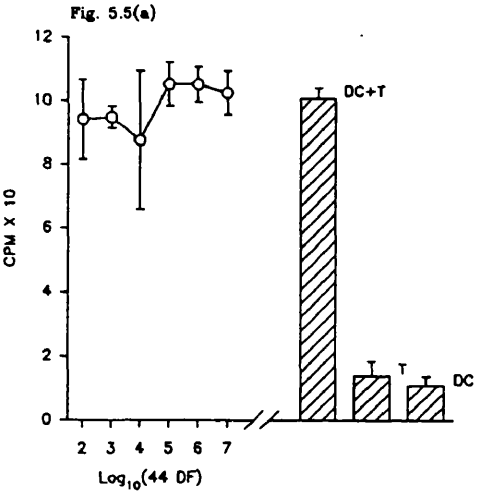


Fig. 5.5(b)

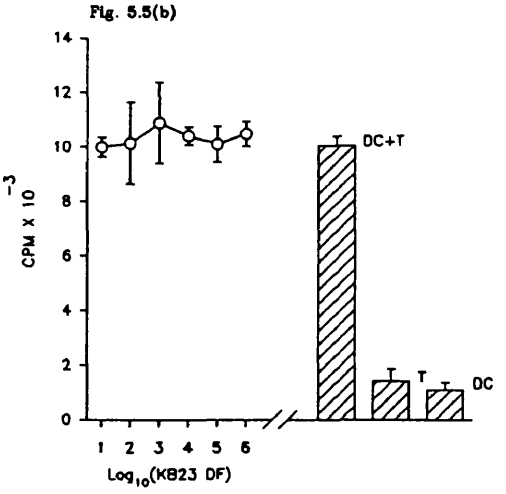
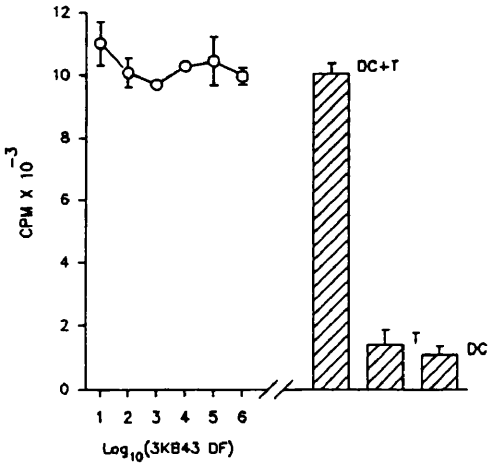


Fig. 5.5(c)



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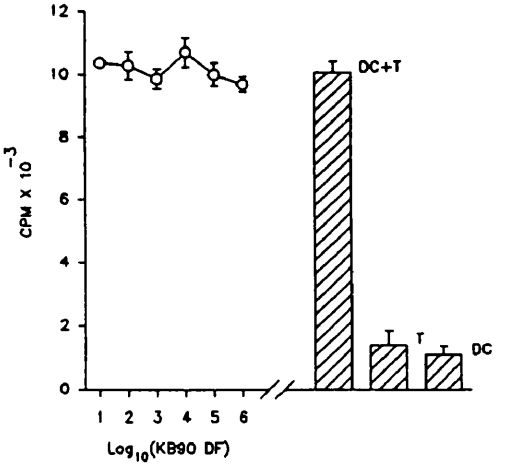
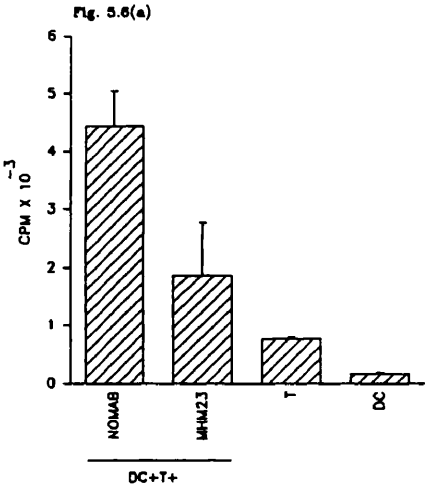


Fig. 5.6(a)



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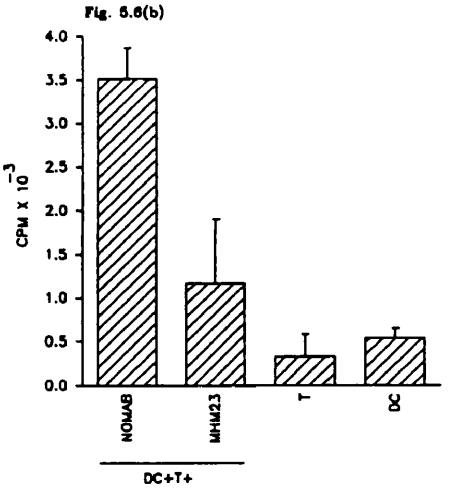


Fig. 5.7

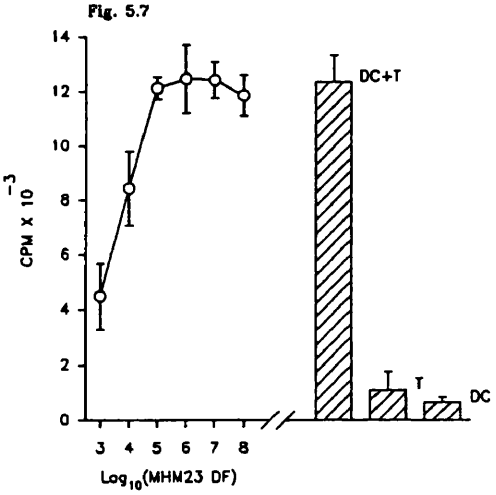
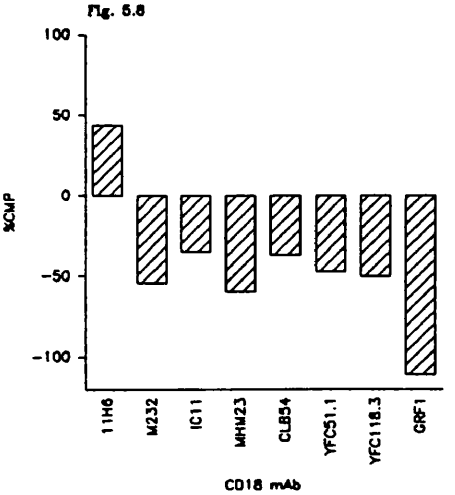


Fig. 5.8



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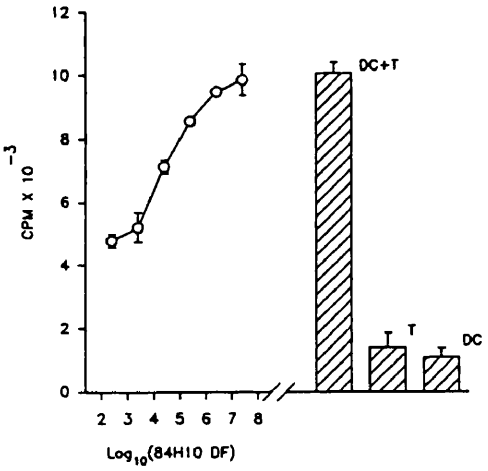
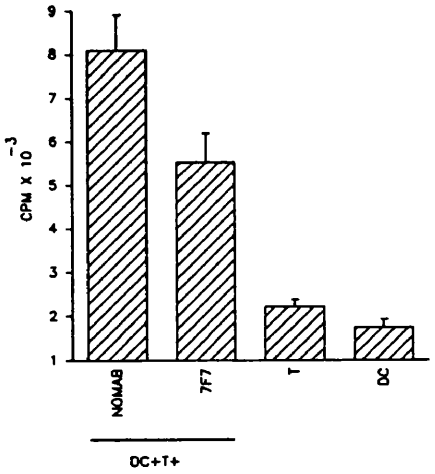


Fig. 5.10



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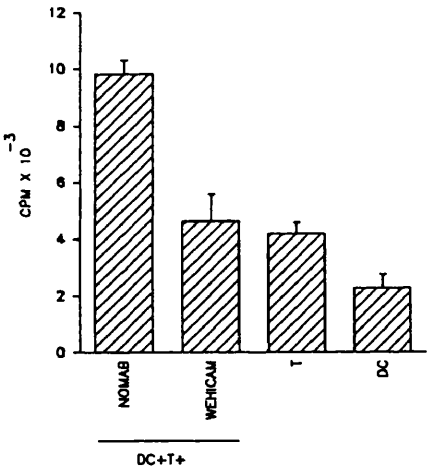

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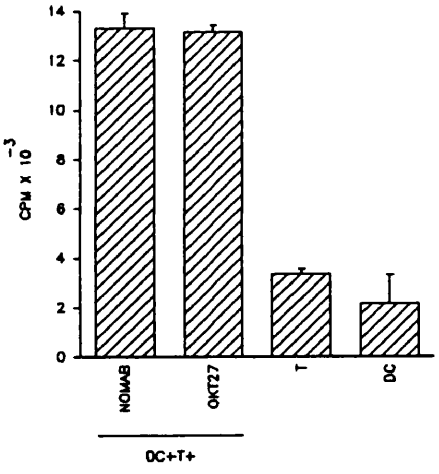


Fig. 5.13

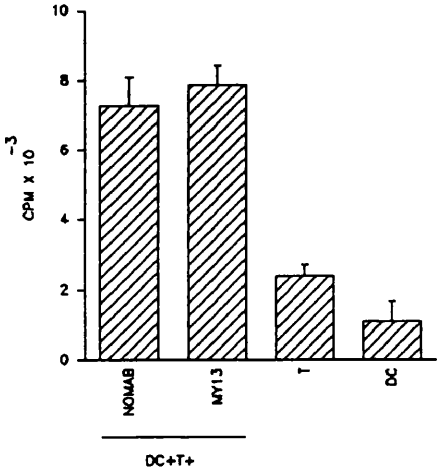
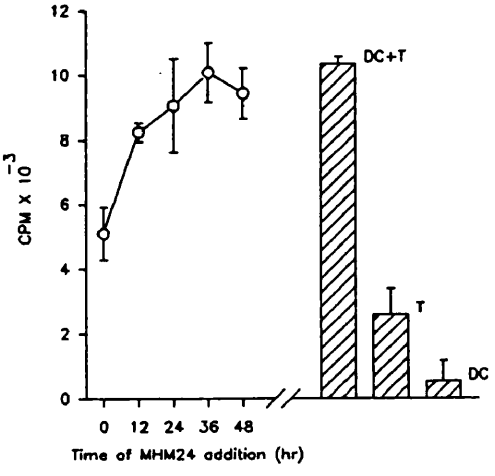


Fig. 5.14(a)



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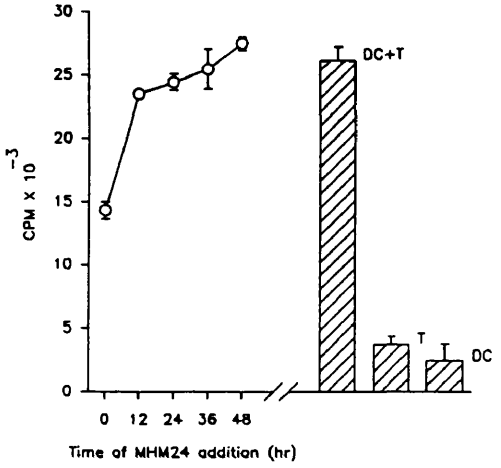


Fig. 5.15

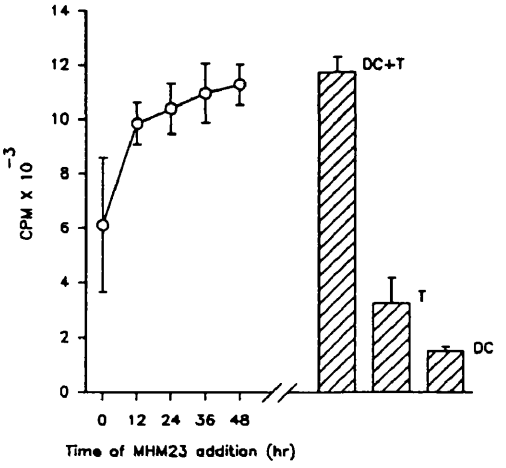


Fig. 5.16

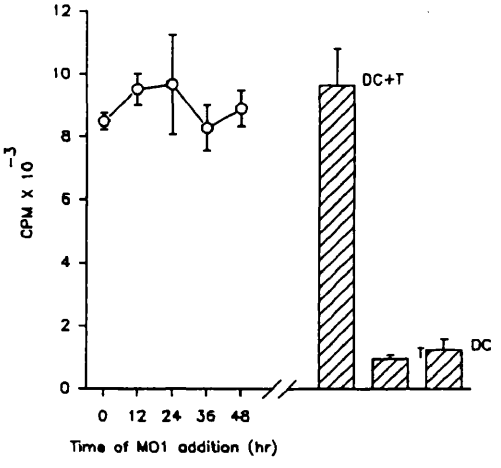


Fig. 5.17

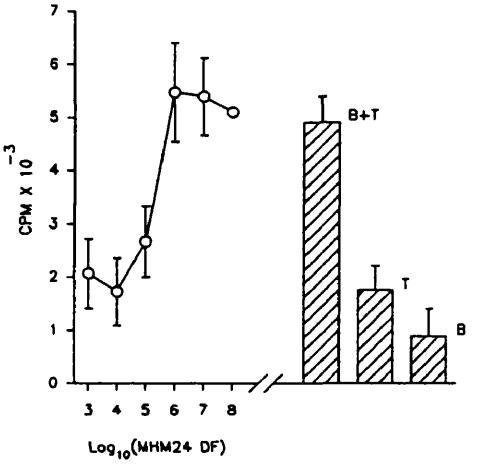
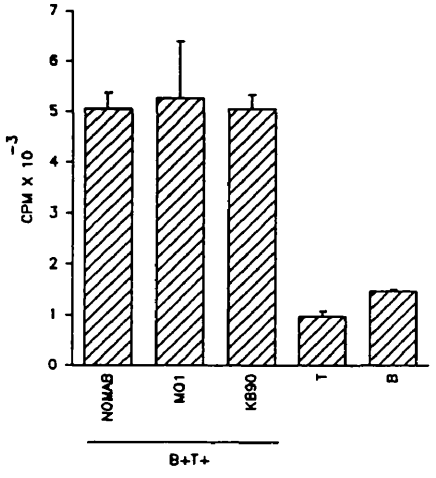


Fig. 5.18



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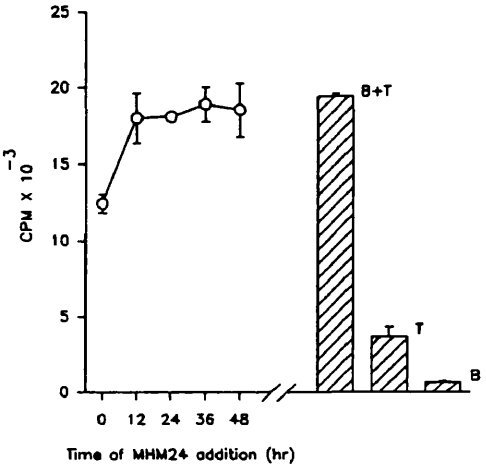

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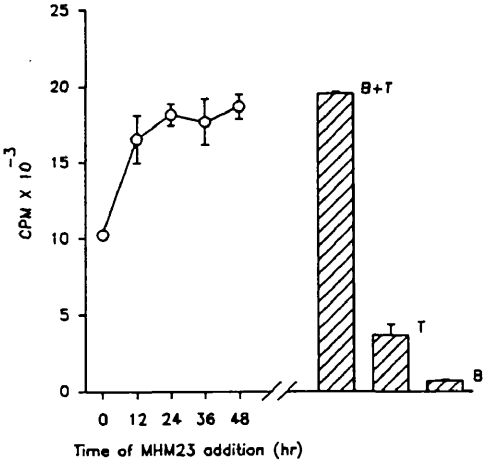
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0
CPM X 10^-3
B+T
T
B
0 12 24 36 48
Time of MHM23 addition (hr)

Fig. 5.20

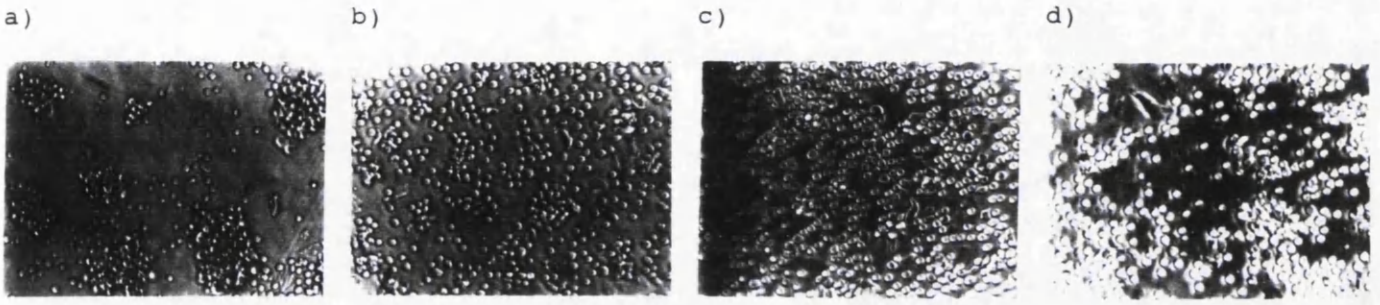


Fig. 5.21

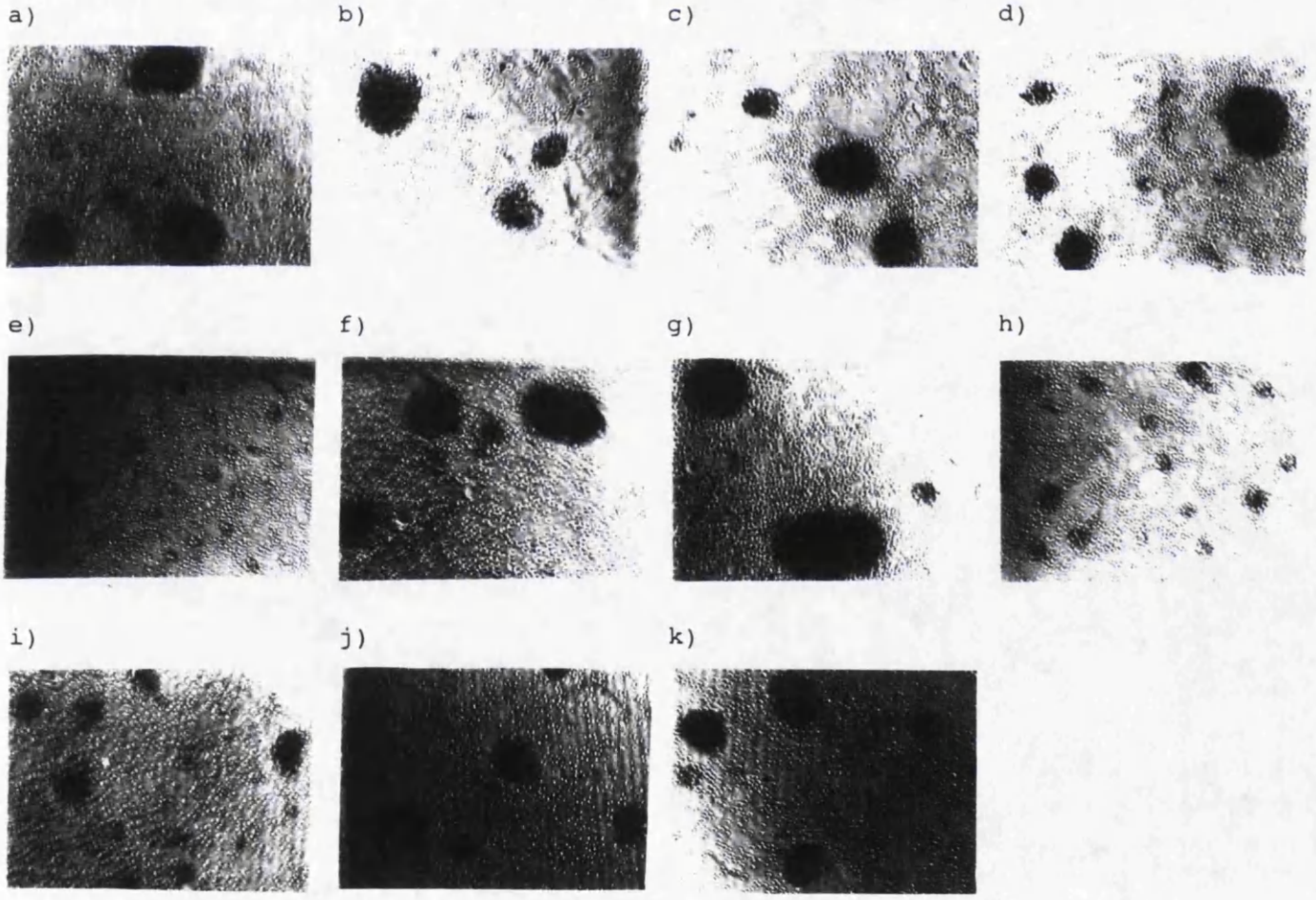


Fig. 5.22

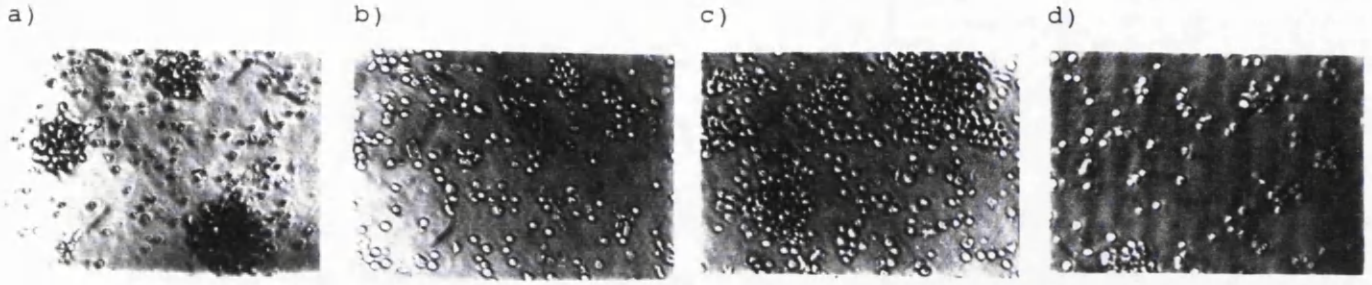


Fig. 5.23

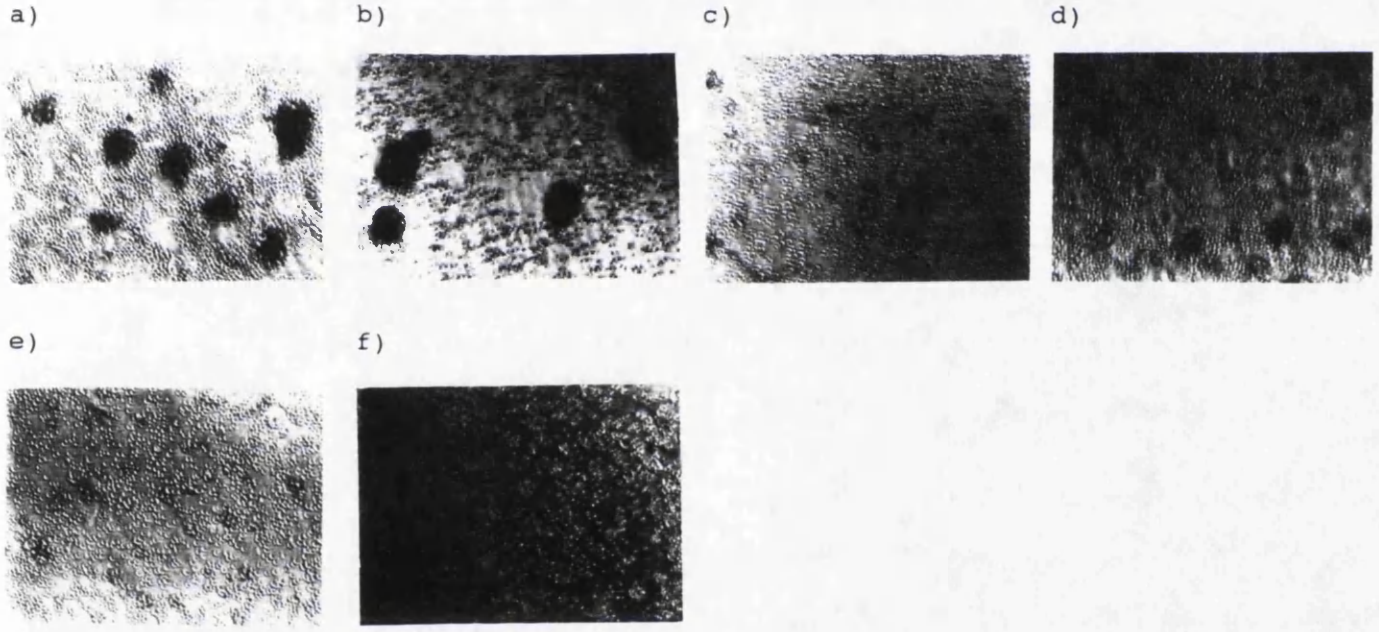


Fig. 5.24

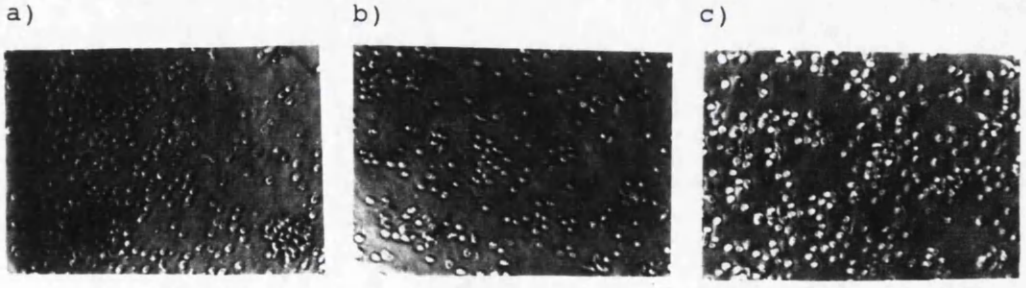


Fig. 5.25

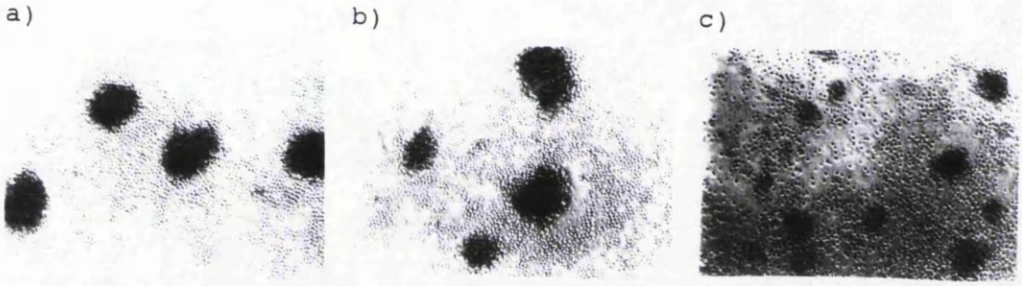


Fig. 5.26

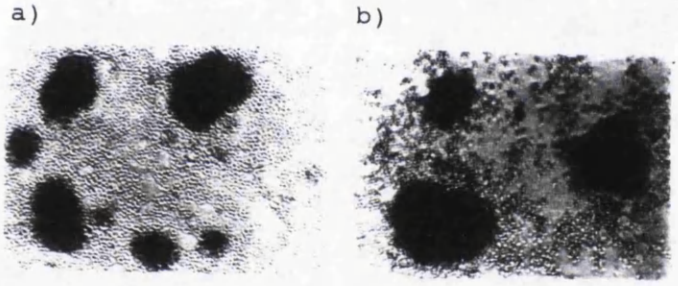


Fig. 5.27

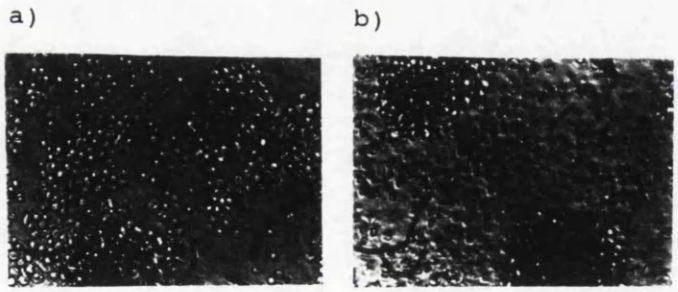


Figure Legends

See Materials and Methods plus:

Fig. 5.1. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well.

Fig. 5.2. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well; all mAb DF - 4 X 10^2; MEM-25, MEM-95, 25.3.1, MHM24, 1524, 2F12, F110.22, TMD3-1, ITM3-2, CC51D7, VIPIIIB1, GRS3 and HI111 inhibitions and CRIS-3, 122-2A5, BU17, MEM-83 and 459 enhancements are SS; MEM-30 and YTH81.5 enhancements are not SS.

Fig. 5.3. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, MO1 DF - 50.

Fig. 5.4. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, 44 DF - 50.

Fig. 5.5(a-d). DC induced oxidative mitogenesis. DC number - 8 X 10^4/well.

Fig. 5.6(a). DC induced oxidative mitogenesis. DC number - 5 X 10^4/well, MHM23 DF - 4 X 10^2.

Fig. 5.6(b). DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, MHM23 DF - 4 X 10^2.

Fig. 5.7. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well.

Fig. 5.8. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well; all mAb DF - 4 X 10^2; M232, IC11, MHM23, CLB54, YFC51.1, YFC118.3 and GRF1 inhibitions are SS; 11H6 enhancement is SS.

Fig. 5.9. DC induced oxidative mitogenesis. DC number - 8 X 10^4/well.

Fig. 5.10. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, 7F7 DF - 4 X 10^2.

Fig. 5.11. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, WEHICAM-1 DF - 4 X 10^2.

Fig. 5.12. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, OKT27 DF - 4 X 10^2.

Fig. 5.13. DC induced oxidative mitogenesis. DC number - 7 X 10^4/well, MY13 DF - 4 X 10^2.

Fig. 5.14(a). DC induced oxidative mitogenesis. DC number - 1 X 10^5/well; MHM24 DF - 10^3; inhibition at 0 and 12 hr time points is SS; inhibition at 24, 36 and 48 hr time points is not SS.

Fig. 5.14(b). DC induced oxidative mitogenesis. DC number - 4 X 10^4/well; MHM24 DF - 4 X 10^2; inhibition at 0 and 12 hr time points is SS; inhibition or enhancement at 24, 36 and 48 hr time points is not SS.

Fig. 5.15. DC induced oxidative mitogenesis. DC number - 5 X 10^4/well; MHM23 DF - 4 X 10^2; inhibition at 0 and 12 hr time points is SS; inhibition at 24, 36 and 48 hr time points is not SS.

Fig. 5.16. DC induced oxidative mitogenesis. DC number - 5 X 10^4/well, MO1 DF - 2 X 10^2.

Fig. 5.17. B lymphocyte induced oxidative mitogenesis. B lymphocyte number - 1 X 10^5/well.

Fig. 5.18. B lymphocyte induced oxidative mitogenesis. B lymphocyte number - 5 X 10^4/well, MO1 DF - 4 X 10^2, KB90 DF - 10^2.

Fig. 5.19(a,b). B lymphocyte induced oxidative mitogenesis. B lymphocyte number - 5 X 10^4/well, MHM24 DF - 4 X 10^2, MHM23 DF - 2 X 10^2, MHM24 inhibition at the 0 hr time point is SS (inhibition at 12, 24, 36 and 48 hr time points is not SS), MHM23 inhibition at 0 and 12 hr time points is SS (inhibition at 24, 36 and 48 hr time points is not SS).

Figs. 5.20-5.27 - DC-T lymphocyte clustering

Fig. 5.20. Oxidative mitogenesis. All photographed at 4 hr, all DC+T+plus (a) NomAb, (b) MHM24 (CD11a), (c) MHM23 (CD18), (d) 84H10 (CD54).

Fig. 5.21. Oxidative mitogenesis. All photographed at 12 hr, all DC+T plus the following CD11a mAb (a) NomAb, (b) CRIS-3, (c) 122-2A5, (d) BU17, (e) MEM-25, (f) MEM-30, (g) MEM-83, (h) MEM-95, (i) 25.3.1, (j) 459, (k) YTH81.5.

Fig. 5.22. Oxidative mitogenesis. All photographed at 12 hr, all DC+T plus the following CD11a mAb (a) NomAb, (b) F110.2, (c) ITM3-2, (d) GRS3.

Fig. 5.23. Oxidative mitogenesis. All photographed at 12 hr, all DC+T plus the following CD18 mAb (a) NomAb, (b) 11H6, (c) M232, (d) IC11, (e) CLB-54, (f) YFC51.1.

Fig. 5.24. Oxidative mitogenesis. All photographed at 12 hr, all DC+T plus the following CD18 mAb (a) NomAb, (b) YFC118.3, (c) GRF1.

Fig. 5.25. Oxidative mitogenesis. All photographed at 12 hr, all DC+T plus the following CD54 mAb (a) NomAb, (b) 7F7, (c) WEHICAM-1.

Fig. 5.26. Oxidative mitogenesis. Photographed at 12 hr, both DC+T plus (a) NomAb, (b) OKT27 (CD54).

Fig. 5.27. Oxidative mitogenesis. Photographed at 12 hr, both DC+T plus (a) NomAb, (b) MY13 (CD54).

Discussion

LFA-1 and ICAM-1 but not Mac-1 and p150,95 play a role in the induction of T lymphocyte proliferation in tonsillar dendritic cell induced oxidative mitogenesis. In this Chapter, the role of LFA-1, Mac-1 and p150,95 molecules in human tonsillar DC induced oxidative mitogenesis has been examined using antibodies that react with their unique alpha chains (CD11a, CD11b and CD11c antibodies respectively) and their common beta chain (CD18 antibodies). Also, the role of ICAM-1 (CD54 antigen) in this response has been analysed using ICAM-1 specific antibodies (CD54 antibodies). Most CD11a antibodies, all CD18 antibodies and most CD54 antibodies influenced T lymphocyte proliferation whereas CD11b and CD11c antibodies did not. These findings are consistent with the notion that LFA-1 and ICAM-1 but not Mac-1 and p150,95 play a role in the induction of T lymphocyte proliferation in tonsillar DC induced oxidative mitogenesis.

A limited number of CD11a, CD11b, CD11c and CD18 antibodies were also tested for their effects upon tonsillar B lymphocyte induced oxidative mitogenesis. Again CD11a and CD18 antibodies affected proliferation (both types inhibited as in the DC induced response) whereas CD11b and CD11c antibodies did not, suggesting that in this response, too, LFA-1 but not Mac-1 and p150,95 play a role in proliferation induction.

Heterogeneity of the effects of LFA-1 and ICAM-1 antibodies upon proliferation and clustering in tonsillar dendritic cell induced oxidative mitogenesis: Distinct epitopes of LFA-1 and ICAM-1 involved in signal transduction and adhesion

LFA-1. LFA-1 antibodies could be divided into four different groups according to their effects upon proliferation and clustering in tonsillar DC induced oxidative mitogenesis, i.e. inhibitors of proliferation and

clustering, enhancers of proliferation and no effect on clustering, inhibitors of proliferation and no effect on clustering, and no effect upon proliferation and clustering (see Tables 5.2-5.4). These results suggest the presence of at least two distinct functional epitopes upon this molecule. One epitope, defined by the LFA-1 antibodies which inhibit proliferation and clustering, would function as an "adhesotope" mediating the binding of DC and T lymphocytes. The other epitope, defined by the LFA-1 antibodies antibodies which influence proliferation but do not affect clustering, would function in signal transduction in this reaction. Presumably, the two LFA-1 (CD11a) antibodies which do not affect proliferation or clustering detect a non-functional epitope(s).

In it's mediation of DC-T lymphocyte clustering the "adhesotope" of LFA-1 probably interacts with ICAM-1 (see below) and an interaction with ICAM-2 is also possible (a variety of criteria indicate that ICAM-1 and ICAM-2 bind the same LFA-1 epitope or adhesotope, 378,379,487-489, predicted in a membrane distal globular domain at the site of close contact between alpha and beta chains, 479,480). In this regard the cluster inhibiting LFA-1 antibodies would achieve their effects by blocking, either directly or indirectly, LFA-1/ICAM interactions.

In it's signal transduction role, the signal transduction epitope of LFA-1 could interact with a hitherto unidentified ligand and that such an interaction is not involved in clustering could be due to any of it's low affinity, weak expression of the ligand at cell surfaces or the possibility that the ligand is a soluble molecule. In this role the signal transduction epitope of LFA-1 can be viewed as part of a receptor site in either a positive or negative signalling pathway. In the former case enhancement of proliferation by LFA-1 antibodies can be viewed as functional mimicry of the unidentified ligand in it's binding to LFA-1 and inhibition of proliferation

as blockade of this interaction without mimicry. In the latter case the exact reverse would apply.

A strong candidate for the signal transduction epitope is a sequence of approximately two hundred amino acids found near the N-terminus of the LFA-1 alpha chain known as the inserted (I) domain (491). I domain homologous sequences have been found in a number of other proteins, e.g. von Willebrands factor (492), complement factor B (493) and cartilage matrix protein (494), where they are either known or are predicted to be directly involved in ligand binding. Further, the I domain as the signal transduction epitope might explain the paucity of CD18 (LFA-1 beta) antibodies that influence proliferation and do not affect clustering. One possible drawback of this model, however, is the juxtaposition of the I domain to the predicted "adhesotope" of LFA-1. This might suggest that binding of ICAMs to the "adhesotope" would prevent, by induction of conformational changes or steric hindrance, binding of an unidentified ligand to the signal transduction epitope, and vice versa. To counter this though, there may be a "division of labour" amongst LFA-1 molecules with some LFA-1 molecules performing signal transduction roles and others performing clustering roles. Indeed, an inability of a single LFA-1 molecule to perform both roles could represent a channelling force which dictates specialisation.

In comparison with other studies of LFA-1 antibodies there are two important points. Firstly, the idea that LFA-1 is involved in signal transduction, in addition to adhesion, is not unique but has been described before, for example in the mouse splenic DC induced primary allo MLR (see page 72) and in studies of CD3 antibody mitogenesis (495-497). None of these previous studies, however, have addressed the issue of a separate signal transduction epitope upon this molecule. Secondly, although there is only partial agreement with physical epitope mapping studies (498,499) there is

almost complete concordance with other functional epitope mapping studies in an adhesion inhibiting versus an adhesion non-inhibiting context. These other systems include each of monocyte-T lymphocyte adhesion (500), PMA induced homotypic aggregation of B and T lymphocyte lines and B lymphocyte adhesion to purified LFA-1 (501).

One other finding that has emerged from the LFA-1 antibody studies is that the proliferation and cluster inhibiting MHM24 (CD11a) and MHM23 (CD18) antibodies only block proliferation in tonsillar DC and B lymphocyte induced oxidative mitogenesis when added to cultures at early time points. This is in contrast to class II MHC antibodies (see Chapter 4) and strongly suggests that the adhesotope of LFA-1 interacts with ICAMs at an early stage, and in a non-equilibriated fashion, in both types of response. Early involvement of LFA-1 in it's interaction with ICAMs is indeed to be expected given that accessory cell-T lymphocyte clustering in oxidative mitogenesis starts around 1 hr and is complete by 6hr of culture.

ICAM-1. CD54 antibodies divided themselves into three different groups - inhibitors of proliferation and clustering, inhibitors of proliferation and no effect on clustering, and no effect upon proliferation and clustering. As with LFA-1, therefore, the best explanation for these observations is the existence of an "adhesotope", a signal transduction epitope and a non-functional epitope(s) respectively upon this molecule.

Almost definitely, in it's mediation of DC-T lymphocyte clustering, the "adhesotope" of ICAM-1 interacts with the "adhesotope" of LFA-1 (see above) and presumably the cluster inhibiting CD54 antibodies interrupt this interaction by binding (or functionally blocking) the most N-terminal Ig-like domain of ICAM-1, which is widely recognised as forming the "adhesotope" of this molecule (489).

There are a number of candidate molecules which could interact with

the signal transduction epitope of ICAM-1. Given that within the Ig superfamily ICAM-1 is most homologous to the CD56 and CD57 antigens (487,488), both of which participate in homophilic interactions in the mediation of neural cell adhesion (502,503), ICAM-1 might interact with itself in this role. Another possibility is that the signal transduction epitope of ICAM-1 interacts with the signal transduction epitope of LFA-1 and in support of this is previous evidence (albeit suggestive) that certain residues in the second domain of ICAM-1 bind LFA-1 in a low affinity interaction (489). If indeed, in tonsillar DC induced oxidative mitogenesis, such low affinity binding occurs then this also identifies the LFA-1 signal transduction epitope ligand and explains more accurately an inability of this interaction to mediate clustering (see above). It might also be important that ICAM-1 has been shown to interact with the CD25 antigen (504), rhinoviruses (505) and P. Falciparum (506) the last two of which may mimic endogenous molecules in their binding to ICAM-1. With whichever molecule the signal transduction epitope of ICAM-1 interacts, however, because the 7F7 antibody (which defines the signal transduction epitope) inhibited proliferation, then this is consistent with most models of the nature of it's involvement (with antibody inhibition or mimicry as appropriate), except that is as a ligand in a negative signalling pathway.

Lastly, there is good agreement between the functional epitope mapping study of ICAM-1 reported here and similar studies reported elsewhere. Thus, the WEHICAM-1 and 84H10 antibodies (both cluster inhibiting) also block many other forms of LFA-1/ICAM-1 dependent adhesion (e.g. 423,487,507) whereas the 7F7 and MY13 antibodies (both no effect upon clustering) generally do not (500,508,509). An exception is the OKT27 antibody (no effect upon clustering) which blocks the PMA induced homotypic aggregation of B lymphocytes (508). The glycosylation pattern of ICAM-1, however, might

explain this difference. ICAM-1 is a heavily glycosylated molecule which exhibits molecular weight heterogeneity between different cell types due to the extent of this glycosylation (392). OKT27, therefore, might bind to a carbohydrate moeity which is present upon the ICAM-1 molecules of some cell types such as B lymphocytes but absent from the ICAM-1 molecules of other cell types such as T lymphocytes and DC. In this hypothesis the carbohydrate moeity would not directly contribute to the "adhesotope" of ICAM-1 but nonetheless OKT27 could effect inhibition of B lymphocyte aggregation secondarily following induction of conformational changes or by steric hindrance.

LFA-1/ICAM-1 mediated dendritic cell-T lymphocyte adhesion and potency of dendritic cell accessory activity. The confirmation that LFA-1 and ICAM-1 mediate DC-T lymphocyte clustering would seem an important first step in defining the potency of DC accessory activity in molecular terms. However, against the obvious hypothesis that potency results from the hyper-expression of these molecules upon DC is the finding that tonsillar DC express only the same levels of LFA-1 and ICAM-1 as tonsillar macrophages (see Chapter 2) which are much weaker accessory cells (see Chapter 3). Alternative possibilities, therefore, are that LFA-1 upon DC is in a permanently high avidity state or that other molecules such as ICAM-2 and LFA-3 participate in accessory cell-T lymphocyte adhesion and it is these that are hyper-expressed upon DC and underlie there potent accessory activity.

High avidity of LFA-1 and hyper-expression of other adhesion molecules not withstanding, as discussed previously (see page 75), the net negative charge of an accessory cell surface might be the most important factor which controls the extent of clustering and hence potency. Along these lines a lower cell surface negative charge upon DC would result in a reduced like-

like charge mediated accessory cell-T lymphocyte repulsion and as such would represent a strong clustering force.

Summary

In this Chapter, LFA-1 and ICAM-1 have been shown to play a role in the induction of T lymphocyte proliferation in tonsillar DC induced oxidative mitogenesis. In contrast, Mac-1 and p150,95 molecules are not involved. LFA-1 and ICAM-1 appear to perform both clustering and signal transduction roles in this response. Furthermore, these different roles appear to be associated with separate epitopes (an "adhesotope and a signal transduction epitope) upon the two molecules. In the mediation of DC-T lymphocyte clustering the "adhesotopes" of LFA-1 and ICAM-1 probably interact with each other at an early stage. The molecules with which the signal transduction epitopes of LFA-1 and ICAM-1 interact is unclear although it is possible that these epitopes, too, interact with each other. Despite the identification of LFA-1 and ICAM-1 as mediators of DC-T lymphocyte clustering neither of these molecules is hyper-expressed upon DC. This is discussed in the context that other factors, such as involvement of additional adhesion molecules in clustering or a low cell surface negative charge upon DC, determines their potent accessory activity.

CHAPTER 6

EFFECT OF T LYMPHOCYTE, B LYMPHOCYTE AND MYELOID ANTIBODIES UPON TONSILLAR DENDRITIC CELL-T LYMPHOCYTE INTERACTION

INTRODUCTION

Apart from MHC and LeuCAM molecules, few other leucocyte cell surface molecules have previously been examined for their involvement in DC accessory function. Studies of purified lymphocytes and of the accessory function of non-DC types, however, predict that a variety of other molecules are likely to play a role and furthermore still other molecules, which have not been implicated in accessory mechanisms before, might too be involved. To examine this in part, in this Chapter, panels of T lymphocyte (CD2, CD3, CD4, CD5, CD8 and CD28), B lymphocyte (CD9, CD10, CD20, CD23, CD24, CD38, CD39, CD40, CD76 and CDw78) and myeloid (CD13, CD14, CD16, CD31, CDw32, CD33, CD34, CD35 and CD64) antibodies have each been tested for their effects upon proliferation and clustering in tonsillar DC induced oxidative mitogenesis.

MATERIALS AND METHODS

Cells. DC, high density T lymphocytes and low density B lymphocytes were isolated from human tonsils as described in Chapter 2. Macrophages were also isolated as described in Chapter 2 with the modifications of this procedure described in Chapter 3.

Proliferation assays. Oxidative mitogenesis assays (2 mM periodate was employed throughout) were performed as indicated in Chapter 3 using varying numbers per well of irradiated DC, B lymphocytes and macrophages as accessory cells and 2×10^5 per well of high density T lymphocytes as responder cells. In addition, T lymphocyte, B lymphocyte and myeloid antibodies were added to accessory cell plus T lymphocyte wells (either at culture initiation or at various time points thereafter) in varying concentrations in 20 ul quantities. Assays were of 64 hr duration and were pulsed with ^{125}IdUrd 16 hr before termination.

Assessment of cellular clustering. Clustering of DC and 2 mM periodate modified autologous high density T lymphocytes, in the presence (added at time zero) and absence of T lymphocyte, B lymphocyte and myeloid antibodies, and clustering of 2 mM periodate modified autologous high density T lymphocytes cultured alone, was assessed visually and photographed at 4 and 12 hr following culture initiation (see Chapter 4). Again, both oxidative mitogenesis assays as well as independent cultures (in which proliferation was not subsequently assessed) were used for this purpose.

Antibodies. Details of M-T910 and TS2/18.1.1 (CD2); T3, UCHT1 and 2AD (CD3); Leu3a, M-T310 and T4 (CD4); T8 and UCHT4 (CD8); FMC56 (CD9); VILA1 (CD10); MEM-15, MEM-18, UCHM1 and VIM-13 (CD14); BW209/2, CLBFcRGranI and GRM1 (CD16); VIBE3 (CD24); KOLT-2 (CD28); SG134 (CD31); 2E1 (CDw32); MY9 (CD33);

MY10 (CD34); E11 (CD35); NOE2/6/C6 (CD38); A1 and OKT28 (CD39); B-E10 (CD40); and MR11 (CDw78) antibodies are given in Table 2.1. Lists of other T lymphocyte, B lymphocyte and myeloid antibodies used in proliferation and clustering assays are shown in Tables 6.1-6.3. With the exception of the CD23 antibodies, anti-P45, EBVCS1, EBVCS2, EBVCS3 and RA23 (510,511), all cluster designations are according to the nomenclature committees of the Third and Fourth International Workshops on Leucocyte Differentiation Antigens. All antibodies were sterile filtered, dialysed and deaggregated, as appropriate (see Chapter 4).

Statistics. See Chapters 3, 4 and 5.

Table 6.1 - Additional T lymphocyte antibodies

Antibody	CD grouping/ specificity	Species of origin[a]	Isotype	mAb/Poly[b]	CS/AF[c]	Source
G19-1	CD4	M		mAb	AF	Q. Sattentau
66.1	CD4	M		mAb	AF	Q. Sattentau
T1LP	CD5	M	G2a	mAb	AF	Workshop 4
LOTact5	CD5	R	M	mAb	AF	Workshop 4
PanL7D	CD7	M	G2a	mAb	AF	Workshop 4
F1	V-beta gene product?	M	G2a	mAb	AF	Workshop 4

a) M - mouse, R - rat, Ra - rabbit.

b) mAb - monoclonal antibody, Poly - polyclonal antibody.

c) CS - culture supernatant, AF - ascites fluid.

Table 6.2 - Additional B lymphocyte antibodies

Antibody	CD grouping	Species of origin	Isotype	mAb/ Poly	CS/AF	Source
HI117	CD9	M	G3	mAb	AF	Workshop 4
B1	CD20	M	G2a	mAb	AF	Workshop 4
anti-P45	CD23	M	-	Poly	-	P. Brickell
EBVCS1	CD23	M	G1	mAb	AF	P. Brickell
EBVCS2	CD23	M	G1	mAb	AF	P. Brickell
EBVCS3	CD23	M	M	mAb	AF	P. Brickell
MHM6	CD23	M	G1	mAb	AF	P. Brickell
RA23	CD23	Ra	-	Poly	-	P. Brickell
HD66	CD76	M	M	mAb	AF	Workshop 4

See footnotes to Table 6.1

Table 6.3 - Additional myeloid antibodies

Antibody	CD grouping/ specificity	Species of origin	Isotype	mAb/ Poly	CS/AF	Source
CLBMonGranII	CD13	M	G2	mAb	AF	A. von dem Borne
MEM-18	CD14	M	G1	mAb	AF	Workshop 3
M5E2	CD14	M	G2a	mAb	AF	Workshop 3
BL-LGL/1	CD16	M	G1	mAb	AF	Workshop 4
B73.1	CD16	M	G1	mAb	AF	Workshop 4
G7E11	CD16	M	M	mAb	AF	Workshop 4
HUNK2	CD16	M	G2a	mAb	AF	Workshop 4
VEP13	CD16	M	M	mAb	AF	Workshop 4
3G8	CD16	M	G1	mAb	AF	Workshop 4
LAK-1	CD31	M	G2a	mAb	AF	Workshop 4
CIKM5	CDw32	M	G1	mAb	AF	Workshop 4
IV.3	CDw32	M	G2b	mAb	AF	Workshop 4
KB61	CDw32	M	G1	mAb	AF	Workshop 4
41H16	CDw32	M	G2a	mAb	AF	Workshop 4
D3HL60	CD33	M		mAb	AF	Workshop 4
P67-5	CD33	M		mAb	AF	Workshop 4
P67-6	CD33	M		mAb	AF	Workshop 4
WM53	CD33	M		mAb	AF	Workshop 4
WM54	CD33	M		mAb	AF	Workshop 4
p115.2	CD34	M	G1	mAb	AF	Workshop 4
TuK3	CD34	M	G3	mAb	AF	Workshop 4
12.8	CD34	M	M	mAb	AF	Workshop 4
BL-3C5	CD34	M	G1	mAb	AF	Workshop 4
J3D3	CD35	M	G1	mAb	AF	Workshop 4
22.2	CD64	M	G1	mAb	AF	Workshop 4
32.2	CD64	M	G1	mAb	AF	Workshop 4

See footnotes to Table 6.1

RESULTS

Effect of T lymphocyte antibodies upon dendritic cell induced oxidative mitogenesis

CD2 and CD3 antibodies. Two different CD2 antibodies were tested for their effects upon T lymphocyte proliferation in DC induced oxidative mitogenesis. Fig. 6.1 shows that the first of these antibodies, TS2/18.1.1, inhibited the T lymphocyte response in a dose dependent fashion when incorporated into cultures from the outset. The effect of the second antibody, M-T910, is shown in Fig. 6.2. M-T910 also inhibited T lymphocyte proliferation in DC induced oxidative mitogenesis.

Fig. 6.2 also shows the effect of a CD3 antibody, T3, upon DC induced oxidative mitogenesis. Like the M-T910 CD2 antibody, T3 inhibited T lymphocyte proliferation in this response. Additional CD3 antibodies were also tested for their effects upon DC induced oxidative mitogenesis. Figs. 6.3 and 6.4 show the results of two different experiments in which the CD3 antibodies 2AD and UCHT1 respectively, were examined. Both of these antibodies inhibited T lymphocyte proliferation and for UCHT1 this effect was shown to be dependent upon the concentration of added antibody.

CD4 and CD8 antibodies. Fig. 6.5 depicts the results of an experiment in which a CD4 antibody (T4) was tested for it's effect upon T lymphocyte proliferation in DC induced oxidative mitogenesis. As shown, when added to cultures at time zero, T4 inhibited proliferation. In another experiment, four other CD4 antibodies (G19-2, 66.1, M-T310 and Leu3a) were tested for their effects upon T lymphocyte proliferation in this response. The results are depicted in Fig. 6.6 which shows that, like T4, each of these antibodies inhibited the response.

The T4 antibody was used to investigate the effect of adding CD4

antibodies at different times to the DC induced oxidative mitogenesis reaction. Fig. 6.7 shows that T4 inhibited T lymphocyte proliferation when added to cultures at both early and intermediate time points i.e. 0, 12, and 24 hr following culture initiation. However, no significant inhibition of proliferation occurred when the antibody was added at the later time points of 36 and 48 hr.

Fig. 6.8 shows the results of a similar time course experiment using the same antibody but in which B lymphocytes rather than DC were used as accessory cells for the oxidative mitogenesis response. As in the DC induced reaction, T4 inhibited T lymphocyte proliferation in B lymphocyte induced oxidative mitogenesis when incorporated into cultures at early and intermediate time points (in this case all time points up to and including 36 hr). Again, however, T4 did not significantly affect T lymphocyte proliferation when added to cultures at the later time point of 48 hr.

Figs. 6.9 and 6.10 show the results of five different experiments in which the CD8 antibody, UCHT4, was examined for it's effect upon DC induced oxidative mitogenesis. When tested in either single concentrations (Fig. 6.9) or in a range of concentrations (Fig. 6.10) UCHT4 failed to affect T lymphocyte proliferation in this response. The effects of another CD8 antibody, T8, upon DC induced oxidative mitogenesis is shown in Fig. 6.11. As with the UCHT4 antibody, T8 had no effect upon T lymphocyte proliferation as determined in two different experiments.

<u>CD5, CD7, CD28 and V-beta gene product antibodies.</u> Fig. 6.12 shows the results of an experiment in which two CD5 antibodies, T1LP and LOTact5, one CD7 antibody, PanL7D, and one possible V-beta gene product antibody, F1, were tested for their effects upon T lymphocyte proliferation in DC induced oxidative mitogenesis. When incorporated into the reaction at the outset, both CD5 antibodies significantly enhanced the T lymphocyte proliferative

response. In contrast, however, the CD7 antibody and the V-beta gene product antibody did not affect proliferation when incorporated into cultures at this same time.

The effect of a CD28 antibody, KOLT-2, upon DC induced oxidative mitogenesis is depicted in Fig. 6.13. Like the CD5 antibodies, KOLT-2 also significantly enhanced T lymphocyte proliferation in this response when added to cultures at time zero.

Effect of B lymphocyte antibodies upon dendritic cell induced oxidative mitogenesis

CD9, CD10, CD20 and CD24 antibodies. The effect of adding two CD9 antibodies (HI117 and FMC56), a CD10 antibody (VILA1) and a CD24 antibody (VIBE3), at time zero, to the DC induced oxidative mitogenesis reaction is shown in Fig. 6.14. None of these antibodies affected the magnitude of the T lymphocyte proliferative response.

Fig. 6.15 shows the results of two different experiments in which the CD20 antibody, B1, was tested for it's effects upon proliferation in DC induced oxidative mitogenesis. B1, also had no effect upon the magnitude of the response.

CD23 antibodies. The effect of a murine CD23 mAb, MHM6, upon DC induced oxidative mitogenesis is shown in Fig. 6.16. MHM6 had no effect upon upon T lymphocyte proliferation when incorporated into the reaction at time zero. Fig. 6.17 shows the effect of titrating this antibody and Fig 6.18 shows the effect of titrating a rabbit CD23 polyclonal antibody, RA23, into the response. Again, MHM6 failed to affect T lymphocyte proliferation and RA23 also had no effect. The effects of four additional CD23 antibodies upon DC induced oxidative mitogenesis are shown in Fig. 6.19. None of these antibodies, which included a murine polyclonal antibody (anti-P45) and three

different murine mAb (EBVCS1, EBVCS2 and EBVCS3), affected the T lymphocyte proliferative response.

The MHM6 antibody was also tested for it's effects upon B lymphocyte and macrophage induced oxidative mitogenesis. Fig. 6.20 shows that, as with the DC induced reaction, MHM6 did not affect T lymphocyte proliferation in either of these responses.

CD38, CD39 and CD40 antibodies. Fig. 6.21 shows the effect of a CD38 antibody, NOE2/6/C6, upon DC induced oxidative mitogenesis. When incorporated cultures at time zero, NOE2/6/C6 had no effect upon the T lymphocyte proliferative response.

The effect of two CD39 antibodies, A1 and OKT28, and one CD40 antibody, B-E10, upon DC induced oxidative mitogenesis is shown in Fig. 6.22. Both CD39 antibodies significantly enhanced T lymphocyte proliferation when added to cultures at culture initiation. In contrast, the CD40 antibody did not affect proliferation when added to cultures at the same time.

CD76 and CDw78 antibodies. Fig. 6.23 shows the effect of a CD76 antibody, HD66, and a CDw78 antibody, MR11, both added at time zero, upon T lymphocyte proliferation in DC induced oxidative mitogenesis. HD66 did not affect proliferation whereas MR11 inhibited proliferation. Fig. 6.24 shows that the inhibitory effect of the MR11 antibody was dependent upon the concentration of added antibody.

Effect of myeloid antibodies upon dendritic cell induced oxidative mitogenesis

CD13 and CD14 antibodies. Fig. 6.25 depicts the results of an experiment in which a CD13 antibody, CLBMonGranII, was titrated into the DC induced oxidative mitogenesis reaction at culture initiation. As shown, CLBMonGranII

did not significantly affect T lymphocyte proliferation in this response at any of the tested concentrations.

The effects of five different CD14 antibodies upon DC induced oxidative mitogenesis are shown in Figs. 6.26-6.29. When incorporated into cultures at time zero none of the antibodies UCHM1 (Fig. 6.26), MEM-18 and M5E2 (Fig. 6.27), MEM-15 (Fig. 6.28) or VIM13 (Fig. 6.29) influenced the magnitude of the T lymphocyte proliferative response.

CD16 antibodies. The effect of CD16 antibodies upon DC induced oxidative mitogenesis was initially investigated using the CLBFcRGranI antibody. When added to cultures in a single concentration at time zero, CLBFcRGranI had no effect upon T lymphocyte proliferation (Fig. 6.30) and, similarly, no effect was observed when the same antibody was incorporated into cultures in a range of concentrations at the same time (Fig. 6.31).

In further experiments, other CD16 antibodies in addition to CLBFcRGranI were tested for their effects upon DC induced oxidative mitogenesis. The results of these experiments are summarised in Fig. 6.32 which shows that none of the CD16 antibodies G7E11, BL-LGL/1, B73.1, CLBFcRGranI, BW209/2, GRM1, 3G8, VEP13 or HUNK2 significantly affected T lymphocyte proliferation.

CD31, CDw32 and CD64 antibodies. Fig. 6.33 depicts the results of an experiment in which two CD31 antibodies, SG134 and LAK-1, and two CDw32 antibodies, 41H16 and IV.3, were tested for their effects upon DC induced oxidative mitogenesis. As shown, none of these antibodies affected T lymphocyte proliferation when incorporated into cultures at time zero.

Other CDw32 antibodies were also tested for their effects upon T lymphocyte proliferation in this response. Fig. 6.34 depicts the results of an experiment in which the 2E1 CDw32 antibody was examined. Like 41H16 and

IV.3, 2E1 also had no effect upon proliferation. In another experiment (Fig. 6.35) 41H16, IV.3 and 2E1 were compared with the KB61 and CIKM5 CDw32 antibodies for their effects upon proliferation. Again, 41H16, IV.3 and 2E1 failed to affect proliferation and KB61 and CIKM5 also had no effect.

The effects of incorporating two CD64 antibodies, 22.2 and 32.2, at time zero into the DC induced oxidative mitogenesis reaction is shown in Fig. 6.36. Neither of these antibodies affected proliferation in this response.

CD33, CD34 and CD35 antibodies. Fig. 6.37 depicts the results of a single experiment in which six different CD33 antibodies (MY9, P67-5, P67-6, D3HL60, WM53 and WM54) and five different CD34 antibodies (BL-3C5, TuK3, p115.2, 12.8 and MY10) were screened for their effects upon T lymphocyte proliferation in DC induced oxidative mitogenesis. None of the antibodies affected proliferation when incorporated into the reaction at time zero.

The effects of two CD35 antibodies, J3D3 and E11, upon DC induced oxidative mitogenesis are shown in Figs. 6.38 and 6.39 respectively. Again, when incorporated into cultures from the outset no inhibitory or enhancing effect upon T lymphocyte proliferation was observed with these antibodies.

Effect of T lymphocyte antibodies upon dendritic cell-T lymphocyte clustering in dendritic cell induced oxidative mitogenesis

The effects of the CD2 antibody, TS2/18.1.1, the CD3 antibodies, 2AD and T3 and the CD4 antibodies, Leu3a and T4 upon DC-T lymphocyte clustering in the oxidative mitogenesis reaction are shown in Figs. 6.40 and 6.41. TS2/18.1.1 inhibited clustering when incorporated into cultures at time zero [Fig 6.40 (a,c)]. However, neither 2AD [Fig. 6.41 (a,b)] and T3 [Fig. 6.41 (a,c)] or Leu3a [Fig. 6.40 (a,d)] and T4 [Fig. 6.41 (a,d)] affected DC-T lymphocyte clustering when incorporated cultures at the same time.

Figs. 6.42 and 6.43 show the effects of CD5, CD7, CD8, CD28 and V-beta gene product antibodies upon DC-T lymphocyte clustering. None of the antibodies LOTact5 (CD5), KOLT-2 (CD28) or F1 (V-beta gene product?) affected clustering (Fig 6.42). Similarly, the antibodies PANL7D (CD7) and T8 (CD8) had no effect upon clustering in this response (Fig. 6.43).

With respect to the other T lymphocyte antibodies that were tested for their effects upon T lymphocyte proliferation in DC induced oxidative mitogenesis, the CD3 antibody, UCHT1, the CD4 antibodies, G19.2, 66.1 and M-T310, the CD5 antibody, T1LP and the CD8 antibody, UCHT4, had no effect upon clustering in this reaction. However, like the TS2/18.1.1 CD2 antibody, the M-T910 CD2 antibody inhibited clustering when incorporated into cultures at time zero (not shown).

Effect of B lymphocyte antibodies upon dendritic cell-T lymphocyte clustering in dendritic cell induced oxidative mitogenesis

The effects of the CD9 antibody (FMC56), the CD10 antibody (VILA1) and the CD24 antibody (VIBE3) upon DC-T lymphocyte clustering in oxidative mitogenesis are shown in Fig. 6.44. None of these antibodies affected clustering when incorporated into cultures at the outset.

Each of the other B lymphocyte antibodies that were tested for their effects upon T lymphocyte proliferation in DC induced oxidative mitogenesis were also tested for their effects upon DC-T lymphocyte clustering in this reaction. Fig. 6.45 shows that neither the B1 CD20 antibody nor the B-E10 CD40 antibody affected clustering and Fig. 6.46 shows that the NOE2/6/C6 CD38 antibody and the A1 and OKT28 CD39 antibodies, too, had no effect. Finally, the CD9 antibody (HI117), the CD23 antibodies (MHM6, RA23, anti-P45, EBVCS1, EBVCS2 and EBVCS3), the CD76 antibody (HD66) and the CDw78 antibody (MR11) did not affect DC-T lymphocyte clustering (not shown).

Effect of myeloid antibodies upon dendritic cell-T lymphocyte clustering in dendritic cell induced oxidative mitogenesis

Fig. 6.47 shows the effects of the CD14 antibody, MEM-15, and the CD31 antibodies, SG134 and LAK-1, upon DC-T lymphocyte clustering in the oxidative mitogenesis reaction. None of these antibodies affected clustering when added to cultures at the outset. Fig. 6.48 shows the effects of the CD14 antibody, VIM-13, and the CD64 antibodies, 22.2 and 32.2, upon clustering. Again no effect upon clustering was observed when the antibodies were incorporated into cultures at time zero.

Of the remaining myeloid antibodies that were tested for their effects upon T lymphocyte proliferation in DC induced oxidative mitogenesis all were also tested for their effects upon DC-T lymphocyte clustering in this response. Consistent with the lack of any inhibitory or enhancing effect upon clustering seen with the myeloid antibodies shown in Figs. 6.47-6.48, the CD13 antibody (CLBMonGranII), the CD14 antibodies (UCHM1, MEM-18 and M5E2), the CD16 antibodies (BL-LGL/1, B73.1, CLBFcRGranI, G7E11, BW209/2, GRM1, 3G8, VEP13 and HUNK2), the CDw32 antibodies (KB61, 2E1, CIKM5, IV.3 and 41H16), the CD33 antibodies (MY9, P67-5, P67-6, D3HL60, WM53 and WM54), the CD34 antibodies (BL-3C5, TuK3, p115.2, 12.8 and MY10) and the CD35 antibodies (J3D3 and E11) also had no effect upon DC-T lymphocyte clustering in oxidative mitogenesis (not shown).

Fig. 6.1

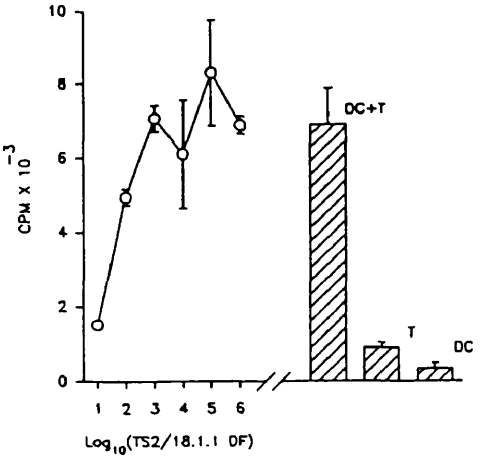


Fig. 6.2

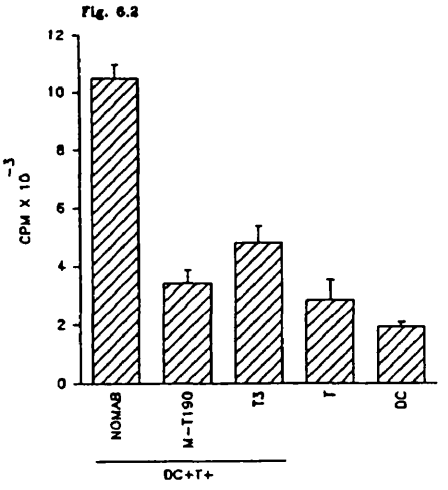


Fig. 6.3

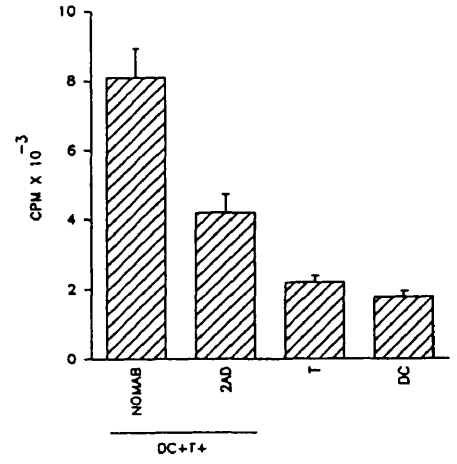


Fig. 6.4

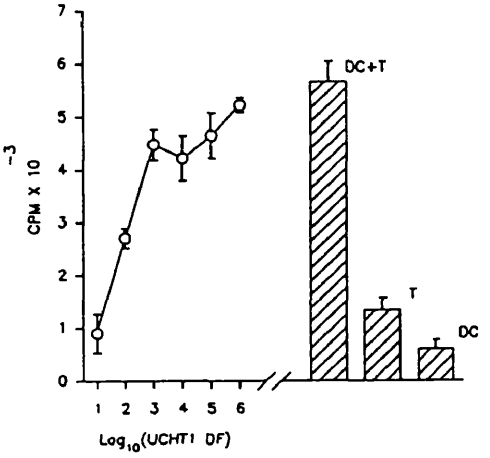


Fig. 6.5

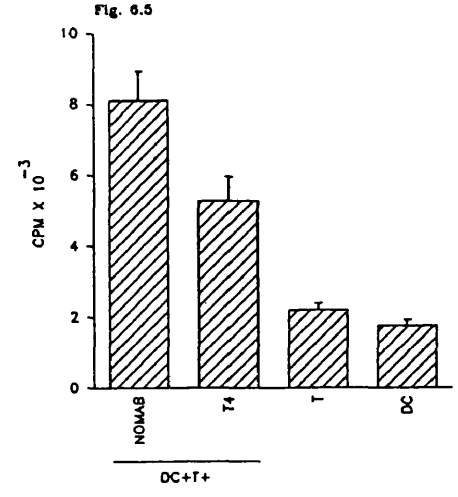


Fig. 6.6

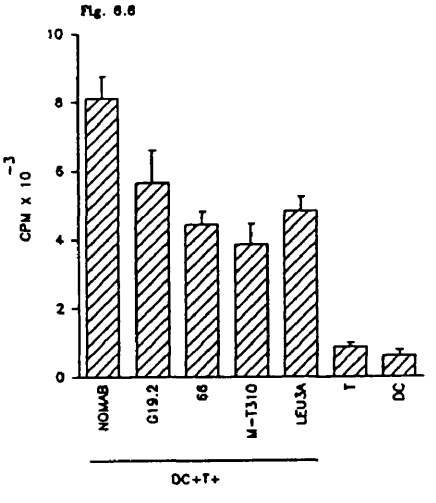


Fig. 6.7

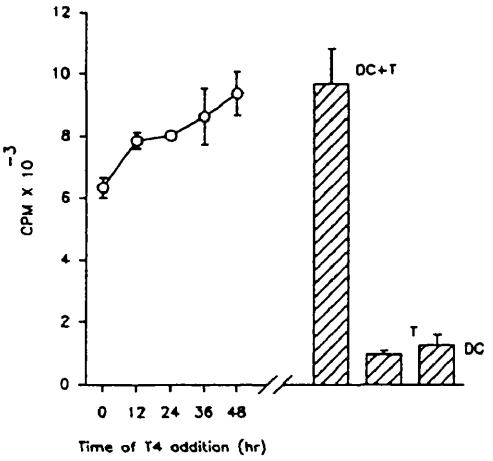


Fig. 6.8

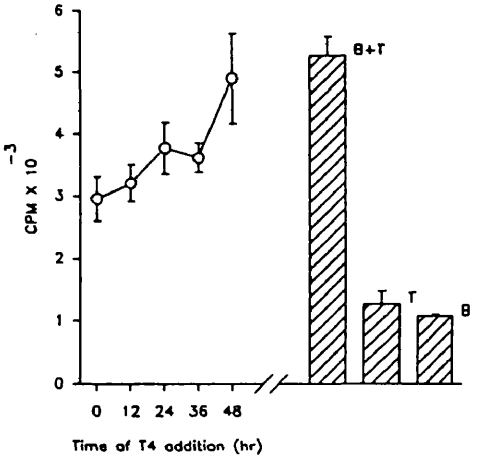


Fig. 6.9(a)

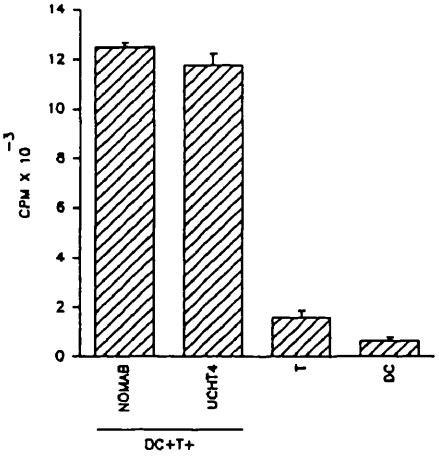


Fig. 6.9(b)

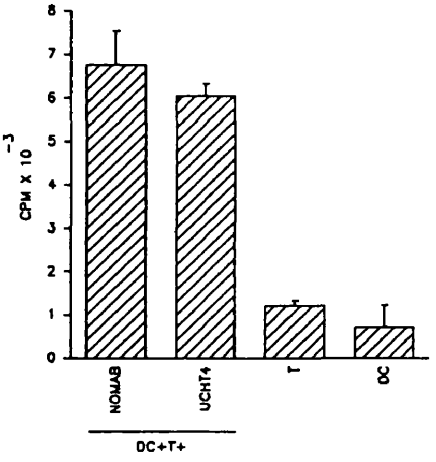


Fig. 6.9(c)

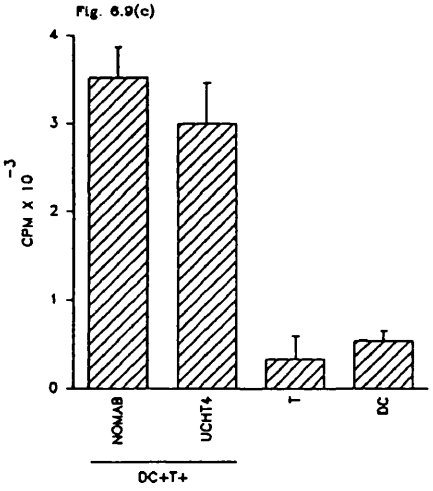


Fig. 6.9(d)

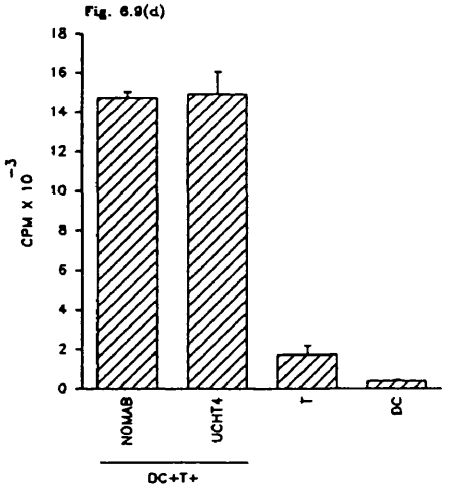


Fig. 6.10

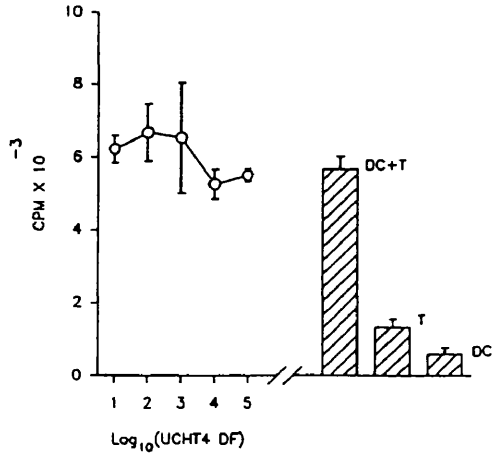


Fig. 6.11(a)

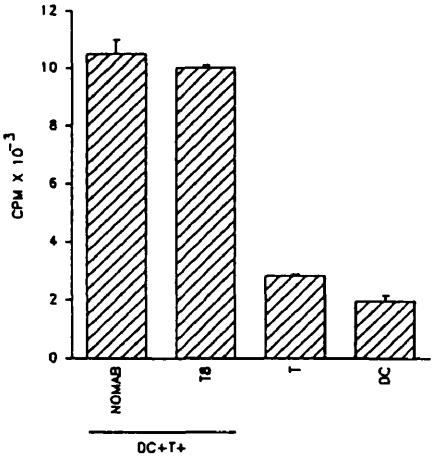


Fig. 6.11(b)

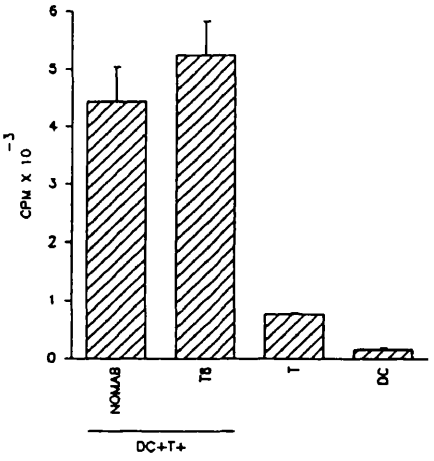


Fig. 6.12

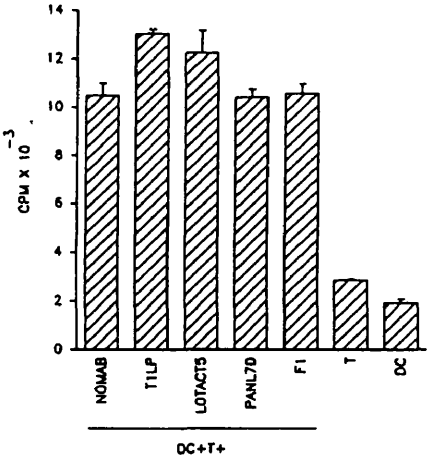


Fig. 6.13

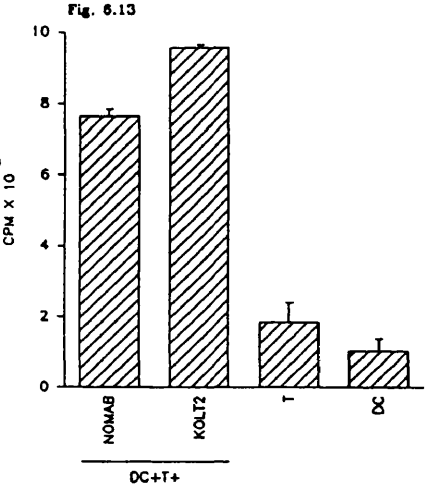


Fig. 6.14

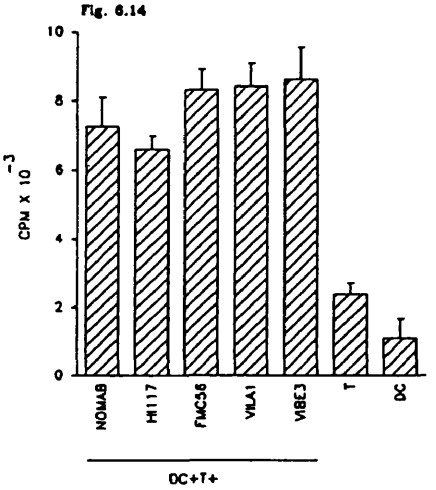


Fig. 6.15(a)

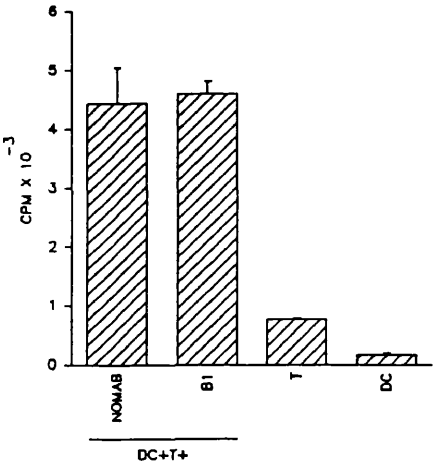


Fig. 6.15(b)

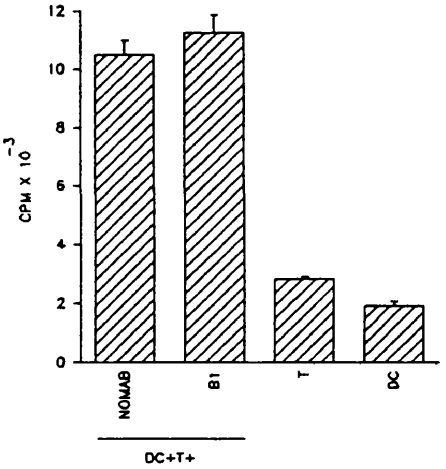


Fig. 6.16

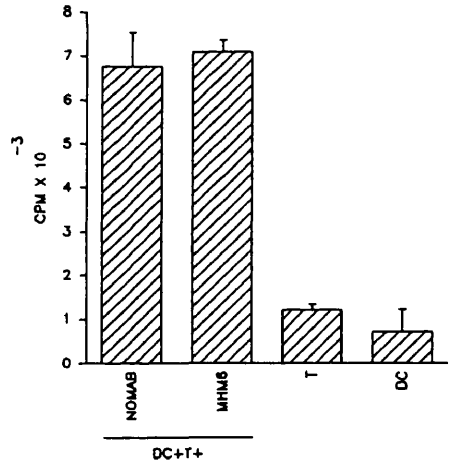


Fig. 6.17

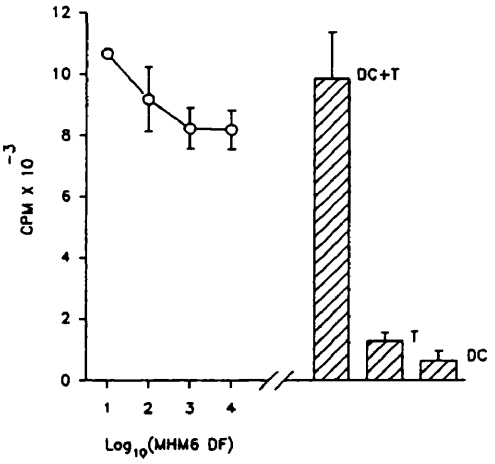


Fig. 6.18

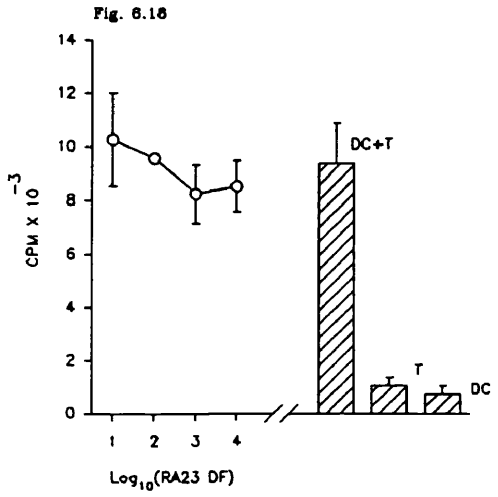


Fig. 6.19

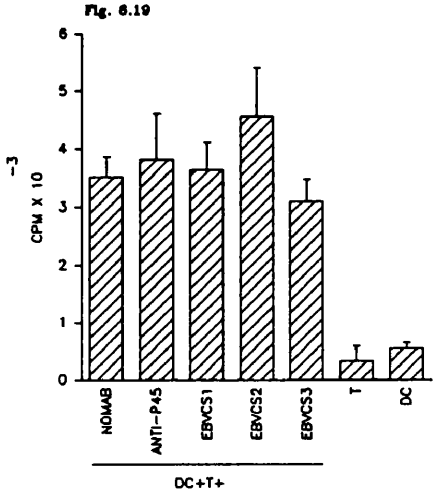


Fig. 6.20

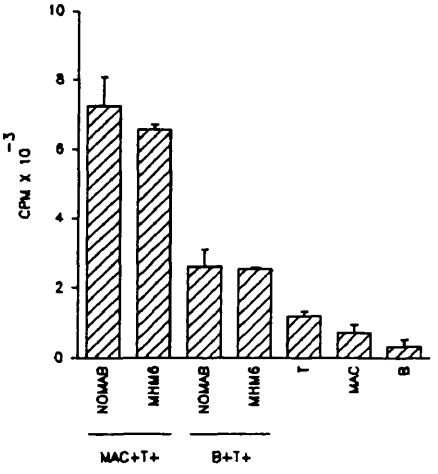


Fig. 6.21

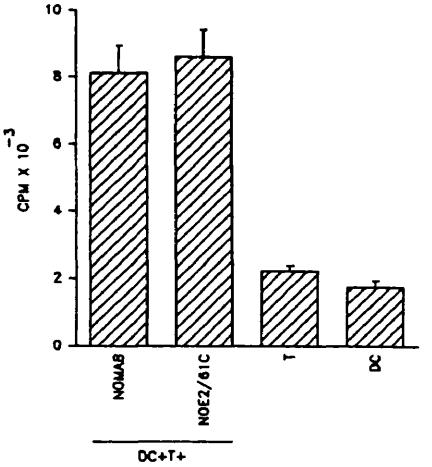


Fig. 6.22

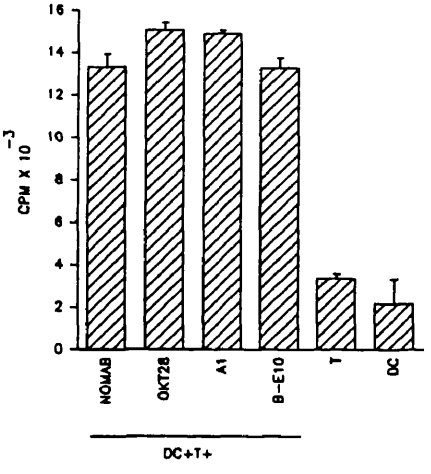


Fig. 6.23

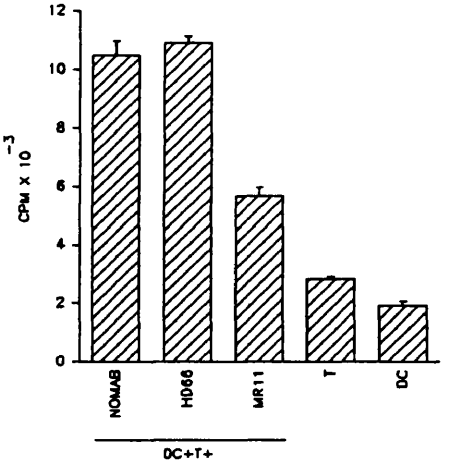


Fig. 6.24

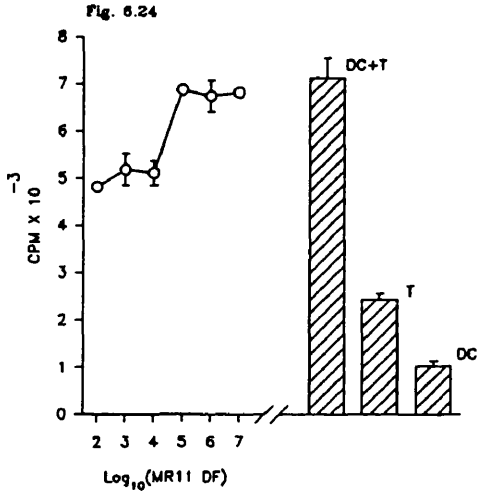


Fig. 6.25

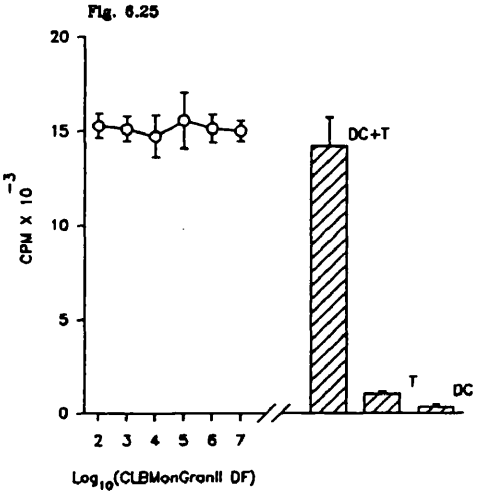


Fig. 6.26

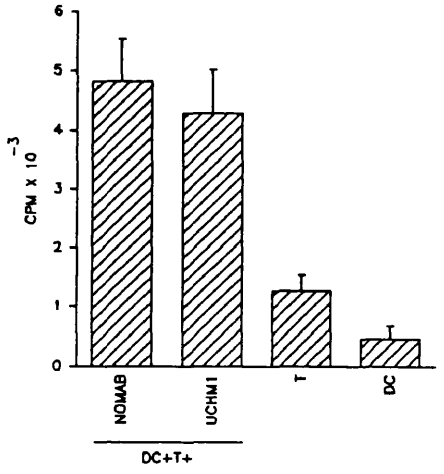


Fig. 6.27

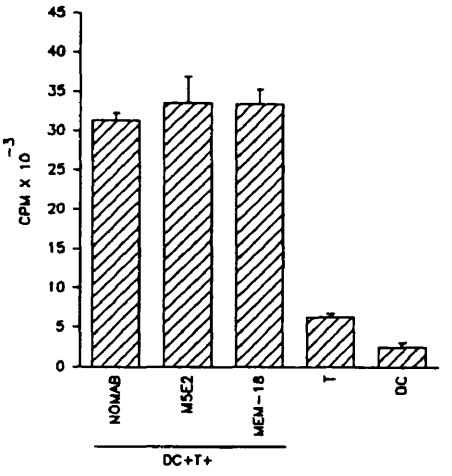


Fig. 6.28

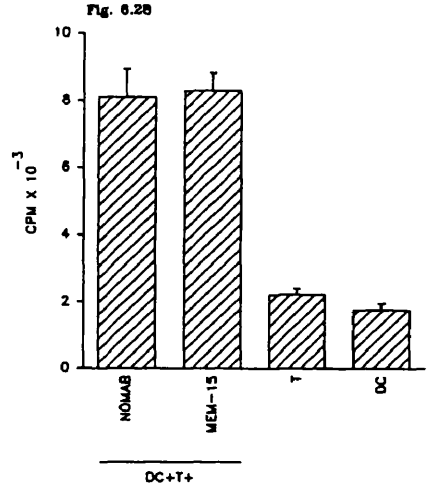


Fig. 6.29

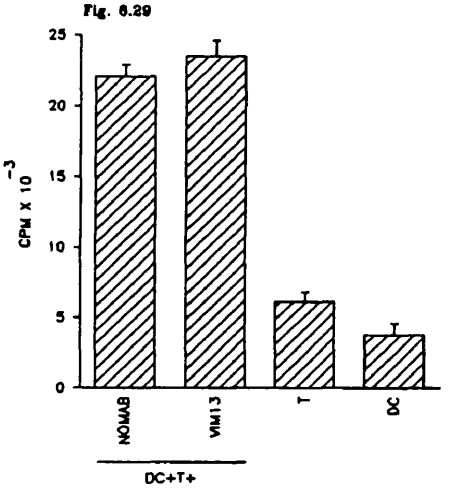


Fig. 6.30

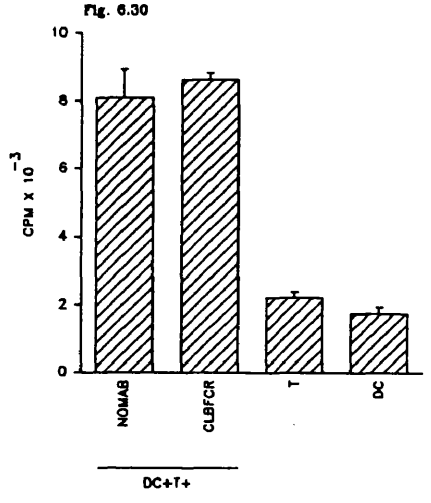
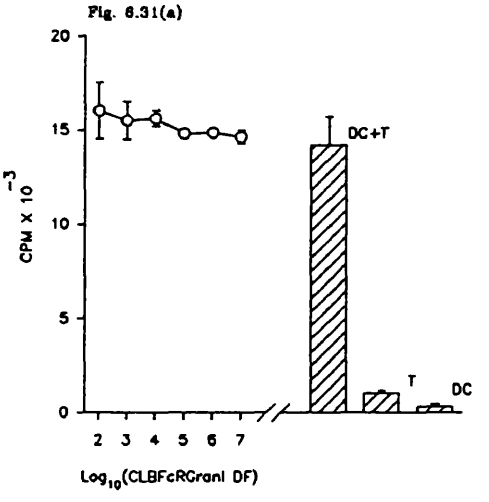


Fig. 6.31(a)



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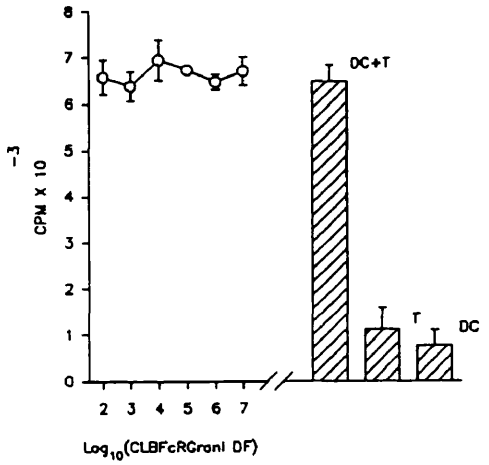


Fig. 6.32

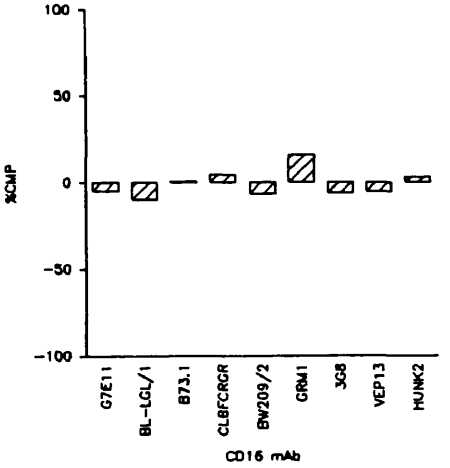
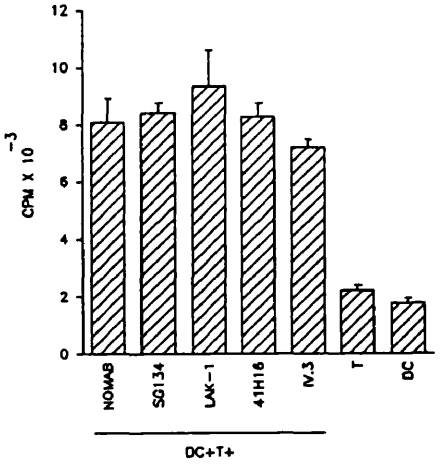


Fig. 6.33



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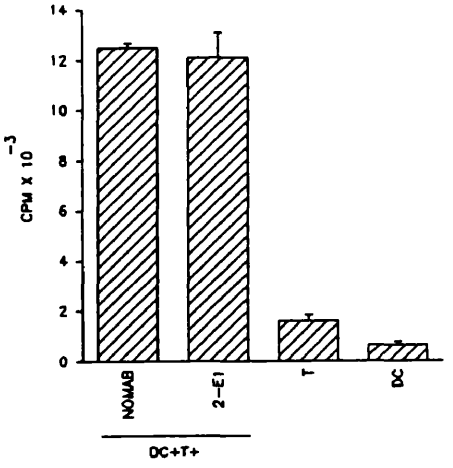

Fig. 6.35

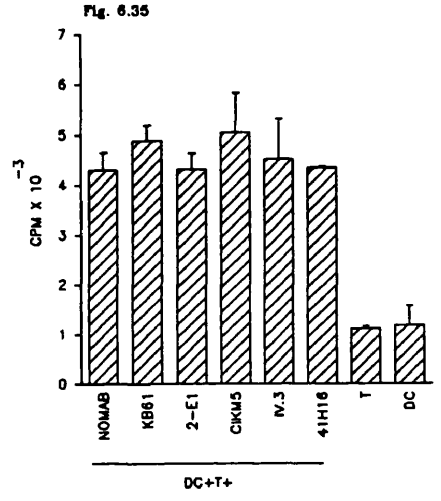

Fig. 6.36

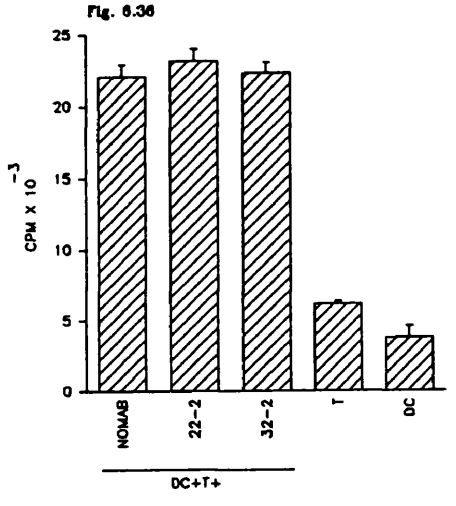

Fig. 6.37

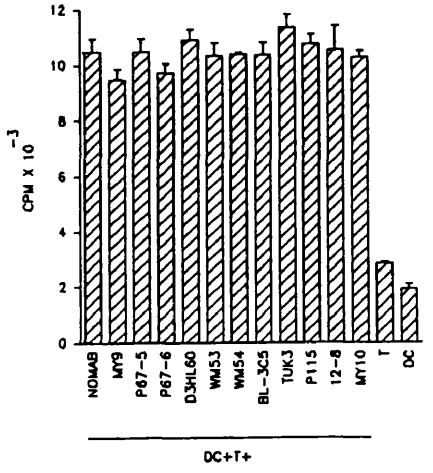

Fig. 6.38

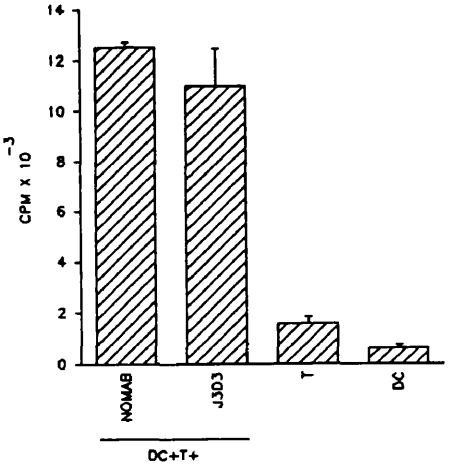

Fig. 6.39

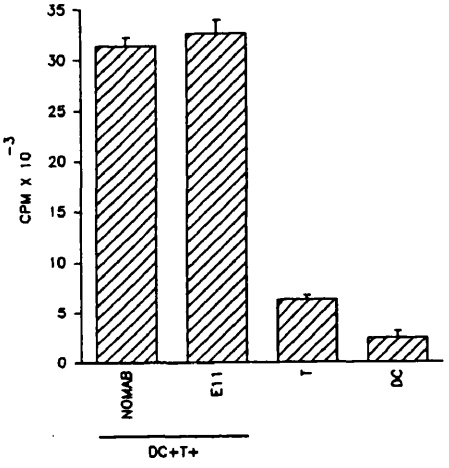

Fig. 6.40

a) b) c) d)

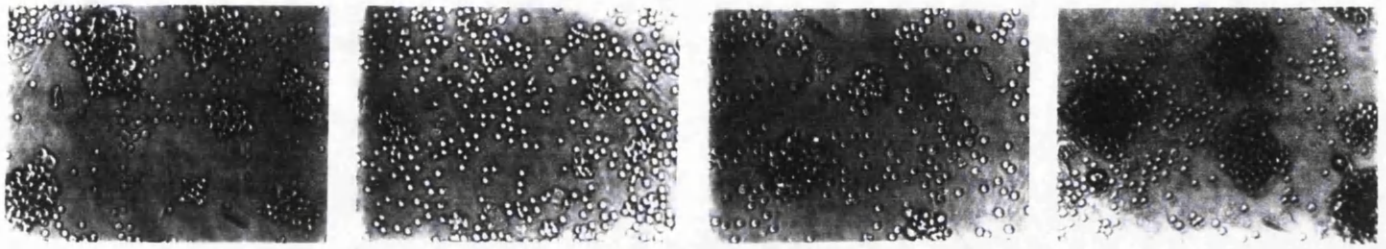

Fig. 6.41

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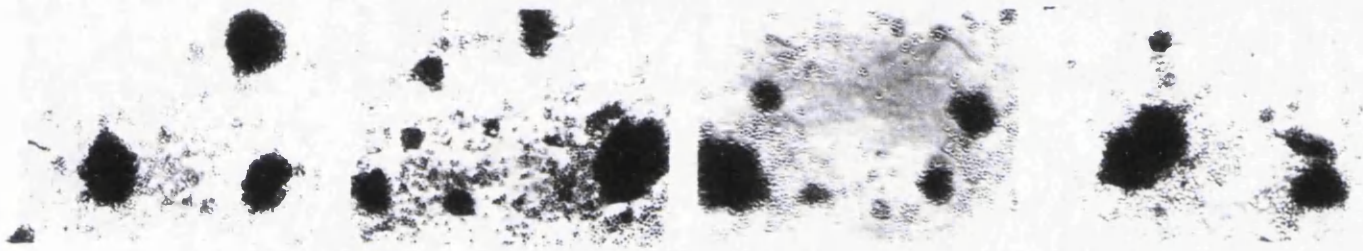

Fig. 6.42

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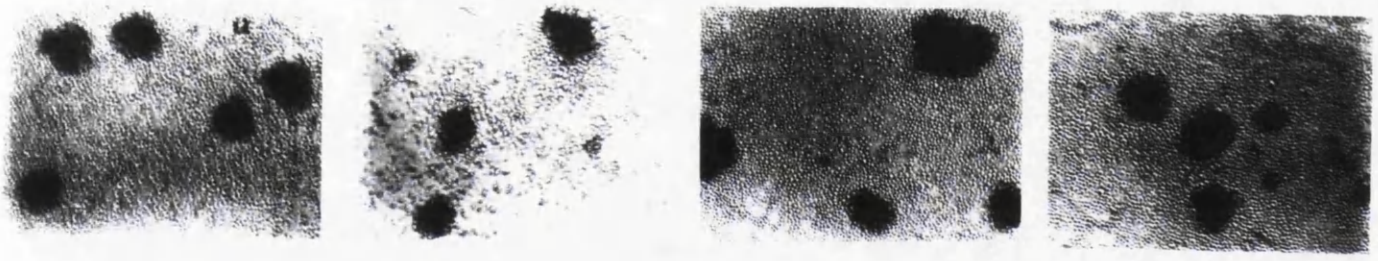


Fig. 6.43

a) b) c)

Fig. 6.44

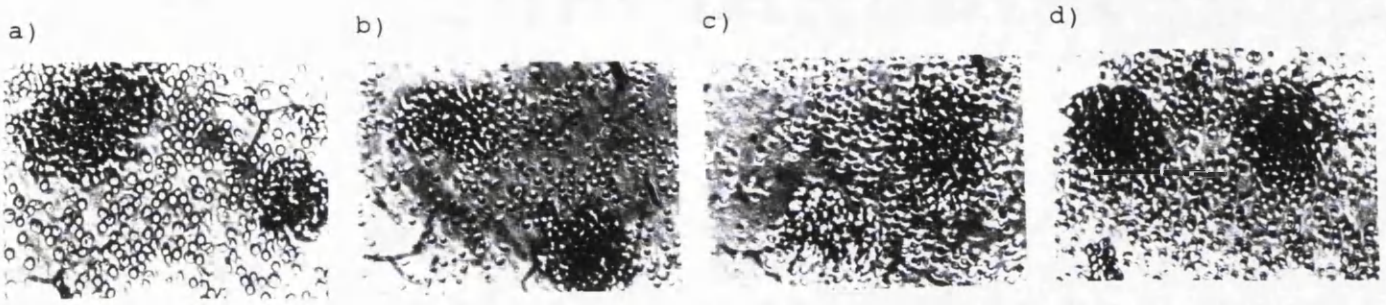


Fig. 6.45

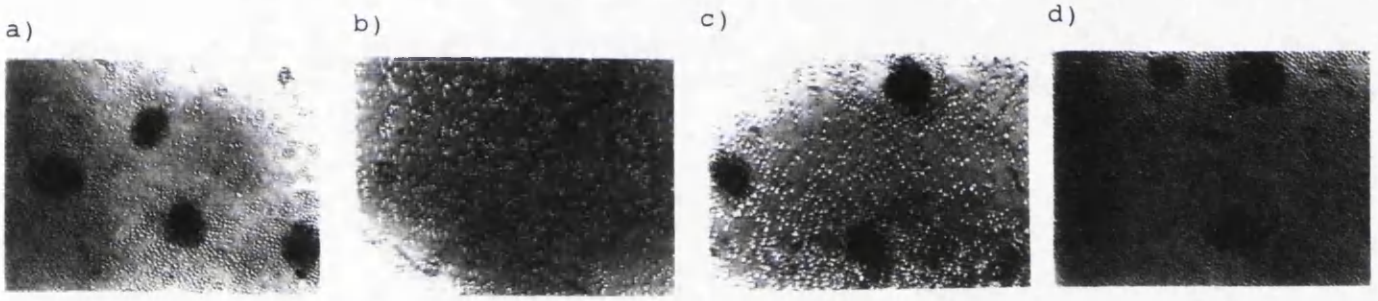


Fig. 6.46

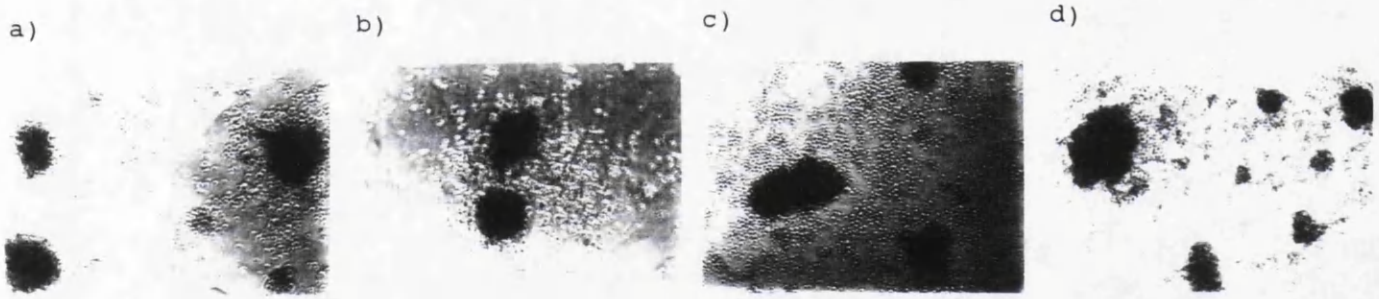


Fig. 6.47

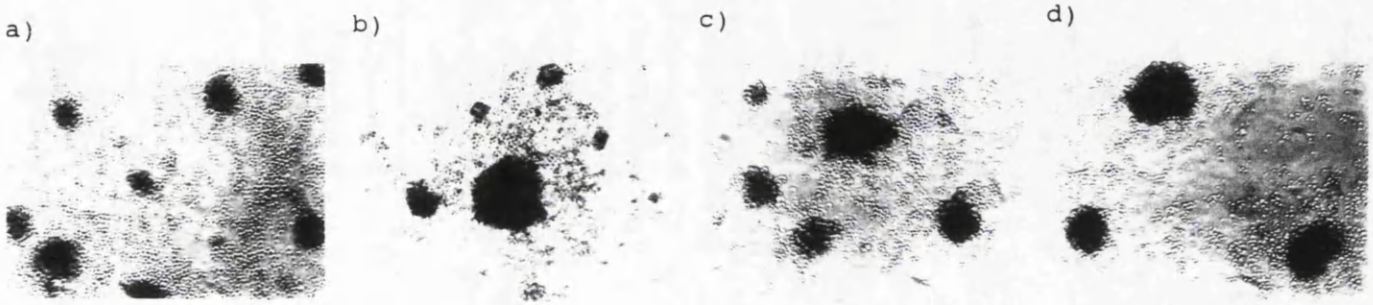


Fig. 6.48

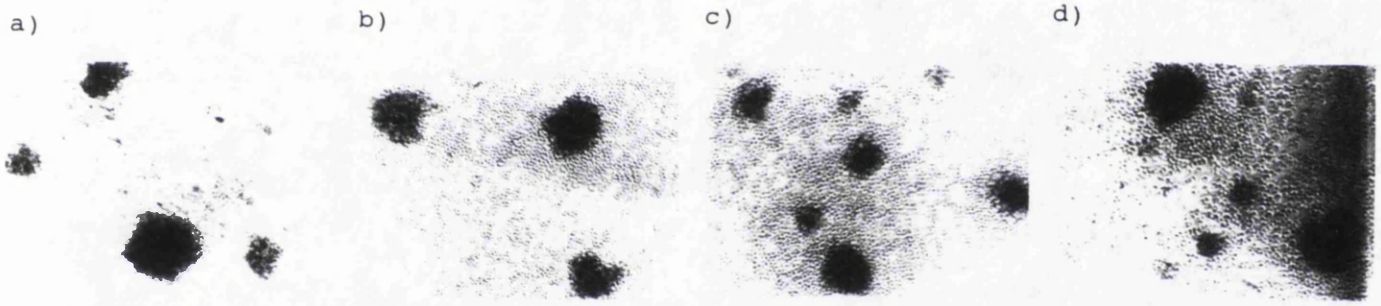


Figure Legends

See Materials and Methods plus:

Fig. 6.1. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well.

Fig. 6.2. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, M-T910 and T3 DF - 4 X 10^2.

Fig. 6.3. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, 2AD DF - 4 X 10^2.

Fig. 6.4. DC induced oxidative mitogenesis. DC number - 2 X 10^4/well.

Fig. 6.5. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, T4 DF - 4 X 10^2.

Fig. 6.6. DC induced oxidative mitogenesis. DC number - 3 X 10^4/well, all mAb DF - 2 X 10^2.

Fig. 6.7. DC induced oxidative mitogenesis. DC number - 5 X 10^4/well; T4 DF - 6 X10^2; inhibition at 0, 12 and 24 hr time points is SS; inhibition at 36 and 48 hr time points is not SS.

Fig. 6.8. B lymphocyte induced oxidative mitogenesis. B lymphocyte number - 5 X 10^4/well; T4 DF - 6 X 10^2; inhibition at 0, 12, 24 and 36 hr time points is SS; inhibition at the 48 hr time point is not SS.

Fig. 6.9(a). DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, UCHT4 DF - 20.

Fig. 6.9(b). DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, UCHT4 DF - 10.

Fig. 6.9(c). DC induced oxidative mitogenesis. DC number - 5 X 10^4/well, UCHT4 DF - 10.

Fig. 6.9(d). DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, UCHT4 DF - 50.

Fig. 6.10. DC induced oxidative mitogenesis. DC number - 2 X 10^4/well.

Fig. 6.11(a,b). DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, T8 DF - 4 X 10^2.

Fig. 6.12. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, all mAb DF - 4 X 10^2, T1LP and LOTact5 enhancements are SS, PANL7D inhibition and F1 enhancement is not SS.

Fig. 6.13. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, KOLT-2 DF - 4 X 10^2.

Fig. 6.14. DC induced oxidative mitogenesis. DC number - 5 X 10^4/well, all mAb DF - 4 X 10^2.

Fig. 6.15(a,b). DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, B1 DF - 4 X 10^2.

Fig. 6.16. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, MHM6 DF - 1 X 10^2.

Fig. 6.17. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well.

Fig. 6.18. DC induced oxidative mitogenesis. DC number - 8 X 10^4/well.

Fig. 6.19. DC induced oxidative mitogenesis. DC number - 5 X 10^4/well, all antibody DF - 40.

Fig. 6.20. Macrophage and B lymphocyte induced oxidative mitogenesis. Macrophage and B lymphocyte number - 1 X 10^5/well, MHM6 DF - 10^2.

Fig. 6.21. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, NOE2/61C6 DF - 4 X 10^2.

Fig. 6.22. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, all mAb DF - 4 X 10^2, OKT28 and A1 enhancements are SS, B-E10 inhibition is not SS.

Fig. 6.23. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, HD66 and MR11 DF - 4 X 10^2.

Fig. 6.24. DC induced oxidative mitogenesis. DC number - 4 X 10^4/well.

Fig. 6.25. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well.

Fig. 6.26. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, UCHM1 (AF) DF - 50.

Fig. 6.27. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, MEM-18 and M5E2 DF - 80.

Fig. 6.28. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, MEM-15 DF - 4 X 10^2.

Fig. 6.29. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, VIM-13 DF - 4 X 10^2.

Fig. 6.30. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, CLBFcRGranI DF 4 X 10^2.

Fig. 6.31(a,b). DC induced oxidative mitogenesis. DC number - 1 X 10^5/well.

Fig. 6.32. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, all mAb DF - 4 X 10^2, all inhibitions and enhancements are not SS.

Fig. 6.33. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, all mAb DF - 4 X 10^2.

Fig. 6.34. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, 2E1 DF - 50.

Fig. 6.35. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, all mAb DF - 4 X 10^2.

Fig. 6.36. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, 22.2 and 32.2 DF - 4 X 10^2.

Fig. 6.37. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, all

mAb DF - 4 X 10^2.

Fig. 6.38. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, J3D3 DF - 50.

Fig. 6.39. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, E11 DF - 50.

Figs. 6.40-6.48 - DC-T lymphocyte clustering

Fig. 6.40. Oxidative mitogenesis. All photographed at 4 hr, (a) DC+T, (b) T alone, (c) DC+T+TS2/18.1.1. (CD2), (d) DC+T+Leu3a (CD4).

Fig. 6.41. Oxidative mitogenesis. All photographed at 12 hr, all DC+T plus (a) NomAb, (b) 2AD (CD3), (c) T3 (CD3), (d) T4 (CD4).

Fig. 6.42. Oxidative mitogenesis. All photographed at 12 hr, all DC+T plus (a) NomAb, (b) LOTact5 (CD5), (c) KOLT-2 (CD28), (d) F1 (TcR V-beta gene product?).

Fig. 6.43. Oxidative mitogenesis. All photographed at 12 hr, all DC+T plus (a) NomAb, (b) PANL7D (CD7), (c) T8 (CD8).

Fig. 6.44. Oxidative mitogenesis. All photographed at 12 hr, all DC+T plus (a) NomAb, (b) FMC56 (CD9), (c) VILA1 (CD10), (d) VIBE3 (CD24).

Fig. 6.45. Oxidative mitogenesis. All photographed at 12 hr, (a) DC+T, (b) T alone, (c) DC+T+B1 (CD20), (d) DC+T+B-E10 (CD40).

Fig. 6.46. Oxidative mitogenesis. All photographed at 12 hr, all DC+T plus (a) NomAb, (b) NOE2/6/C6 (CD38), (c) A1 (CD39), (d) OKT28 (CD39).

Fig. 6.47. Oxidative mitogenesis. All photographed at 12 hr, all DC+T plus (a) NomAb, (b) MEM-15 (CD14), (c) SG134 (CD31), (d) LAK-1 (CD31).

Fig. 6.48. Oxidative mitogenesis. All photographed at 12 hr, all DC+T plus (a) NomAb, (b) VIM-13 (CD14), (c) 22.2 (CD64), (d) 32.2 (CD64).

Discussion

Functional roles for CD2, CD3, CD4, CD5, CD28, CD39 and CDw78 antigens in tonsillar dendritic cell induced oxidative mitogenesis. In this Chapter it has been demonstrated that CD2, CD3, CD4 and CDw78 antibodies inhibit proliferation and that CD5, CD28 and CD39 antibodies enhance proliferation in tonsillar DC induced oxidative mitogenesis. In contrast, none of a particular V-beta gene product, CD7, CD8, CD9, CD10, CD13, CD14, CD16, CD20, CD23, CD24, CD31, CDw32, CD33, CD34, CD35, CD38, CD40, CD64 and CD76 antibodies had any effect. Also, it has been shown that CD2 antibodies inhibit DC-T lymphocyte clustering in this reaction whereas none of the other antibodies that were tested had any influence upon clustering. These findings, therefore, suggest that the CD2 antigen functions at least as an adhesion molecule and that the TcR-CD3 antigen complex (albeit not those carrying one particular V-beta gene product), CD4, CD5, CD28, CD39 and CDw78 antigens function in signal transduction in this response. None of the antigens recognised by the other antibodies, however, appear to play any role.

Involvement of an additional adhesion molecule pair in dendritic cell-T lymphocyte clustering. The identification of the CD2 antigen as a mediator of DC-T lymphocyte clustering readdresses the issue of the potency of DC accessory activity (see Chapter 5). Because the CD2 antigen is restricted in it's expression to T lymphocytes and NK cells (see also Chapter 2), one hypothesis would be that the well recognised ligand of the CD2 antigen, LFA-3, is hyper-expressed upon DC; and in support of this is the recent observation that a CD2 antigen/LFA-3 interaction plays no role in human monocyte-T lymphocyte adhesion (500). In fact, however, in this study LFA-3 was found to be comparably expressed upon tonsillar DC and macrophages (see

Chapter 2) suggesting that some other factor controls an enhanced ability of DC to cluster T lymphocytes. Also, this finding, if taken with the assumption that the CD2 antigen and LFA-3 play no role in tonsillar macrophage-T lymphocyte clustering either, suggests that in tonsillar DC-T lymphocyte clustering some other factor, apart from the level of expression of LFA-3 upon DC, permits a CD2 antigen/LFA-3 interaction to occur.

Once again, in both of these cases, it is tempting to speculate that a low cell surface negative charge upon DC is this important other factor. How a low cell surface negative charge might mitigate in favour of potent accessory activity has been discussed before (see page 75 and Chapter 5) but further to this, because of the relatively short lengths of the CD2 antigen and LFA-3 (512), then a smaller degree of like-like charge mediated repulsion between DC and T lymphocytes might be a requirement for the interaction of these two molecules at least to such an extent that they participate in accessory cell-T lymphocyte adhesion.

In general, CD2 antibodies appeared to cause less inhibition of DC-T lymphocyte clustering than LFA-1 and ICAM-1 antibodies (see Chapter 5 and Fig. 6.40) suggesting that CD2 antigen and LFA-3 play only a minor role in clustering in contrast to LFA-1 and ICAM-1 which play a major role. The reasons for this might be several fold. For example, owing to residual charge repulsion between DC and T lymphocytes the full potential of a CD2 antigen/LFA-3 interaction may not be realised. Also, a lower affinity of a CD2 antigen/LFA-3 interaction versus an LFA-1/ICAM-1 interaction could be responsible as might a lower level of expression of CD2 antigen and LFA-3 versus LFA-1 and ICAM-1 at DC and/or T lymphocyte surfaces (see Chapter 2). On this last point, the absence of CD2 antigen from DC would mean that an interaction of this molecule with LFA-3 could only be unidirectional, i.e. from T lymphocyte to DC, in contrast to an LFA-1/ICAM-1 interaction which

has the potential for bi-directionality.

There is evidence that the CD2 antigen is also involved in signal transduction in tonsillar DC induced oxidative mitogenesis. Thus, although CD2 antibodies only partially inhibit clustering they almost completely block proliferation. Most likely this signal transduction role is as a receptor for LFA-3 "first signals" (see page 4). In addition, however, based upon evidence that LFA-3 antibodies induce the release of IL-1 from monocytes (513), the CD2 antigen might also function as a ligand for LFA-3 and induce the release of this cytokine from DC.

Role of the TcR-CD3 antigen complex: CD3 antibodies probably block tonsillar dendritic cell induced oxidative mitogenesis indirectly. The finding that the CD3 antigen is involved in signal transduction in tonsillar DC induced oxidative mitogenesis provides strong confirmatory evidence that TcR complex recognition of "first signals" in the form of MHC plus antigen, is an important event in this response (see Chapters 3 and 4). How CD3 antibodies achieve inhibition of proliferation, however, is unclear although the fact that the TcR complex exists preformed upon the surface of T lymphocytes and the fact that the CD3 antigen is conceived of as the signal relaying element of the complex (see page 49) suggests that the inhibition is probably indirect.

One way in which the CD3 antibodies could effect indirect inhibition is by antibody mediated cell surface down regulation of the TcR complex (514,515). As well as blockading the T lymphocyte acceptance of MHC plus antigen "first signals", this down regulation would also result in the blockade of the T lymphocyte acceptance of LFA-3 "first signals" ("first signals" delivered through the CD2 antigen are known to be dependent upon cell surface expression of the TcR complex, 514,515). Further, if a TcR complex/MHC plus antigen interaction leads to the release of "second

signals" from DC then down regulation of the TcR complex would also result in the blockade of this process.

Other ways in which the CD3 antibodies could achieve indirect blockade are by the induction of conformational changes in or by steric hindrance of the TcR complex. This might occur for the MHC plus antigen binding pocket in the TcR such that MHC plus antigen can no longer be recognised. Alternatively, CD3 antibodies may induce conformational changes in CD3 antigen components such that effective signal relaying by the TcR complex is prevented. In both of these cases this would result in the blockade of the acceptance of MHC plus antigen "first signals" and in the former case again possibly the induction of "second signals" from DC.

It might also be important to consider the evidence that in their augmentation of MHC plus antigen "first signals" (see Chapter 4 and below), CD4 and CD8 antigens, by virtue of their association with the tyrosine kinase $p56^{lck}$, tyrosine phosphorylate the zeta chain of the CD3 antigen and that in this role binding to the zeta chain is required (516-520). Therefore, CD3 antibodies may inhibit proliferation by blocking an incorporation of the CD4 and CD8 antigens into the TcR complex thus preventing zeta chain phosphorylation; and that such blockade is probably also indirect is suggested by the fact that the great majority of CD3 antibodies studied to date bind the epsilon and not zeta chain of the CD3 antigen (521,522).

A common feature of each of the above models is a blockade of CD3 antigen function. Hence, it would seem unlikely that the TcR complex plays an indirect role in DC-T lymphocyte clustering in this response, that has perhaps not been detected by the CD3 antibodies used here, by increasing the avidity of LFA-1 for ICAMs (assuming that in such a putative role the TcR complex would also use CD3 antigen components to relay information). That

the TcR complex plays a direct role in clustering, however, is not completely excluded. Thus, in two of the above models, i.e. that CD3 antibodies prevent a signal relaying function of the CD3 antigen per se or by blocking an integration of the CD4 and CD8 antigens into the TcR complex, CD3 antibodies can be viewed as inhibiting CD3 antigen function without affecting either the level of cell surface expression or conformation of the TcR.

Finally, it is worth a mention that despite the functional effects of CD3 antibodies upon tonsillar DC induced oxidative mitogenesis an antibody (F1) which possibly reacts with a particular TcR V-beta gene product did not affect proliferation (or clustering) in this response. This could indicate either that T lymphocytes which express the F1 V-beta gene product do not proliferate in tonsillar DC induced oxidative mitogenesis or that the frequency of F1 expressing T lymphocytes is too small to allow the detection of functional effects.

The dichotomy between the effects of CD4 and CD8 antibodies. The demonstration that the CD4 antigen is involved in signal transduction in tonsillar DC induced oxidative mitogenesis adds weight to the concept that a class II MHC/CD4 antigen interaction plays role in this response, serving to augment "first signals" delivered through the TcR (see Chapter 4). Each of CD4 antibodies used in this study have been shown to bind to a region of the CD4 antigen involved in class II MHC binding (486,523-526) and hence all probably block this function of CD4 antigen directly.

With regards the time course inhibition studies, CD4 antibodies were different from class II MHC antibodies in that they only inhibited tonsillar DC and B lymphocyte induced oxidative mitogenesis when added to cultures at early and intermediate time points. This may seem surprising if the CD4 antigen and class II MHC interact with each other in these responses but can

be explained in terms of an inability of the particular class II MHC antibodies used to block not only an interaction of class II MHC with the CD4 antigen but also with the TcR. In this view, further to a previous discussion (see page 133), it is a class II MHC/TcR interaction that would be of low affinity (i.e. equilibriated) and might well occur at an early stage, in contrast to a class II MHC/CD4 antigen interaction which would be of relatively high affinity (i.e. non-equilibriated) and would occur at a later stage (around 24-36 hr post culture initiation).

The finding that CD8 antibodies did not affect proliferation in tonsillar DC induced oxidative mitogenesis would seem to cast further doubt upon an analogous interaction between class I MHC and the CD8 antigen involved in the activation of CTL (see Chapter 4). Rather than completely excluding the notion of such a functional interaction, however, there are two alternative explanations that can be forwarded to explain this observation. Firstly, as previously discussed, CD8 antigen binding to class I MHC might induce the release of a suppressor factor from DC. Therefore, although CD8 antibodies might block "first signals" delivered to CTL this effect could be balanced by an inhibition of the release of this suppressor factor. Secondly, it is possible that both of the CD8 antibodies used here detect non-functional epitopes upon the CD8 antigen, and certainly that this might be the case for the UCHT4 antibody is suggested by it's inability to block the cytotoxic activity of an alloreactive CTL clone (527)

The role of CD5 and CD28 antigens as receptors for "second signals" in tonsillar dendritic cell induced oxidative mitogenesis. It is now widely accepted that cytokines are not the only "second signals" for T lymphocytes. In this context antibodies against several different T lymphocyte cell surface molecules have been shown to boost T lymphocyte proliferative responses initiated by suboptimal concentrations of immobilised CD3

antibodies or pairwise combinations of CD2 antibodies but themselves (and unlike CD3 or CD2 antibodies) are unable to induce proliferation. Two such molecules are the CD5 (528,529) and CD28 (531-534) antigens and the inference is thus that each acts a receptor for alternative T lymphocyte "second signals" (in this view the antibodies can be thought of as functionally mimicking these alternative "second signals").

Given this then, function as T lymphocyte "second signal" receptors is a possible signal transduction role of the CD5 and CD28 antigens in tonsillar DC induced oxidative mitogenesis (again functional mimicry of CD5 and CD28 antigen ligands is envisaged). One problem with this interpretation, however, is the recent identification of the CD28 antigen ligand as the B lymphocyte restricted BB1/B7 antigen (535,536). This raises the question as to whether or not CD5 and CD28 antigens are are genuinely involved in tonsillar DC induced oxidative mitogenesis or whether effects observed with CD5 and CD28 antibodies are unrelated to normal proliferation induction events in this response. In the case of the CD28 antigen, though, perhaps it is too early to exclude a "second signal" receptor role for although the BB1/B7 antigen is described as B lymphocyte restricted it's expression upon DC has not yet been examined.

Activation induction of the CD39 antigen at the T lymphocyte surface and of the CDw78 antigen at the dendritic cell surface probably accounts for the effect of CD39 and CDw78 antibodies upon tonsillar dendritic cell induced oxidative mitogenesis. In most studies the CD39 antigen is described as B lymphocyte restricted (537,538). In fact, this molecule is expressed upon activated T lymphocytes as well (539,540) and, moreover, in this study was shown to be expressed upon tonsillar DC (see Chapter 2). In tonsillar DC induced oxidative mitogenesis, therefore, in it's signal transduction role the CD39 antigen could act at either or both of the T lymphocyte and DC

level, although the fact that both CD39 antibodies similarly enhanced proliferation yet only one of these (A1) stained DC favours a role upon the former cell type only. The CD39 antigen has been implicated in accessory function before (541) but clues as to it's precise signal transduction role have not yet been forthcoming. In tonsillar DC-T lymphocyte interaction enhancement by CD39 antibodies means that any of a role as a receptor in a positive signalling pathway or a receptor or ligand in a negative signalling pathway are possible.

In contrast to the CD39 antigen the "B lymphocyte restricted" CDw78 antigen (542,543) is not expressed upon T lymphocytes (resting or activated) and was also found not to be expressed upon isolated tonsillar DC (see Chapter 2). A preliminary report, however, has described the expression of this antigen upon tonsillar and splenic interdigitating DC in situ (544) and thus one possibility is that the CDw78 antigen is induced upon DC during the course of their interaction with T lymphocytes, whereupon it executes it's signal transduction role. The CDw78 antigen has not previously been implicated in accessory function and in this interaction that CDw78 antibodies inhibited proliferation leaves open the possibilities of function as a receptor or ligand in a positive signalling pathway or a receptor in a negative signalling pathway.

Summary

In this Chapter it has been demonstrated that CD2, CD3, CD4, CD5, CD28, CD39 and CDw78 antigens play a role in T lymphocyte proliferation induction in tonsillar DC induced oxidative mitogenesis. In contrast, CD7, CD8, CD9, CD10, CD13, CD14, CD16, CD20, CD23, CD24, CD31, CDw32, CD33, CD34, CD35, CD38, CD40, CD64 and CD76 antigens are not involved. The function of the CD2 antigen relates to the mediation of DC-T lymphocyte clustering and in this role the CD2 antigen likely interacts with LFA-3. The CD2 antigen, however, again via an interaction with LFA-3, is probably also involved in signal transduction in this response acting as both a receptor for T lymphocyte "first signals" and a ligand which induces the release of "second signals" from DC. The other implicated molecules function in signal transduction only in tonsillar DC induced oxidative mitogenesis. For the CD3 and CD4 antigens this signal transduction role is again likely to be T lymphocyte acceptance of "first signals", i.e. acceptance per se as a component of the TcR complex and phosphorylation of the CD3 antigen respectively; and for the CD4 antigen in its interaction with class II MHC in this role (in tonsillar DC and B lymphocyte induced oxidative mitogenesis) there is evidence that this is a relatively late event. The signal transduction roles of the CD5 and CD28 antigens, by contrast, is probably as T lymphocyte "second signal" receptors. Lastly, the signal transduction roles of the CD39 and CDw78 antigens is unclear. Both of these molecules, however, probably perform their roles following their activation induction upon DC or T lymphocytes.

CHAPTER 7

EFFECT OF NON-LINEAGE RESTRICTED ANTIBODIES UPON TONSILLAR DENDRITIC CELL-T LYMPHOCYTE INTERACTION

Non-lineage restricted antigens have also not been examined in DC induced T lymphocyte responses but again the results of many other studies predict that several might play important roles (see Introduction to Chapter 6). In particular, the CD45/LCA family of antigens might be expected to function in signal transduction and the CD44 antigen might be expected to function in clustering and signal transduction. Also, previous studies as well as studies presented in Chapter 6, suggest a role for the CD58 antigen/LFA-3 molecule in clustering and signal transduction. In this Chapter, therefore, to examine the role of non-lineage restricted antigens in tonsillar DC induced oxidative mitogenesis, panels of CD43, CD44, CD45, CD45-like, CD45RA, CD45RO, CD45R, CD46, CD47, CD48, CDw52, CD53, CD55, CD56, CD57, CD58 and CD59 antibodies have each been tested for their effects upon proliferation and DC-T lymphocyte clustering in this response.

MATERIALS AND METHODS

Cells. DC and high density T lymphocytes were isolated from human tonsils as described in Chapter 2.

Proliferation assays. Oxidative mitogenesis assays were performed as indicated in Chapter 3 using varying numbers per well of irradiated DC as accessory cells and 2 X 10^5 per well of 2 mM periodate modified autologous high density T lymphocytes as responder cells. Non-lineage restricted antibodies were also added to wells (all at culture initiation) in varying concentrations in 20 ul quantities. All assays were pulsed with ^{125}IdUrd at 48 hr and terminated at 64 hr.

Assessment of cellular clustering. Clustering of DC and 2 mM periodate modified autologous high density T lymphocytes, in the presence (added at time zero) and absence of non-lineage restricted antibodies, and clustering of 2 mM periodate modified autologous high density T lymphocytes cultured alone, was assessed visually and photographed at 4 and 12 hr following culture initiation (see Chapter 4). Both oxidative mitogenesis assays and independent cultures were used for this purpose.

Antibodies. Details of G19-1 and OTH71C5 (CD43); F10-44-2 (CD44); F10-89-4, 136-4B5 and 562/10D3 (CD45); UCHL1 (CD45RO); 4KB5 (CD45R); HuLym5 (CD46); BRIC126 (CD47); WM68 (CD48); 097 (CDw52); MEM-53 (CD53); 143-30 (CD55); NKH1a (CD56); L183 (CD57); and TS2/9 (CD58) antibodies are given in Table 2.1. A list of other non-lineage restricted mAb used in proliferation and clustering assays is shown in Table 7.1. All of these antibodies were obtained as AF preparations from the Fourth International Workshop on Leucocyte Differentiation Antigens. Cluster designations are according to the Third and Fourth Workshops. All antibodies were sterile filtered,

dialysed and deaggregated as appropriate (see Chapter 4).

Statistics. See Chapters 3,4 and 5.

Table 7.1 - Additional non-lineage restricted antibodies

Antibody	CD grouping/ specificity	Species of origin[a)]	Isotype
GRHL1	CD44	M	G1
AA14	CD45	M	G1
AB103	CD45	M	G1
AB187	CD45	M	G1
BMAC1	CD45	M	G1
BMAC2	CD45	M	G1
BMAC3	CD45	M	G1
BRA55	CD45	M	G1
D3/9	CD45	M	G1
GB3	CD45	M	G1
GRT2	CD45	M	G1
GRT3	CD45	M	G1
GRT4	CD45	M	G1
HI30	CD45	M	G1
HI73	CD45	M	G2a
HuLym3	CD45	M	G2a
IOR-L3	CD45	M	G1
NU-LPAN	CD45	M	G1
RP1/10	CD45	M	
TL-1	CD45	M	G1
Tu116	CD45	M	G2b
T2/48	CD45	M	G1
T29/33	CD45	M	M
U87	CD45	M	G1
X16	CD45	M	
YTH54.12	CD45	R	G2b
135-4C5	CD45	M	G2b
135-4H9	CD45	M	G2b
138-3	CD45	M	M
144-2	CD45	M	M
155-2	CD45	M	M
71.5	CD45	M	G2a
80.2	CD45	M	G1
MEM-58	CD45-like	M	G1
Tu150	CD45-like	M	M
73.5	CD45RA	M	G1
F8-11-13	CD45RA	M	G1
G1-15	CD45RA	M	G1
BL-4D4	CD45R	M	G1
ITM-C1	CD45R	M	G1
KD3	CD45R	M	G1
MB1	CD45R	M	G1
MEM-93	CD45R	M	G1
MT2	CD45R	M	G1
RP2/3	CD45R	M	G2b
TM-H5	CD45R	M	G2a
WR16	CD45R	M	G1
X148	CD45R	M	
YTH80.103	CD45R	R	G2b
J48	CD46	M	G1

Antibody	CD grouping/ specificity	Species of origin	Isotype
122-2	CD46	M	G1
CIKM1	CD47	M	G1
J4-57	CD48	M	G1
LO-MN25	CD48	R	G2a
Tu145	CD48	M	M
WM63	CD48	M	G2a
YTH361.10	CDw52	R	G2c
YTH66.9	CDw52	R	M
HI29	CD53	M	G1
BRIC128	CD55	M	M
FP2-11.4	CD56	M	G2a
Leu-19	CD56	M	G1
NKH1	CD56	M	G1
T-199	CD56	M	G1
Leu-7	CD57	M	M
L186	CD57	M	M
BRIC5	CD58	M	G2a
G26	CD58	M	M
YTH53.1	CD59	R	G2b

a) M - mouse, R - rat

RESULTS

Effect of non-lineage restricted antibodies upon dendritic cell induced oxidative mitogenesis

CD43 and CD44 antibodies. Fig. 7.1 shows the results of an experiment in which two different CD43 antibodies, OTH71C5 and G19-1, were tested for their effects upon DC induced oxidative mitogenesis. Neither antibody significantly affected T lymphocyte proliferation when added to the reaction at time zero.

The effect of two CD44 antibodies, GRHL1 and F10-44-2, upon DC induced oxidative mitogenesis is shown in Fig. 7.2. In contrast to the CD43 antibodies, both CD44 antibodies significantly enhanced T lymphocyte proliferation when added to cultures at time zero.

CD45, CD45-like, CD45RA, CD45RO and CD45R antibodies. The effect of a panel of CD45 antibodies upon DC induced oxidative mitogenesis is summarised in Fig. 7.3. In total, thirty five different CD45 antibodies were tested. Of these, twelve antibodies inhibited proliferation (138-3, 144-2, 155-2, GRT3, GRT2, T29/33, AB187, BRA55, IOR-L3, YTH54.12, 80.2 and 71.5), two antibodies enhanced proliferation (HI73 and 562/10D3) and twenty one antibodies did not affect proliferation (135-4C5, 135-4H9, 136-4B5, U87, AB103, AA14, X16, GRT4, HI30, TL-1, F10-89-4, BMAC1, BMAC2, BMAC3, RP1/10, D3/9, GB3, T2/48, Tu116, HuLym3 and NU-LPAN) when incorporated into cultures at time zero.

Two CD45-like antibodies (MEM-58 and Tu150), three CD45RA antibodies (F8-11-13, G1-15 and 73.5), one CD45RO antibody (UCHL1) and twelve CD45R antibodies (BL-4D4, WR16, X148, 4KB5, MEM-93, RP2/3, YTH80.103, MB1, MT2, KD3, TM-H5 and ITM-C1) were also screened for their effects upon DC induced oxidative mitogenesis. The results are summarised in Fig. 7.4 which shows that one CD45-like antibody (Tu150) inhibited proliferation in contrast to

the other antibodies which did not affect proliferation.

CD46, CD47 and CD48 antibodies. Fig. 7.5 shows the results of an experiment in which two CD46 antibodies (122-2 and J48), two CD47 antibodies (BRIC126 and CIKM1) and two CD48 antibodies (Tu145 and WM63) were screened for their effects upon DC induced oxidative mitogenesis. When incorporated into cultures at the outset, the CD46 and CD47 antibodies had no effect upon T lymphocyte proliferation. However, both CD48 antibodies strongly inhibited the proliferative response.

In another experiment (Fig. 7.6) the effects of an additional CD46 antibody (HuLym5) and three additional CD48 antibodies (WM68, LO-MN25 and J4-57) were examined. With respect to the CD46 antibody, again, no inhibitory or enhancing effect upon proliferation was apparent. However, in contrast to the proliferation inhibiting effects of the Tu145 and WM63 CD48 antibodies (Fig. 7.5), each of the CD48 antibodies tested in this experiment also had no significant effect upon T lymphocyte proliferation.

CDw52 and CD53 antibodies. Figs. 7.7 and 7.8 depict the results of two different experiments in which CDw52 and CD53 antibodies were incorporated into the DC induced oxidative mitogenesis reaction at time zero. As shown, none of the three CDw52 antibodies [097 and YTH66.9 (Fig. 7.7) and YTH361.10 (Fig. 7.8)] that were tested and neither of the CD53 antibodies [HI29 (Fig. 7.7) and MEM-53 (Fig. 7.8)] that were tested significantly affected T lymphocyte proliferation.

CD55, CD56 and CD57 antibodies. The effect of a CD55 antibody, 143-30, upon DC induced oxidative mitogenesis is shown in Fig. 7.9. 143-30 failed to affect T lymphocyte proliferation in this response when incorporated into cultures at the time of culture initiation. Also shown in Fig. 7.9 is the effect of a CD56 antibody, NKH1, in the same experiment. NKH1, too, failed

to affect T lymphocyte proliferation and another CD56 antibody, FP2-11.4, also had no effect as determined in a separate experiment (Fig. 7.10).

The results of an experiment in which additional CD55 and CD56 antibodies were compared with CD57 antibodies for their effects upon DC induced oxidative mitogenesis are shown in Fig. 7.11. Again, neither the CD55 antibody (BRIC128) nor the CD56 antibodies (T-199, NKH1a and Leu-19) that were tested affected T lymphocyte proliferation and each of the CD57 antibodies (L186, Leu-7 and L183) that were examined also had no effect.

CD58 and CD59 antibodies. The effects of two CD58 antibodies, TS2/9 and BRIC5, upon DC induced oxidative mitogenesis are shown in Figs. 7.12 and 7.13 respectively. When incorporated into cultures at time zero both antibodies inhibited T lymphocyte proliferation and for TS2/9 this effect was shown to be dependent upon the concentration of added antibody.

Fig. 7.14 shows the results of another experiment in which an additional CD58 antibody, G26, and a CD59 antibody, YTH53.1, were compared for their effects upon T lymphocyte proliferation in this response. Like TS2/9 and BRIC5, the G26 CD58 antibody inhibited proliferation. In contrast, the CD59 antibody had no effect on proliferation.

Effect of non-lineage restricted antibodies upon dendritic cell-T lymphocyte clustering in dendritic cell induced oxidative mitogenesis

CD43 and CD44 antibodies. The effects of the CD43 antibodies, OTH71C5 and G19-1, and the CD44 antibodies, GRHL1 and F10-44-2, upon DC-T lymphocyte clustering in oxidative mitogenesis are shown in Figs. 7.15 and 7.16 respectively. Neither of the CD43 antibodies affected DC-T lymphocyte clustering when incorporated into cultures at time zero. Similarly, the CD44 antibodies also had no effect upon clustering.

CD45, CD45-like, CD45RA, CD45RO and CD45R antibodies. Fig. 7.17 shows the effects of the BRA55, BMAC3, IOR-L3, RP1/10, D3/9, 71.5, Tu116, T29/33, HuLym3 and NU-LPAN CD45 antibodies upon DC-T lymphocyte clustering in oxidative mitogenesis. RP1/10, D3/9 and 71.5 enhanced clustering when incorporated into cultures at time zero (a,e-g). In contrast, BRA55, BMAC3, IOR-L3, Tu116, T29/33, HuLym3 and NU-LPAN had no effect upon clustering when incorporated into cultures at the same time (a-d,h-k).

Each of the other CD45 antibodies that were tested for their effects upon T lymphocyte proliferation in DC induced oxidative mitogenesis were also tested for their effects upon DC-T lymphocyte clustering in this response. Again, antibodies either enhanced clustering, i.e. 135-4C5, 135-4H9, 136-4B5, U87, AA14, X16, GRT4, HI30, TL-1, F10-89-4, BMAC1, 138-3, 144-2, 155-2, GRT3, GRT2 and 80.2 antibodies, or did not affect clustering, i.e. AB103, BMAC2, GB3, T2/48, AB187, YTH54.12, HI73 and 562/10D3 antibodies (not shown).

The effects of the whole panel of CD45 antibodies upon both proliferation and clustering in DC induced oxidative are summarised in Table 7.2. Of the twelve antibodies that inhibited proliferation, seven enhanced clustering (138-3, 144-2, 155-2, GRT3, GRT2, 80.2 and 71.5) and five did not affect clustering (T29/33, AB187, BRA55, IOR-L3 and YTH54.12); of the twenty one antibodies that did not affect proliferation, thirteen enhanced clustering (135-4C5, 135-4H9, 136-4B5, U87, AA14, X16, GRT4, HI30, TL-1, F10-89-4, RP1/10, D3/9 and BMAC1) and eight antibodies did not affect clustering (AB103, BMAC3, BMAC2, GB3, T2/48, Tu116, HuLym3 and NU-LPAN); and finally of the two antibodies that enhanced proliferation (HI73 and 562/10D3) both had no effect upon clustering.

CD45-like, CD45RA, CD45RO and CD45R antibodies, all of which did not affect T lymphocyte proliferation in DC induced oxidative mitogenesis (with

Table 7.2 - Grouping of CD45 antibodies according to their effects upon proliferation and clustering in dendritic cell induced oxidative mitogenesis

Group 1 - Inhibitors of proliferation enhancers of clustering	Group 2 - Inhibitors of proliferation no effect on clustering
138-3, 144-2, 155-2, GRT3, GRT2, 80.2, 71.5	T29/33, AB187, BRA55, IOR-L3, YTH54.12

Group 3 - No effect on proliferation enhancers of clustering	Group 4 - No effect on proliferation or clustering
135-4C5, 135-4H9, 136-4B5, U87, AA14, X16, GRT4, HI30, TL-1, F10-89-4, RP1/10, D3/9, BMAC1	AB103, BMAC3, BMAC2, GB3, T2/48, Tu116, HuLym3, NU-LPAN

Group 5 - Enhancers of proliferation no effect on clustering

HI73, 562/10D3

the exception of the Tu150 CD45-like antibody which inhibited proliferation), were also tested for their effects upon DC-T lymphocyte clustering in this response. The Tu150 antibody and the CD45RO antibody, UCHL1, both enhanced clustering (not shown). The MEM-58 CD45-like antibody and the BL-4D4, WR16, X148, 4KB5 and MEM-93 CD45R antibodies (all Fig. 7.18), however, did not affect clustering and the CD45RA antibodies G1-15, F8-11-13 and 73.5 and the CD45R antibodies RP2/3, YTH80.103, MB1, MT2, KD3, TM-H5 and ITM-C1 also had no effect (not shown).

CD46, CD47, CD48, CDw52 and CD53 antibodies. The effects of the CD46, CD47, CD48, CDw52 and CD53 antibodies upon DC-T lymphocyte clustering in oxidative mitogenesis are depicted in Figs. 7.19 and 7.20. As shown, when incorporated into cultures at the outset, all three CD46 antibodies [HuLym5 (Fig. 7.19 a,b), 122-2 and J48 (Fig. 7.20 a-c)], one of the CD47 antibodies [BRIC126 (Fig. 7.20 a,d)], all five CD48 antibodies [including WM68, LO-MN25 and J4-57 (Fig. 7.19 c-e) which had no effect upon proliferation and Tu145 and WM63 (Fig. 7.20 a,f,g) which inhibited proliferation], all three CDw52 antibodies [097 and YTH66.9 (Fig. 7.19 a,f,g) and YTH361.10 (Fig. 7.20 a,h)] and both of the CD53 antibodies [HI29 (Fig. 7.19 a,h) and MEM-53 (Fig. 7.20 a,i)] had no effect upon DC-T lymphocyte clustering. However, the CIKM1 CD47 antibody enhanced DC-T lymphocyte clustering in this response when incorporated into cultures at the outset (Fig. 7.20 a,e).

CD55, CD56, CD57, CD58 and CD59 antibodies. Fig. 7.21 shows the effects of the CD55 antibody, 143-30, the CD56 antibody, FP2-11.4 and the CD59 antibody, YTH53.1 upon DC-T lymphocyte clustering in oxidative mitogenesis. None of these antibodies affected clustering when incorporated into cultures at time zero.

The effect of the CD55 antibody, BRIC128, the CD56 antibodies, T-199,

NKH1a and Leu-19, and the CD57 antibodies, L186, Leu-7 and L183 upon clustering are shown in Fig. 7.22. Again, the CD55 and CD56 antibodies did not affect clustering and the CD57 antibodies also had no effect.

Fig. 7.23 shows the effect of the TS2/9 CD58 antibody upon clustering. TS2/9 inhibited clustering when incorporated into cultures at time zero.

With respect to the other CD56 antibody, NKH1, and the two other CD58 antibodies, BRIC5 and G26, that were tested for their effects upon T lymphocyte proliferation in DC induced oxidative mitogenesis, the NKH1 antibody had no effect upon clustering. However, like the TS2/9 CD58 antibody, BRIC5 and G26 inhibited DC-T lymphocyte clustering (not shown).

Fig. 7.1

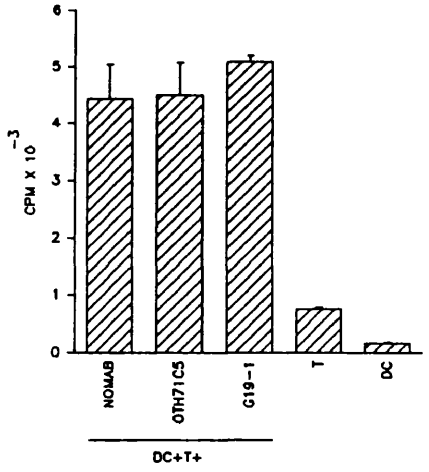


Fig. 7.2

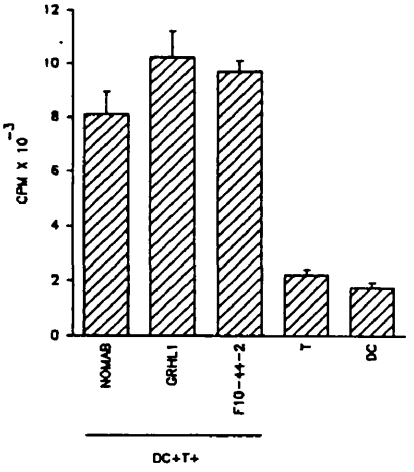


Fig. 7.5

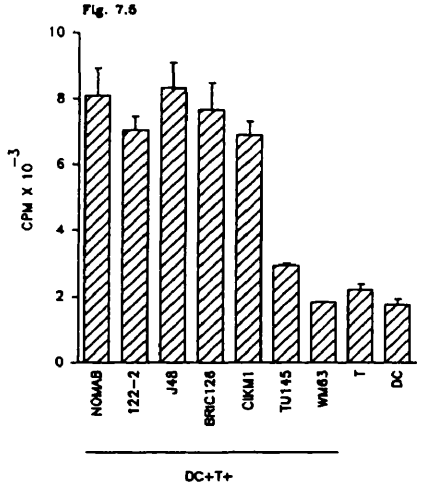


Fig. 7.6

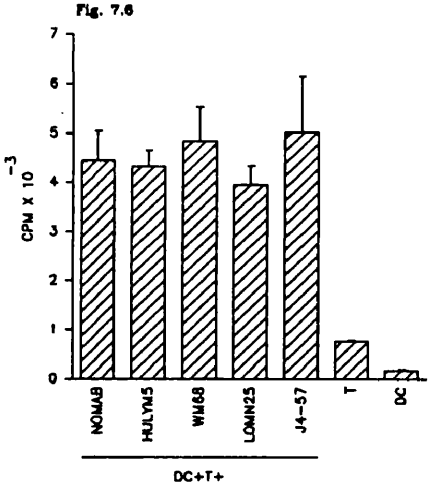
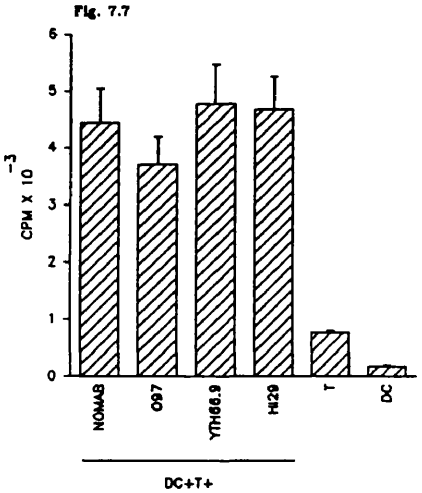


Fig. 7.7



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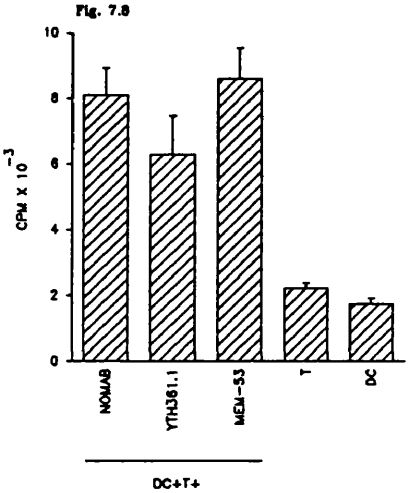
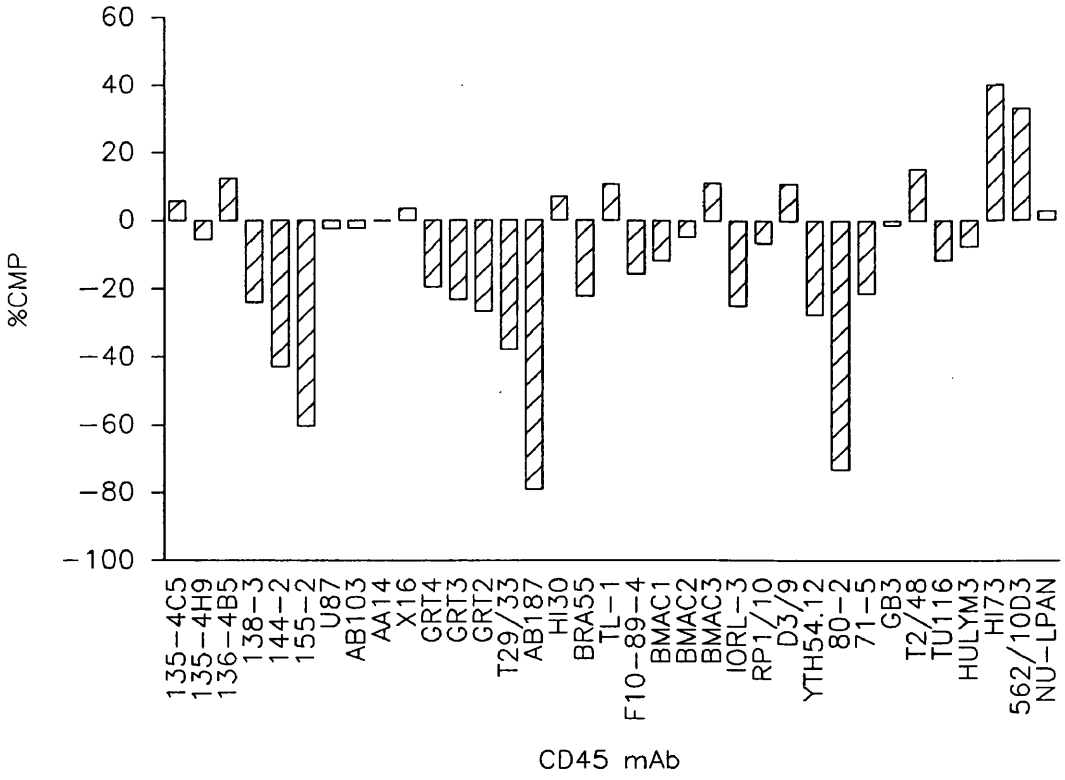


Fig. 7.3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AB187
HI30
BRA55
TL−1
F10−89−4
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BMAC2
BMAC3
IORL−3
RP1/10
D3/9
YTH54.12
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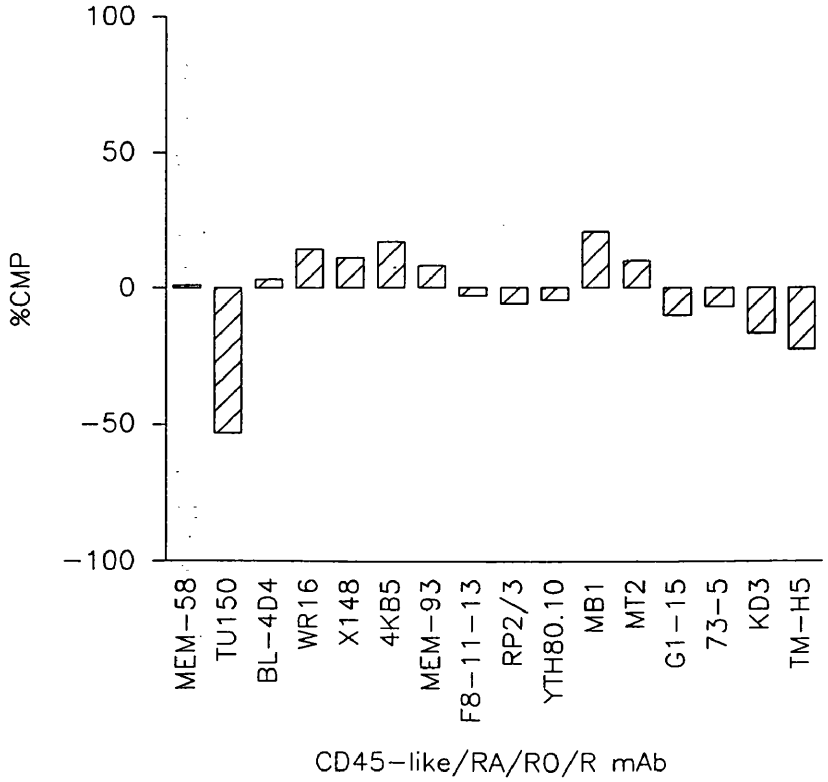
NU−LPAN
CD45 mAb

Fig. 7.4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TM−H5
CD45−like/RA/RO/R mAb

Fig. 7.9

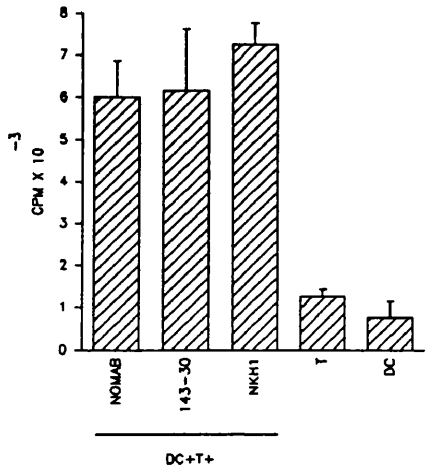


Fig. 7.10

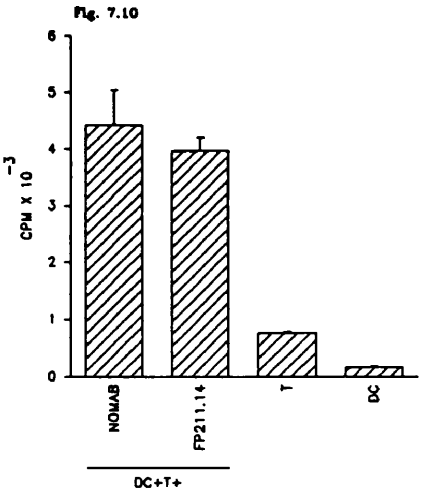


Fig. 7.11

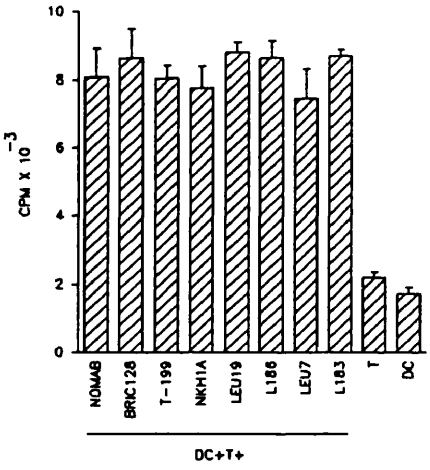


Fig. 7.12

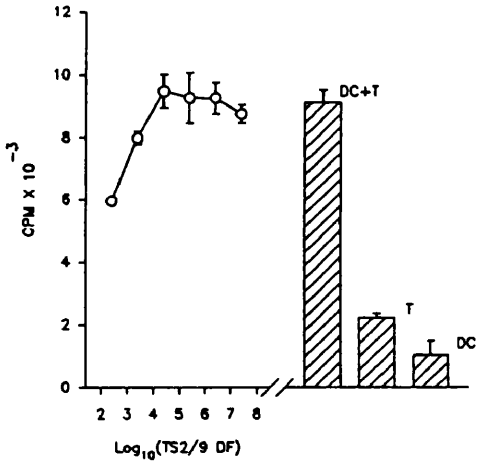


Fig. 7.13

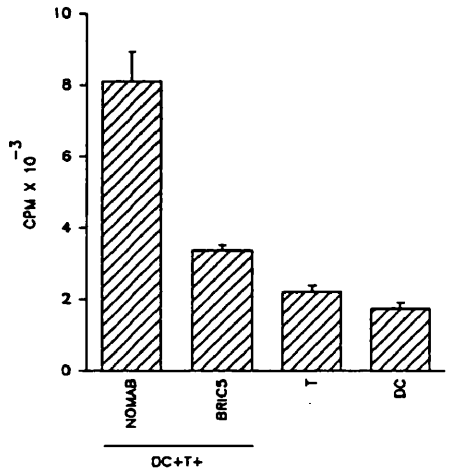


Fig. 7.14

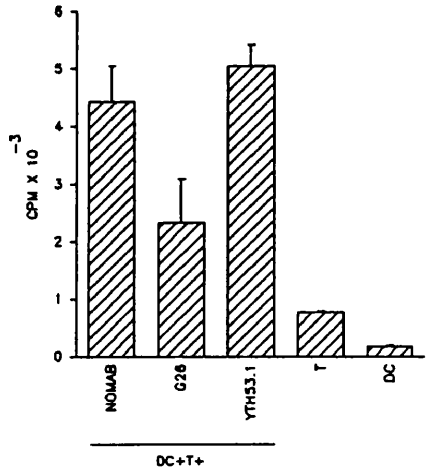


Fig. 7.15

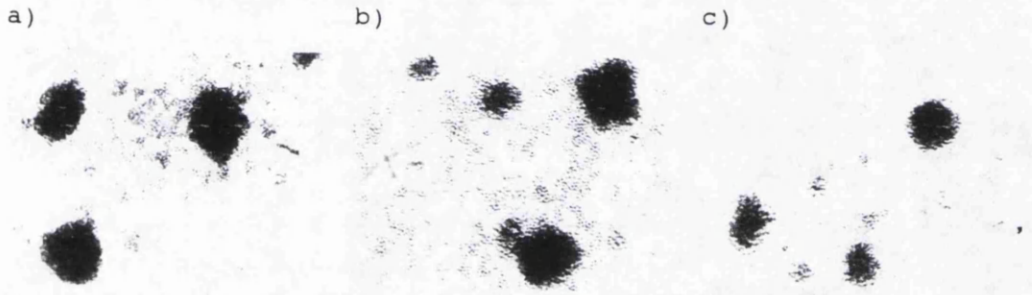


Fig. 7.16

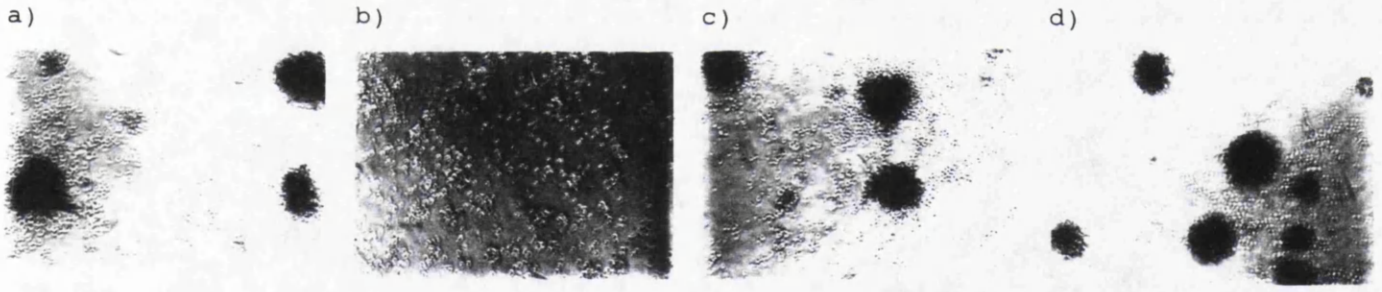


Fig. 7.17

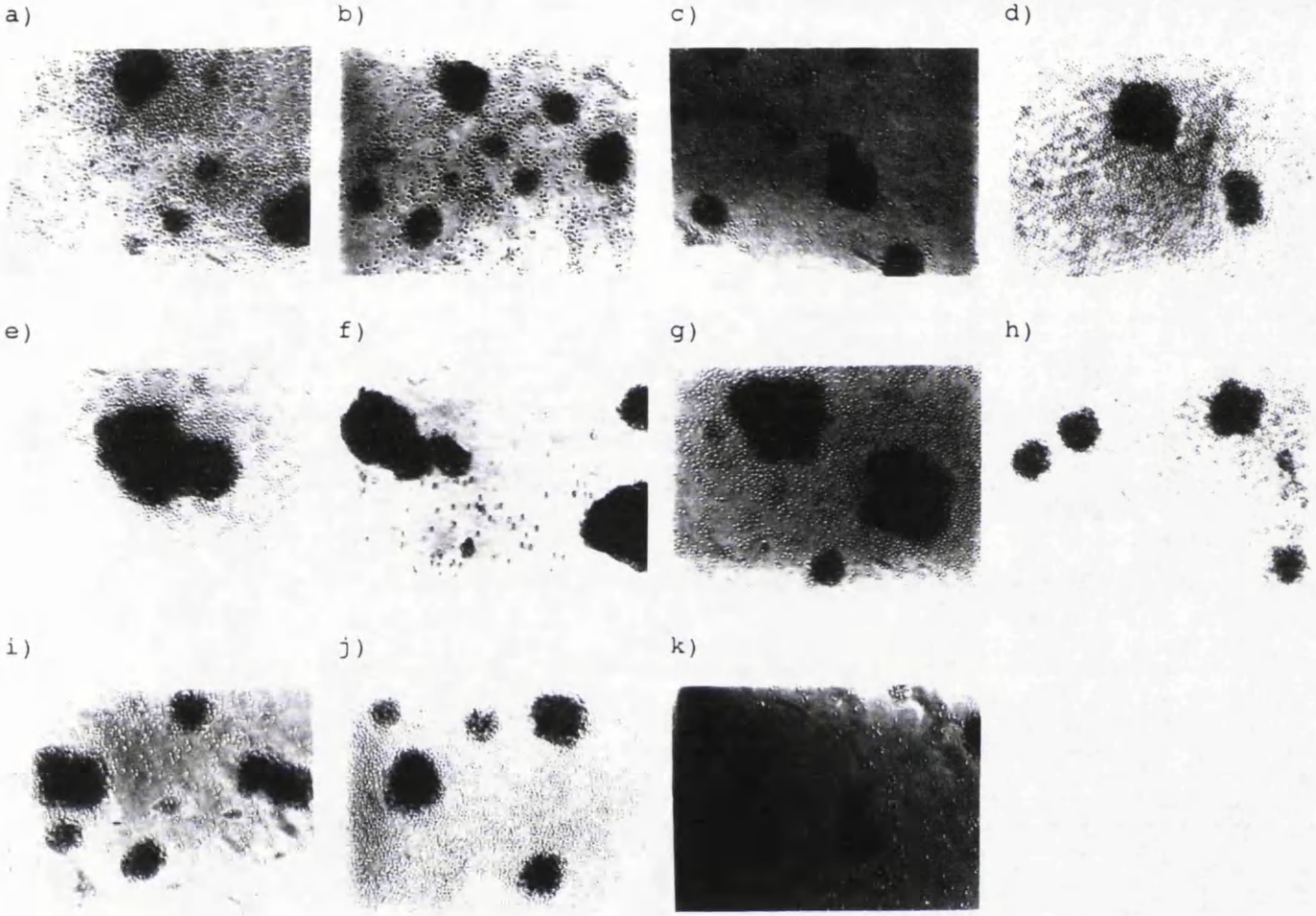


Fig. 7.18

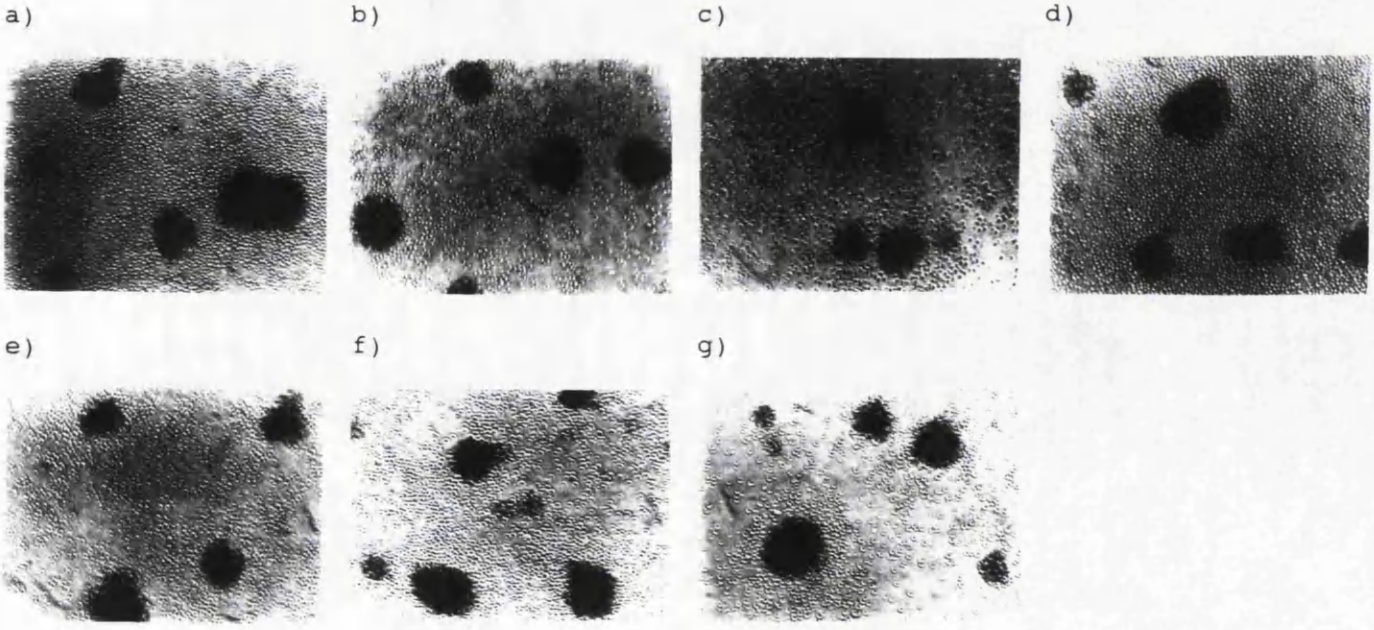


Fig. 7.19

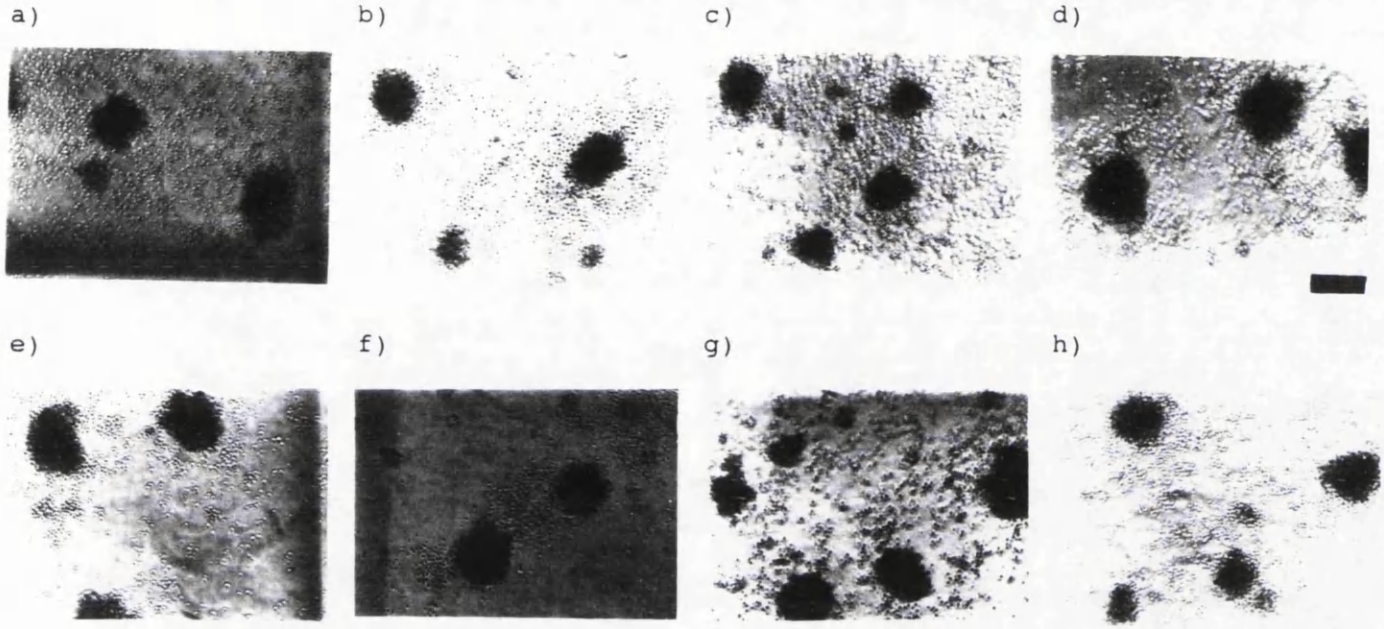


Fig. 7.20

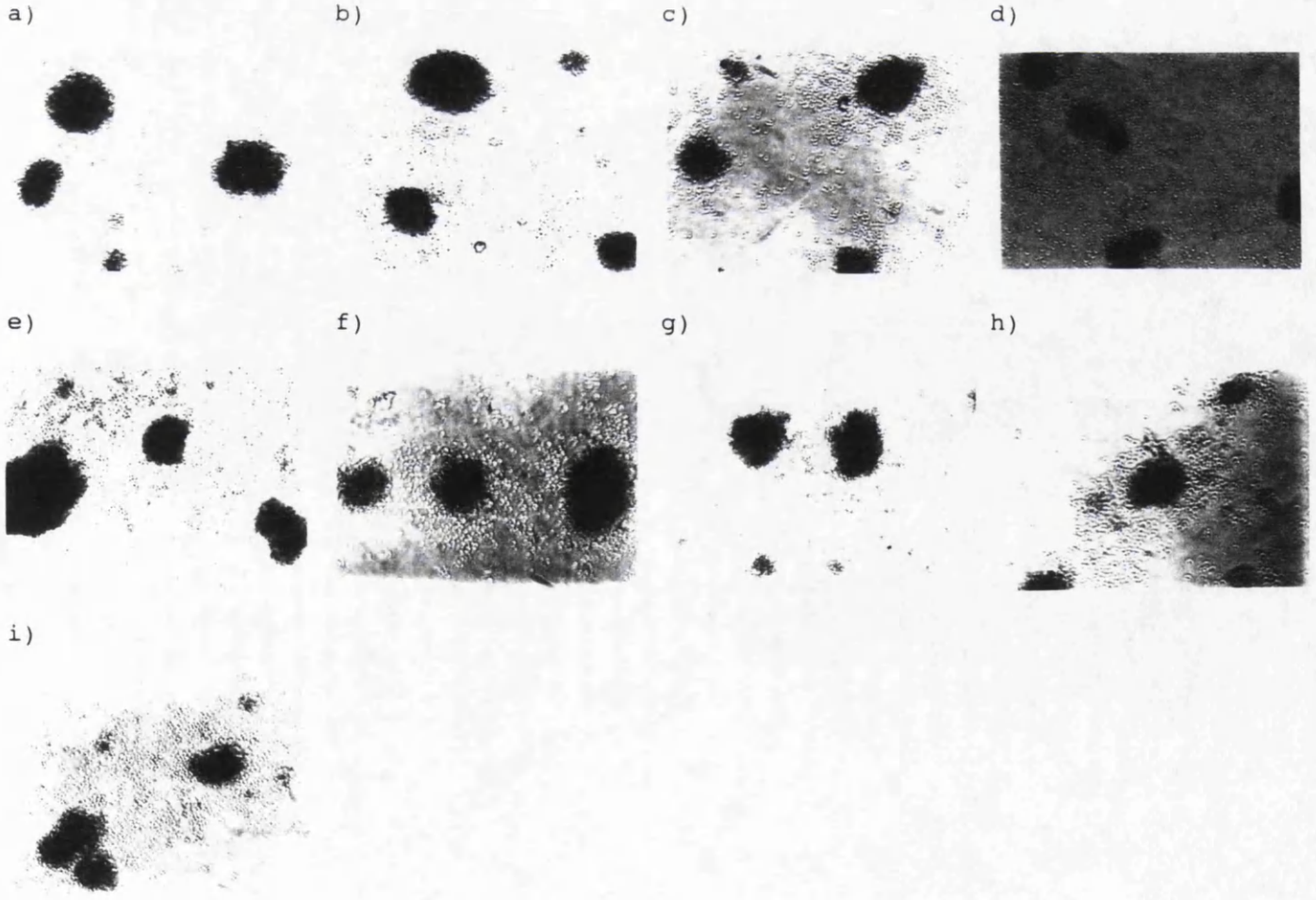


Fig. 7.21

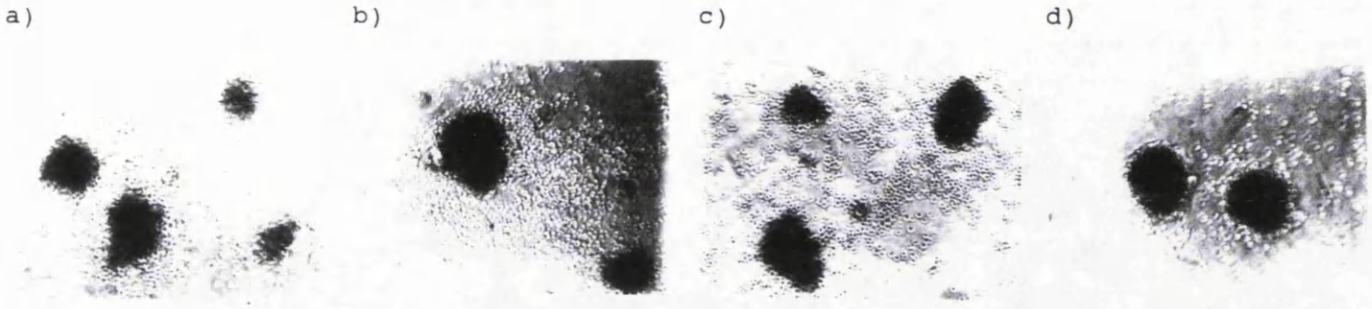


Fig. 7.22

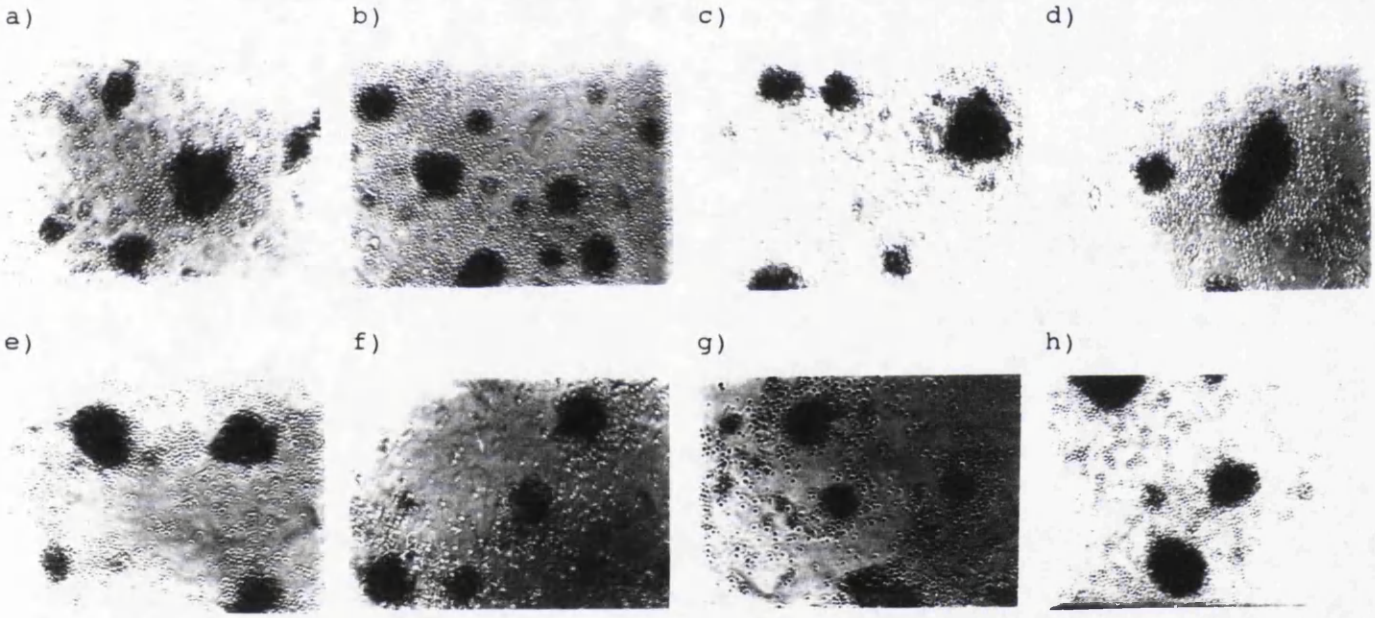


Fig. 7.23

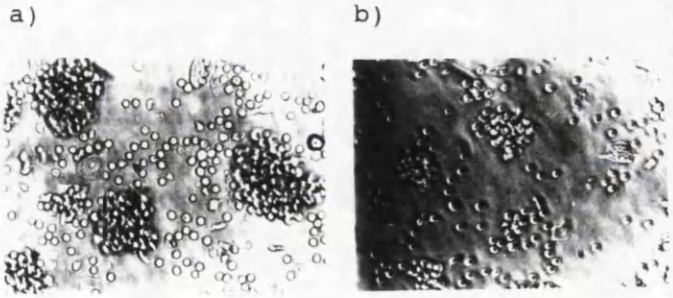


Figure Legends

See Materials and Methods plus:

Fig. 7.1. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, OTH71C5 and G19-1 DF - 4 X 10^2.

Fig. 7.2. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, GRHL1 and F10-44-2 DF - 4 X 10^2.

Fig. 7.3. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well; all mAb DF - 4 X 10^2; 138-3, 144-2, 155-2, GRT3, GRT2, T29/33, AB187, BRA55, IOR-L3, YTH54.12, 80.2 and 71.5 inhibitions and HI73 and 562/10D3 enhancements are SS; 135-4C5, 135-4H9, 136-4B5, U87, AB103, AA14, X16, GRT4, HI30, TL-1, F10-89-4, BMAC1, BMAC2, BMAC3, RP1/10, D3/9, GB3, T2/48, Tu116, HuLym3 and NULPAN inhibitions or enhancements are not SS.

Fig. 7.4. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well; all mAb DF - 4 X 10^2, Tu150 inhibition is SS, inhibition or enhancement with other antibodies is not SS (UCHL1 - AF).

Fig. 7.5. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well; all mAb DF - 4 X 10^2; Tu145 and WM63 inhibition is SS, 122-2, J48, BRIC126 and CIKM1 inhibition or enhancement is not SS.

Fig. 7.6. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, all mAb DF - 4 X 10^2.

Fig. 7.7. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, all mAb DF - 4 X 10^2.

Fig. 7.8. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, YTH361.10 and MEM-53 DF - 4 X 10^2.

Fig. 7.9. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, 143-30 and NKH1 DF - 4 X 10^2.

Fig. 7.10. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, FP2-11.4 DF - 4 X 10^2.

Fig. 7.11. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, all mAb DF - 4 X 10^2.

Fig. 7.12. DC induced oxidative mitogenesis. DC number - 5 X 10^4/well, TS2/9 - CS.

Fig. 7.13. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, BRIC5 DF - 4 X 10^2.

Fig. 7.14. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, G26 and YTH53.1 DF - 4 X 10^2, G26 inhibition is SS, YTH53.1 enhancement is not SS.

Figs. 7.15-7.23 - DC-T lymphocyte clustering

Fig. 7.15. Oxidative mitogenesis. All photographed at 12 hr, all DC+T plus the following CD43 mAb (a) NomAb, (b) OTH71C.5, (c) G19-1.

Fig. 7.16. Oxidative mitogenesis. All photographed at 12 hr, (a) DC+T, (b) T alone, (c) DC+T+GRHL1 (CD44), (d) DC+T+F10-44-2 (CD44).

Fig. 7.17. Oxidative mitogenesis. All photographed at 12 hr, all DC+T plus the following CD45 mAb (a) NomAb, (b) BRA55, (c) BMAC3, (d) IORL3, (e) RP1/10, (f) D3/9, (g) 71.5, (h) Tu116, (i) T29/33, (j) HuLym3, (k) NULPAN.

Fig. 7.18. Oxidative mitogenesis. All photographed at 12 hr, all DC+T plus (a) NomAb, (b) MEM-58 (CD45-like), (c) BL-4D4 (CD45R), (d) WR16 (CD45R), (e) X148 (CD45R), (f) 4KB5 (CD45R), (g) MEM-93 (CD45R).

Fig. 7.19. Oxidative mitogenesis. All photographed at 12 hr, all DC+T plus (a) NomAb, (b) HuLym5 (CD46), (c) WM68 (CD48), (d) LOMN25 (CD48), (e) J4D7 (CD48), (f) 097 (CDw52), (g) YTH66.9 (CDw52), (h) HI29 (CD53).

Fig. 7.20. Oxidative mitogenesis. All photographed at 12 hr, all DC+T plus (a) NomAb, (b) 122-2 (CD46), (c) J48 (CD46), (d) BRIC126 (CD47), (e) CIKM1 (CD47), (f) Tu145 (CD48), (g) WM63 (CD48), (h) YTH361.10 (CDw52), (i) MEM-53 (CD53).

Fig. 7.21. Oxidative mitogenesis. All photographed at 12 hr, all DC+T plus (a) NomAb, (b) 143-30 (CD55), (c) FP2-11.4 (CD56), (d) YTH53.1 (CD59).

Fig. 7.22. Oxidative mitogenesis. All photographed at 12 hr, all DC+T plus (a) NomAb, (b) BRIC128 (CD55), (c) T199 (CD56), (d) NKH1a (CD56), (e) Leu19 (CD56), (f) L186 (CD57), (g) Leu7 (CD57), (h) L183 (CD57).

Fig. 7.23. Oxidative mitogenesis. Photographed at 4 hr, both DC+T plus (a) NomAb, (b) TS2/9 (CD58).

Involvement of non-lineage restricted antigens in tonsillar dendritic cell induced oxidative mitogenesis. In this Chapter CD44, CD45, CD45-like, CD48 and CD58 antibodies have been shown to influence proliferation and CD43, CD45RA, CD45RO, CD45R, CD46, CD47, CDw52, CD53, CD55, CD56, CD57 and CD59 antibodies have been shown to have no effect upon proliferation in tonsillar DC induced oxidative mitogenesis. In some cases, given the effects of some of the non-lineage restricted antibodies upon tonsillar DC-T lymphocyte clustering, as well as other factors (see below), interpretation of these results is difficult but tentatively these findings suggest that the CD44, CD45 (possibly some isoforms and not others - see below), CD48 and CD58 antigens are involved in proliferation induction in this response whereas the antigens recognised by the other antibodies are not.

Signal transduction roles for the CD44 and CD48 antigens. Both of the two CD44 antibodies that were tested enhanced proliferation. In contrast, of the five CD48 antibodies tested, two inhibited proliferation and three had no affect upon proliferation. All CD44 and CD48 antibodies, however, did not affect tonsillar DC-T lymphocyte clustering thus pointing to a signal transduction role for these molecules.

Like the CD5 and CD28 antigens (see Chapter 6), studies of purified T lymphocytes have implicated the CD44 antigen as another "second signal" receptor upon these cells (545,546). Therefore, function as a T lymphocyte "second signal" receptor is the likely signal transduction function of the CD44 antigen in tonsillar DC induced oxidative mitogenesis (with CD44 antibody mimicry of a "second signal" being envisaged). The nature of the "second signal" with which the CD44 antigen interacts, however, is unclear. Recently, the CD44 antigen has been shown to function as a receptor for the

glycosaminoglycan hyaluronic acid (547-549) and thus this molecule is a strong candidate. There are other possibilities though. Hence, purified CD44 antigen from a B lymphocyte line binds a mucosal vascular adressin known as gp58-66 (550). Further, there is evidence that the CD44 antigen binds another glycosaminoglycan - chondroitin sulphate (547). Given that chondroitin sulphate modified forms of the CD44 antigen have been detected upon several different cell types (551,552) this could indicate that the CD44 antigen interacts with itself in it's role as a "second signal" receptor. An attraction of this last possibility is the expression of the CD44 antigen upon tonsillar DC (see Chapter 2) and this in turn raises the issue of a receptor like role of the CD44 antigen at this cellular level too. Denning et al have demonstrated that CD44 antibodies can induce the release of IL-1 from peripheral blood monocytes (553). Therefore, upon tonsillar DC, the CD44 antigen, like the CD58 antigen (see Chapter 6 and below), might function as a receptor which controls their release of IL-1 (enhancement of proliferation again being interpreted as functional mimicry of a physiological ligand).

Non-involvement of the CD44 antigen in DC-T lymphocyte clustering might seem surprising in light of the function of the CD44 antigen as an intercellular adhesion molecule in other systems, i.e. lymphocyte homing (554), lymphocyte/stromal cell adhesion (548) and T lymphocyte-RBC rosetting (545). Also, in still other model systems, CD44 antibodies have been shown to enhance or induce cellular aggregation (500,555,556). Lack of direct involvement in clustering could be explained in terms of a low level of CD44 antigen and CD44 antigen ligand expression in this system or alternatively that the CD44 antibodies used in this study do not detect an "adhesotope" upon the CD44 antigen (for instance F10-44-2 does not block T lymphocyte-RBC rosetting, 545). Non-involvement of the CD44 antigen in clustering in an

indirect manner (as is implicit in the observation that CD44 antibodies in some situations can enhance clustering), however, is more complex and will be discussed in detail in Chapter 9.

The finding that the CD48 antigen is involved in signal transduction in tonsillar DC induced oxidative mitogenesis is the first description of a functional role for this molecule. Because of the paucity of information concerning the CD48 antigen any discussion as to it's precise signal transduction role in this response must be speculative. However, the fact that the CD48 antigen is expressed upon tonsillar T lymphocytes (557) and DC (see Chapter 2) indicates that it might be implicated at either or both of these cellular levels. In addition, the fact that CD48 antibodies inhibited proliferation is consistent with a function of the CD48 antigen as any of a ligand or receptor in a positive signalling pathway or a receptor in a negative signalling pathway (presumably the three CD48 antibodies that do not affect proliferation detect non-functional epitopes). In this last context, that the CD48 antigen is a phosphotidyl-Inositol linked membrane antigen (558) might be of relevance, favouring a ligand rather than a receptor role.

Signal transduction and clustering roles for the CD45 antigen: Reappraisal of the relationship between clustering and proliferation. The CD45 antigen is a complex family of high molecular weight transmembrane glycoproteins consisting of different isoforms (with differing polypeptide sequences) of the same molecule (559,560). Glycosylation modifications are extensive and contribute to a diversity of CD45 antigen structures. Differenr isoforms result from the alternative splicing of a primary transcript such that three adjacent exons, labelled A, B and C, which encode extreme N-terminal amino acids, are subject to inclusion or exclusion from a mature mRNA species. In this way there are eight possible different isoforms although hitherto only

five have been identified corresponding to the sequences ABC (220 KD), AB (205 KD), BC (205 KD), B (190 KD) and none of A, B or C (180 KD).

Serologically, several different groups of antibodies against the CD45 antigen have been defined (561-565) as follows: 1) CD45 antibodies per se react with all leucocytes, immunoprecipitate all molecular weight bands and are known to recognise epitopes encoded by constant region exons; 2) CD45RA antibodies exhibit a restricted pattern of staining amongst leucocytes, immunoprecipitate 205 and 220 KD bands only and are known to react with epitopes encoded by variable region exon A; 3) CD45R antibodies are identical to CD45RA antibodies only reactivity with exon A encoded sequences remains to be demonstrated; 4) CD45-like antibodies stain leucocytes in a pattern distinct from that seen with CD45 and CD45R(RA) antibodies, immunoprecipitate all or only selected molecular weight bands and their exon product reactivity status is unknown; and 5) CD45RO antibodies also have a distinct pattern of staining, immunoprecipitate the 180 KD band only and react with a conformational epitope created by the juxtaposition of a sequence encoded by an exon immediately upstream of the A exon and an exon immediately downstream of the C exon (i.e. exon D). As implicit in the different staining patterns of these different antibodies not all CD45 antigen isoforms are expressed upon all leucocytes. There are two general rules. Firstly, CD45R(RA) and CD45RO antigen expression are mutually exclusive (441,443,566,567). Secondly, in any one lineage, the CD45R(RA)-/CD45RO+ phenotype is a feature of more differentiated "end" cells (443,444). There are, however, important exceptions to this last generalisation, most notably within the T lymphocyte lineage. Thus, although in the peripheral T lymphocyte pool the CD45R(RA)+/CD45RO- and CD45R(RA)-/CD45RO+ phenotypes are thought to demarcate virgin and memory T lymphocytes respectively, thymocytes, like memory T lymphocytes, are also CD45R(RA)-

/CD45RO+ (441,442,568).

It is upon T lymphocytes that the function of the CD45 antigen has been most examined. Thus many studies have shown that CD45 antibodies profoundly influence T lymphocyte proliferation attesting to an important functional role of the CD45 antigen in the activation of these cells (e.g. 320,569-571). In the most informative antibody study, however, cross-linking the CD45 antigen to the CD3 antigen in CD3 antibody mitogenesis was shown to block proliferation and calcium mobilisation in purified responding T lymphocytes. Also, and in contrast, intracellular calcium mobilisation in the same cells, induced by CD4 antigen cross-linking, was enhanced by cross-linking to the CD4 antigen CD45 antigen isoforms (572). Based upon these findings and the finding that the large cytoplasmic tail of the CD45 antigen possesses protein tyrosine phosphatase (PTPase) activity (572-574) Clark and Ledbetter have proposed a model for the involvement of the CD45 antigen in physiological T lymphocyte activation (572-574). In this model a CD45 antigen ligand with specificity only for variable exon encoded sequences (i.e. high molecular weight isoforms) is envisaged to be expressed upon accessory cells, physically associated with MHC antigens. As a consequence, upon virgin T lymphocytes (but not upon memory T lymphocytes) the CD45 antigen would become cross-linked to the TcR complex which would cause a down regulation of the activation of these cells in turn as a result of the dephosphorylation of phospho-tyrosyl residues in the zeta chain of the CD3 antigen. In addition, this model also proposes that upon memory T lymphocytes (but not upon virgin T lymphocytes) CD45 antigen isoforms associate with the CD4 or CD8 antigens resulting in an augmented activation which would be mediated by both tyrosine dephosphorylation of the protein tyrosine kinase $p56^{lck}$ associated with the CD4 and CD8 antigens (causing an increase of kinase activity) and phosphorylation of the cytoplasmic tail of

the CD45 antigen (causing a reduction of PTPase activity). More than simply providing a theoretical basis for the enhanced responsiveness of memory over virgin T lymphocytes, with time, this model, particularly the CD4/CD8 antigen and CD45 antigen interaction component, is gaining credence. Thus, studies of CD45 antigen minus T lymphocyte mutants have shown that the CD45 antigen dephosphorylates (573) and activates $p56^{lck}$ (576) and that cell surface expression of the CD45 antigen is required for memory T lymphocyte activation (577). Furthermore, purified CD45 antigen has been shown to directly activate the kinase activity of $p56^{lck}$ in vitro (576).

Against this background then is the effect of CD45 antigen family antibodies upon tonsillar DC induced oxidative mitogenesis. With regards to proliferative effects, twelve CD45 and one CD45-like antibody inhibited proliferation; two CD45 antibodies enhanced proliferation; and twenty one CD45, one CD45-like, three CD45RA, one CD45RO and twelve CD45R antibodies did not affect proliferation. Intriguingly, however, seven of the CD45 antibodies which inhibited proliferation, twelve of the CD45 antibodies which did not affect proliferation, the proliferation inhibiting CD45-like antibody and the CD45RO antibody enhanced clustering. These findings indicate that the CD45 antigen is involved in clustering and signal transduction in this response and in addition raise a number of other issues, not least the relationship between clustering and proliferation.

Drawing upon the Clark/Ledbetter model to explain the signal transduction role of the CD45 antigen, in this view the CD45 antibodies which define this role could act by influencing either or both of an association of low molecular weight isoforms with the CD4/CD8 antigens upon the surface of memory T lymphocytes or an association of high molecular weight isoforms with the TcR complex upon the surface of virgin T lymphocytes. However, against the idea that high molecular weight isoforms

are involved in signal transduction is the inability of CD45RA and CD45R antibodies to influence proliferation in this response; and one likely basis for this is an inability of virgin T lymphocytes, due to their low expression of important adhesion molecules (see pages 71 and 73), to adhere to DC.

There are several different ways in which CD45 antigen family antibodies could enhance clustering. Firstly, these antibodies could act by simply cross-linking DC to T lymphocytes. This, however, seems unlikely as antibodies against several other antigens, e.g. CD44, CD48, CDw52 and CD53 antigens, which are also expressed upon tonsillar DC and T lymphocytes, do not enhance clustering. Moreover, firmer evidence that the CD45 antibodies do not enhance clustering via this route is presented in Chapter 9.

Secondly, the CD45 antigen might act as an anti-adhesion molecule in this cellular interaction and the cluster enhancing antibodies may thus achieve their effects by abrogating this anti-adhesion. Both passive and active anti-adhesion are possible. Passive anti-adhesion could be a consequence of a high degree of negative charge upon CD45 antigen isoforms (associated with the carboxyl moieties of sialic acids) which could effect anti-adhesion by mediating a like-like charge repulsion between DC and T lymphocytes. Exactly how negation of this type of anti-adhesion might occur is uncertain but cell surface down regulation, redistribution or steric hindrance of the CD45 antigen are all possible. Active anti-adhesion, by contrast, could result from the role of the CD45 antigen as a receptor in a signalling pathway which serves to inhibit DC-T lymphocyte adhesion by blocking the function of adhesion molecules. This blockade could involve a reduction of adhesion molecule cell surface expression. Alternatively there may be a reduction of adhesion molecule avidity following dephosphorylation of tyrosine residues in their cytoplasmic domains by the CD45 antigen (the

cytoplasmic domains of the LFA-1 beta chain and ICAM-1 contain tyrosine residues, 487,488,578) and this in turn could follow an increase in the PTPase activity of the CD45 antigen secondary to ligand induced CD45 antigen-CD45 antigen cross-linking with concomitant mutual dephosphorylation of isoforms. Whatever, in this view, the cluster enhancing antibodies can be thought of as inhibiting a CD45 antigen-ligand interaction.

Thirdly, the CD45 antigen, might function as a receptor in a signalling pathway which serves to enhance adhesion molecule function. As a direct opposite of the active anti-adhesion model, there may be an upregulation of adhesion molecule expression or an increase in adhesion molecule avidity. In this interpretation, however, rather than blocking a CD45 antigen-ligand interaction, the cluster enhancing antibodies can be viewed as functionally mimicking a CD45 antigen ligand.

To address the issue of why cluster enhancing CD45 antigen family antibodies do not enhance but instead inhibit or do not affect proliferation one basis for this may be a requirement of T lymphocytes to de-adhere from DC. In this regard, as well as causing additional T lymphocytes to adhere to DC, hence allowing their recruitment into cell cycle, the cluster enhancing antibodies might also "tighten" the adhesion of already clustered T lymphocytes to such an extent that at an appropriate point they fail to detach from DC and consequently are unable to proliferate. Therefore, a balance between these two different effects might explain no effect upon proliferation and a predominance of the latter effect over the former (resulting from the fact that at some point upon a continuum DC would become saturated with T lymphocytes) might explain inhibition of proliferation. This would predict that the proliferation inhibiting antibodies are more effective enhancers of clustering than the antibodies which do not affect proliferation and on this theme it is interesting that if cluster

enhancement depends upon CD45 antigen cross-linking four out of nine inhibitory antibodies are IgMs whereas all of the fourteen antibodies which do not affect proliferation are IgGs.

Another possible reason for the distinction between the two types of cluster enhancing antibodies is that the proliferation inhibiting antibodies block an interaction of the CD45 antigen with the CD4 and CD8 antigens upon memory T lymphocytes and thus also interfere with the purely signal transduction function of this molecule. This raises two other issues - 1) why certain CD45 and CD45-like antibodies did not affect clustering and why some of these did not affect proliferation either and 2) why none of the CD45RA or CD45R antibodies affected clustering? In answer to the first question, this can be attributed to the epitope specificities of these antibodies. Thus, CD45/CD45-like antibodies which do not affect clustering might simply not bind epitopes of the CD45 antigen that are associated with cluster formation and the CD45/CD45-like antibodies which do not affect proliferation either might also not bind epitopes involved with a purely signal transduction role of this molecule.

The issue of why none of the CD45RA and CD45R antibodies affected DC-T lymphocyte clustering is more involved. In the passive anti-adhesion model it could be argued that the effect of CD45RA/R antibodies is to reduce the negative charge of virgin T lymphocytes only (high molecular weight isoform expression is restricted to virgin T lymphocytes in this system) and this might not result in enhancement of adhesion owing to the weak expression of adhesion molecules upon these cells. Likewise, in both active anti-adhesion and active adhesion models, if the CD45 antigen were to function only at the DC level, then the restricted expression of high molecular weight isoforms to virgin T lymphocytes would explain an inability of CD45RA/R antibodies to enhance DC-T lymphocyte clustering. If, however,

in these last two models the CD45 antigen were to function only at the T lymphocyte level, then assuming that a CD45 antigen epitope that is involved in the regulation of cluster formation is encoded by a constant region exon, CD45RA/R antibodies may not influence clustering simply because, by definition, they bind sequences that are encoded by variable region exons. At least, in the active adhesion model, this last explanation holds true if enhancement is not mediated through CD45 antigen isoform cross-linking, for if it were it is again necessary to envisage that CD45RA/R antibodies do not enhance clustering because of the weak expression of adhesion molecules upon virgin T lymphocytes.

Confirmation that a CD2 antigen/LFA-3 interaction is involved in clustering and signalling in tonsillar dendritic cell induced oxidative mitogenesis. The demonstration that three different CD58 antibodies inhibit clustering in tonsillar DC induced oxidative mitogenesis adds weight to the notion that a CD2 antigen/LFA-3 interaction functions in the mediation of clustering in this response (see Chapter 6). In addition, that the CD58 antibodies only partially inhibited clustering yet one of these (BRIC5) almost completely blocked proliferation adds further support to the idea that a CD2 antigen/LFA-3 interaction is also involved in signal transduction in tonsillar DC induced oxidative mitogenesis, i.e. in the delivery of "first signals" to T lymphocytes and the release of IL-1 "second signals" from DC (also see Chapter 6). Why the two other CD58 antibodies only partially inhibit proliferation is unclear. Possibly, however, both mimic the CD2 antigen in its binding to LFA-3.

Non-involvement of the CD47, antigen in tonsillar dendritic cell induced oxidative mitogenesis. Although neither of the CD47 antibodies that were tested affected proliferation in tonsillar DC induced oxidative mitogenesis,

one of these antibodies (CIKM1) enhanced clustering. The mechanism of this enhancement is also unclear but it is interesting that the CD47 antigen, like the CD45 antigen, is heavily glycosylated (579) suggesting a passive anti-adhesive role for this molecule (presumably CIKM1 negates this passive anti-adhesion whereas the other CD47 antibody does not). As with certain CD45 antibodies, one basis for an inability of CIKM1 to affect proliferation is that the effect of increasing the number of T lymphocytes that adhere to DC is balanced by the effect of inhibiting the de-adhesion of T lymphocytes already clustered to DC. This is important for it questions the conclusion that the CD47 antigen is not involved in proliferation induction. Alternatively then, the CD47 antigen is involved, merely that the balance between a negative role (inhibition of T lymphocyte adherence) and a positive role (facilitation of de-adherence) mask an effect of CD47 antibodies upon T lymphocyte proliferation.

Summary

In this Chapter, CD44, CD45, CD48 and CD58 antigens but not CD43, CD46, CD47, CDw52, CD53, CD55, CD56, CD57 and CD59 antigens have been shown to play a role in tonsillar DC induced oxidative mitogenesis. The CD44 and CD48 antigens function exclusively in signal transduction in this response. Probably the function of the CD44 antigen is as another T lymphocyte "second signal" receptor and a role as a receptor which induces "second signals" themselves from DC is also a possibility. The signal transduction role of the CD48 antigen is less clear but a ligand-like role for this molecule is favoured. In contrast to the CD44 and CD48 antigens, the CD58 antigen is involved in signal transduction and clustering, thus confirming the role of a CD2 antigen/LFA-3 interaction in both signalling and in the mediation of DC-T lymphocyte clustering in this response. Likewise, the CD45 antigen also plays a role in signal transduction and clustering in tonsillar DC induced oxidative mitogenesis. The signal transduction role of this molecule probably involves an interaction with the CD4 and CD8 antigens upon memory T lymphocytes. The clustering role of this molecule, however, is indirect and in this role the CD45 antigen could function as any of a passive or active anti-adhesion molecule or an active adhesion molecule.

CHAPTER 8

EFFECT OF ACTIVATION ANTIGEN ANTIBODIES, IL-1 ANTIBODIES AND IL-1 UPON TONSILLAR DENDRITIC CELL-T LYMPHOCYTE INTERACTION

Activation antigens, by definition, are antigens whose expression upon cells is induced or increased upon cellular activation and thus one might expect these antigens to play important roles in T lymphocyte responses. Indeed, the function of several different activation antigens [e.g. CD25 antigen (alpha chain of the high affinity IL-2R) and CD71 (transferrin receptor)] in T lymphocyte proliferation induction is well documented. Despite this, however, few studies have examined the role of activation antigens in DC induced responses. Therefore, to examine the role of activation antigens in tonsillar DC induced oxidative mitogenesis, in the first section of this Chapter, panels of CD25, CD26, CD30, CD69, CDw70 and CD71 antibodies have been screened for their effects upon proliferation and clustering in this response.

In contrast to activation antigens, the role of IL-1 in DC induced T lymphocyte responses has been studied extensively. With regards to an endogenous involvement of IL-1 there are conflicting findings (see page 65). A consistent finding, however, is that exogenous IL-1 can boost DC induced T lymphocyte responses (see page 66) and this has been described for each of rat veiled cells, mouse thymic DC and mouse splenic DC where in the last case there is evidence that the IL-1 acts at the DC level and functions to enhance their T lymphocyte clustering capacity. In view of this then, in the second section of this Chapter, in an attempt to clarify whether or not IL-1 plays an endogenous role in DC induced responses, the effect of IL-1 antibodies upon proliferation and clustering in human tonsillar DC induced oxidative mitogenesis has also been examined. In addition, to confirm that IL-1 can boost human DC induced responses, and to determine the mechanism of this, recombinant human IL-1 (rhIL-1) has, too, been tested for it's effects upon proliferation and clustering in this reaction.

MATERIALS AND METHODS

Mice. Five to six week old female Balb/c mice, kept under pathogen free conditions, were obtained from the Middlesex Hospital Pathology Department's animal breeding facility.

Cells. DC, high density T lymphocytes and low density B lymphocytes were isolated from human tonsils as described in Chapter 2. Macrophages were isolated from human tonsils as described in Chapter 3.

Murine thymocytes (for IL-1 co-stimulator assays) were prepared by pushing mouse thymi through 125 uM nylon mesh. Briefly, mice were killed by cervical dislocation, excised thymi were rinsed in 70% ethanol and then RPMI 1640 and pooled thymi were pushed through mesh as described for human tonsils in Chapter 2 (only a collagenase digestion step was omitted). Collected thymocytes were then rinsed four times in RPMI 1640 before resuspension in 5% complete medium and use in assays.

Proliferation assays. Oxidative mitogenesis assays (2 mM periodate was used throughout) and auto MLRs were performed as indicated in Chapter 3 using varying numbers per well of DC, B lymphocytes and macrophages as accessory cells and 2 X 10^5 per well of high density T lymphocytes as responder cells. In addition, activation antigen antibodies, IL-1 antibodies and IL-1 were added to wells (at culture initiation or at various time points thereafter) in varying concentrations in either 20 ul quantities when only one reagent was tested or in 10 ul quantities when two reagents were tested. Oxidative mitogenesis assays and auto MLRs were of 64 hr and 96 hr duration respectively. Both types of assay were pulsed with ^{125}IdUrd 16 hr before termination.

Mouse thymocyte IL-1 co-stimulator assays were performed in 96 well plates in 5% complete medium. All wells received 1 X 10^6 thymocytes in 100

ul of 5% complete medium plus 100 ul of 5% complete medium containing 5 ug/ml of PHA (Sigma, i.e. the final PHA concentration was 2.5 ug/ml) and some wells additionally received IL-1 antibodies and IL-1 in varying concentrations in 10 or 20 ul quantities as above. Assays were of a total of 72 hr duration with a final 6 hr pulse of ^{125}IdUrd. Culture conditions, pulsing conditions and harvesting of assays were as described previously (see Chapter 3).

Assessment of cellular clustering. Clustering of DC and unmodified or 2 mM periodate modified autologous high density T lymphocytes, in the presence (added at time zero) and absence of activation antigen antibodies, IL-1 antibodies and IL-1 was assessed visually and photographed at 12 hr following culture initiation (see Chapter 4). All cultures were the same as those used for the assessment of proliferation in oxidative mitogenesis assays and auto MLRs.

Antibodies. Details of anti-Tac and 7G7/B6 (CD25); 134-4C2 (CD26); Ber-H2, Ber-H8 and Ki-1 (CD30); MLR3 (CD69); HNC142 (CDw70); and BU55 (CD71) activation antigen antibodies are given in Table 2.1. Additional anti-Tac was obtained as AF from the Fourth International Workshop. A list of other activation antigen mAb used in proliferation and clustering assays is shown in Table 8.1 and a list of IL-1 antibodies used in proliferation, clustering and thymocyte assays is shown in Table 8.2. All of the activation antigen antibodies shown in Table 8.1 were obtained as AF preparations from the Fourth International Workshop. Cluster designations are according to the nomenclature committees of the Third and Fourth Workshops. All antibodies were sterile filtered, dialysed and de-aggregated as appropriate (see Chapter 4).

Cytokines. rhIL-1 alpha (Dainippon Corporation, Osaka, Japan) was supplied

at 4.22 X 10^7 U/ml (1 U/ml being defined as the concentration of rhIL-1 alpha that stimulates half maximal murine thymocyte proliferation). rhIL-1 beta and additional rhIL-1 alpha were gifts of Dr. B. Champion (Glaxo, Greenford, Middlesex, UK) and both of these cytokines were supplied at 100 ug/ml of protein.

Statistics. See Chapters 3,4 and 5.

Table 8.1 - Additional activation antigen antibodies

Antibody	CD grouping/ specificity	Species of origin[a)]	Isotype
B-B10	CD25	M	G1
B-E1	CD25	M	M
B-F2	CD25	M	G1
B-G3	CD25	M	G1
B-G8	CD25	M	G1
BL-DD1	CD25	M	G3
CD25-3G10	CD25	M	G1
CD25-4E3	CD25	M	G2b
CD25-8D8	CD25	M	G1
CD25-9G8	CD25	M	G1
IL7.9L	CD25	M	G1
LO-Tact1	CD25	R	G2b
LO-Tact2	CD25	R	G2b
L54	CD25	M	G1
L61	CD25	M	G1
L62	CD25	M	G1
YTH906.9	CD25	R	G2b
11H2.7	CD25	R	G2a
143-13	CD25	M	M
143-24	CD25	M	M
2A3	CD25	M	G1
TS145	CD26	M	G1
Ber-H4	CD30	M	G1
Ber-H6	CD30	M	G1
Ber-H10	CD30	M	G1
HRS1	CD30	M	G2a
HRS4	CD30	M	G1
BL-Ac/p26	CD69	M	G3
FN50	CD69	M	G1
FN61	CD69	M	G1
L78	CD69	M	G1
HNE51	CDw70	M	G1
Ki-24	CDw70	M	
200-4C2	CDw70	M	G1
BU54	CD71	M	G1
JML-H9	CD71	M	G
MEM-75	CD71	M	G1
Nu-TfR2	CD71	M	G2a
Q1/71	CD71	M	
120-2A3	CD71	M	G1
138-18	CD71	M	G

a) M - mouse, R - rat, Ra - rabbit, Sh - sheep

Table 8.2 - IL-1 antibodies

Antibody	Specificity	Species of origin	Isotype	mAb/Poly[a]	CS/AF[b]	Source[c]
HIL1190	IL-1 alpha	M	G1	mAb	AF	Dainippon Corporation
S76	IL-1 alpha	Sh	-	Poly	-	B. Champion
ANOC205	IL-1 beta	M	G1	mAb	AF	Otsuka Pharmaceut.
OCT204	IL-1 beta	Ra	-	Poly	-	Otsuka Pharmaceut.
S77	IL-1 beta	Sh	-	Poly	-	B. Champion

See footnote to Table 8.1 plus

a) mAb - monoclonal antibody, Poly - polyclonal antibody

b) CS - culture supernatant, AF - ascites fluid

c) Otsuka Pharmaceuticals, Tokushima, Japan

Effect of CD25 antibodies upon dendritic cell induced oxidative mitogenesis. The anti-Tac CD25 antibody was first used to investigate the effect of such reagents upon DC induced oxidative mitogenesis. Fig. 8.1 shows that anti-Tac inhibited mitogenesis when incorporated into cultures at time zero.

Subsequently, a panel of CD25 antibodies (including anti-Tac) was screened for it's effects upon T lymphocyte proliferation in this response. Fig. 8.2 shows that out of a total of twenty three different CD25 antibodies tested, ten antibodies inhibited proliferation (L54, 2A3, LOTact1, LOTact2, anti-Tac, YTH906.9, CD25-4E3, CD25-9G8, CD25-3G10 and CD25-8D8) and thirteen antibodies (7G7/B6, L62, L61, BL-DD1, 143-24, 143-13, B-E1, B-B10, B-G8, B-F2, 11H2.7, B-G3 and IL7.9L) had no effect on proliferation.

Effect of CD25 antibodies upon dendritic cell induced oxidative mitogenesis when added at different times. Fig. 8.3 depicts the results of a single experiment in which the effect of adding a CD25 antibody, CD25-3G10, at different times, to the DC induced oxidative mitogenesis reaction was examined. As shown, CD25-3G10 inhibited T lymphocyte proliferation in this response when incorporated into cultures at all time points up to and including 48 hr after culture initiation (albeit with a decreasing degree of inhibition).

Effect of CD26 and CD30 antibodies upon dendritic cell induced oxidative mitogenesis. The effects of two CD26 antibodies, 134-2C2 and TS145, upon DC induced oxidative mitogenesis, are shown in Figs. 8.4 and 8.5 respectively. When incorporated into cultures at time zero, the 134-2C2 antibody enhanced the T lymphocyte proliferative response. In contrast, however, the TS145 antibody had no effect upon proliferation.

Fig. 8.6 summarises the effects of eight different CD30 antibodies

(HRS1, HRS4, Ber-H2, Ber-H4, Ber-H6, Ber-H8, Ber-H10 and Ki-1) upon DC induced oxidative mitogenesis. As shown, none of these antibodies significantly affected T lymphocyte proliferation in this response when incorporated into cultures at time zero.

Effect of CD69 and CDw70 antibodies upon dendritic cell induced oxidative mitogenesis. Fig. 8.7 shows the results of an experiment in which a CD69 antibody, MLR3, was incorporated into the DC induced oxidative mitogenesis reaction at time zero. MLR3 had no effect upon T lymphocyte proliferation in this response when added to cultures at this time.

The results of an experiment in which four more CD69 antibodies (FN61, FN50, L78 and BL-Ac/p26) were compared with four CDw70 antibodies (2D0-4C2, HNE51, HNC142 and Ki-24), for their effects upon DC induced oxidative mitogenesis, are shown in Fig. 8.8. Like MLR3, none of the CD69 antibodies tested in this experiment affected T lymphocyte proliferation. In contrast, each of the CDw70 antibodies that were tested inhibited proliferation.

The HNC142 CDw70 antibody was chosen to test the effect of titrating this type of reagent into the reaction. Fig. 8.9 confirms the inhibitory effect of HNC142 upon DC induced oxidative mitogenesis and shows further that inhibition was dependent upon the concentration of added antibody.

Effect of CDw70 antibodies upon dendritic cell induced oxidative mitogenesis when added at different times. The HNC142 antibody was also used to investigate the effect of adding CDw70 antibodies at different times to the DC induced oxidative mitogenesis reaction. Fig. 8.10 shows that, like the CD25 antibody, CD25-3G10, the proliferation inhibiting effect of HNC142 diminished with an increasing time delay between culture initiation and antibody addition to cultures. However, also like CD25-3G10, at all time points examined, from zero to 48 hr following culture initiation, HNC142

significantly inhibited T lymphocyte proliferation.

Effect of CDw70 antibodies upon B lymphocyte induced oxidative mitogenesis. Fig. 8.11 shows the effect of titrating the HNC142 antibody into the B lymphocyte induced oxidative mitogenesis reaction at the time of culture initiation. As with the DC induced reaction, HNC142 inhibited T lymphocyte proliferation in this response in a dose dependent fashion.

Effect of CDw70 antibodies upon the dendritic cell and B lymphocyte induced auto MLRs. The effect of incorporating CDw70 antibodies, at time zero, into the DC and B lymphocyte induced auto MLRs was also examined. Fig. 8.12 shows that, in contrast to the DC and B lymphocyte induced oxidative mitogenesis reactions, HNC142 had no effect upon T lymphocyte proliferation in the DC and B lymphocyte induced auto MLRs when tested over a range of concentrations.

Effect of CD71 antibodies upon dendritic cell induced oxidative mitogenesis. Eight different CD71 antibodies were tested for their effects upon DC induced oxidative mitogenesis. The results are summarised in Fig. 8.13 which shows that six of the antibodies (Q1/71, 120-2A3, MEM-75, BU54, BU55 and JML-H9) enhanced T lymphocyte proliferation and two of the antibodies (138-18 and NuTfR2) had no effect upon proliferation.

Effect of activation antigen antibodies upon dendritic cell-T lymphocyte clustering in dendritic cell induced oxidative mitogenesis. Fig. 8.14 shows the effects of seven of the CD25 antibodies and two of the CD69 antibodies, that were tested for their effects upon T lymphocyte proliferation in DC induced oxidative mitogenesis, upon DC-T lymphocyte clustering in this response. None of the CD25 antibodies (including anti-Tac, L54 and 2A3 which inhibited proliferation and 7G7/B6, L62, L61 and BL-DD1 which had no effect

upon proliferation) and neither of the CD69 antibodies (L78 and BL-Ac/p26) affected clustering when incorporated into cultures at time zero.

The effects of two CDw70 antibodies, HNC142 and HNE51, and a CD71 antibody, 120-2A3, upon clustering are shown in Fig. 8.15. As with the CD25 and CD69 antibodies shown in Fig. 8.14, the CDw70 antibodies and the CD71 antibody (which inhibited proliferation in this response) had no effect upon clustering.

All of the other activation antigen antibodies, that were tested for their effects upon T lymphocyte proliferation in DC induced oxidative mitogenesis, were also tested for their effects upon DC-T lymphocyte clustering in this reaction. None of these antibodies, which included the other proliferation inhibiting CD25 antibodies (LOTact1, LOTact2, YTH906.9, CD25-4E3, CD25-9G8, CD25-3G10 and CD25-8D8), the other CD25 antibodies that had no effect upon proliferation (143-24, 143-13, B-E1, B-B10, B-G8, B-F2, 11H2.7, B-G3 and IL7.9L), the proliferation enhancing CD26 antibody (134-2C2), the CD26 antibody that had no effect upon proliferation (TS145), the CD30 antibodies (HRS1, HRS4, Ber-H2, Ber-H4, Ber-H6, Ber-H8, Ber-H10 and Ki-1), the other CD69 antibodies (MLR3, FN61 and FN50), the other CDw70 antibodies (200-4C2 and Ki-24), the other proliferation enhancing CD71 antibodies (Q1/71, MEM-75, BU-54, BU55 and JML-H9) and the CD71 antibodies that had no effect upon proliferation (138-18 and NuTfR2) affected DC-T lymphocyte clustering (not shown).

Effect of IL-1 alpha and IL-1 beta antibodies upon dendritic cell induced oxidative mitogenesis. Two different IL-1 alpha antibodies were tested for their effects upon DC induced oxidative mitogenesis. Figs. 8.16-8.18 show that a murine monoclonal IL-1 alpha antibody, HIL1190, had no effect upon proliferation when incorporated into cultures in either single concentrations (Figs. 8.16 and 8.17) or in a range of concentrations (Fig.

8.18) at time zero, despite the ability of this antibody to inhibit murine thymocyte proliferation in response to 5 U/ml rhIL-1 alpha and PHA (Fig. 8.19). Similarly, a sheep polyclonal IL-1 alpha antibody, S76, had no effect upon proliferation when examined in a range of concentrations (Fig. 8.20).

The effects of three different IL-1 beta antibodies upon DC induced oxidative mitogenesis are shown in Figs. 8.17 and 8.20-8.23. A rabbit polyclonal IL-1 beta antibody, OCT204, had no effect upon proliferation when tested in either a single concentration (Fig. 8.17) or in a range of concentrations (Fig. 8.21) and a murine monoclonal IL-1 beta antibody, ANOC205 (Fig. 8.22), and a sheep polyclonal IL-1 beta antibody, S77 (Fig. 8.20), also had no effect over a range of concentrations.

IL-1 alpha and IL-1 beta antibodies were also tested in combination for their effects upon DC induced oxidative mitogenesis. Figs. 8.23 and 8.24 confirm that the IL-1 alpha antibody, HIL1190, and the IL-1 beta antibodies, OCT204 and ANOC205, have no effect upon proliferation when tested separately in either single concentrations (HIL1190 and OCT204 - Fig. 8.23) or in a range of concentrations [HIL1190, OCT204 and ANOC205 - Fig. 8.24 (a-c)]. Further, when the HIL1190 antibody and the OCT204 antibody were tested together in single concentrations there was no inhibitory or enhancing effect upon proliferation (Fig. 8.23) and similarly no effect upon proliferation occurred when the HIL1190 antibody and the OCT204 antibody or the HIL1190 antibody and the ANOC205 antibody were tested together over a range of concentrations [Fig. 8.24 (d-f) and Fig. 8.24 (g-i) respectively].

Effect of IL-1 alpha and IL-1 beta antibodies upon the dendritic cell induced auto MLR. Fig. 8.25 shows the effects of the IL-1 alpha antibody, HIL1190, and the IL-1 beta antibodies, ANOC205 and OCT204, upon the DC induced auto MLR. In contrast to the DC induced oxidative mitogenesis reaction all three of these antibodies inhibited T lymphocyte proliferation

in the DC induced auto MLR.

Effect of IL-1 alpha and IL-1 beta antibodies upon the macrophage and B lymphocyte induced oxidative mitogenesis reactions. The HIL1190 IL-1 alpha antibody and the OCT204 IL-1 beta antibody were also tested for their effects upon the macrophage and B lymphocyte induced oxidative mitogenesis reactions. Fig. 8.26 shows that neither HIL1190 or OCT204 affected T lymphocyte proliferation in macrophage induced oxidative mitogenesis when added to cultures in single concentrations at time zero and for HIL1190 this result was confirmed when the antibody was tested in a range of concentrations (Fig. 8.27). Similarly, neither HIL1190 (Fig. 8.28) or OCT204 (Fig. 8.29) affected T lymphocyte proliferation in B lymphocyte induced oxidative mitogenesis when added to cultures in a range of concentrations at time zero.

Effect of rhIL-1 alpha and rhIL-1 beta upon the dendritic cell induced oxidative mitogenesis reaction and auto MLR. Figs. 8.30 and 8.31 show the effect of adding rhIL-1 alpha (from the same source as that used in Fig. 8.19), at time zero, to the DC induced oxidative mitogenesis reaction and auto MLR respectively. At all of the concentrations tested, from 1×10^{-4} to 10 U/ml, rhIL-1 alpha enhanced T lymphocyte proliferation in the oxidative mitogenesis reaction. In contrast, however, rhIL-1 alpha had no effect upon T lymphocyte proliferation in the auto MLR when tested at a concentration of 1 U/ml, despite the dose dependent inhibitory effect of the HIL1190 IL-1 alpha antibody as confirmed in the same experiment.

rhIL-1 alpha from another source and rhIL-1 beta were also tested for their effects upon the DC induced oxidative mitogenesis reaction and auto MLR. The activity of these cytokines was confirmed in an experiment depicted in Fig. 8.32 which shows that both significantly enhance murine thymocyte

proliferation in a co-stimulator assay with PHA when used at concentrations at or above 1 X 10^{-4} ug/ml. Both cytokines also enhanced T lymphocyte proliferation in the DC induced oxidative mitogenesis reaction [although higher concentrations of each were required for this effect i.e. greater than 1 X 10^{-2} ug/ml (Fig. 8.33 a)]. However, in the same experiment, neither of the cytokines affected T lymphocyte proliferation in the DC induced auto MLR at any of the concentrations tested, i.e. 1 X 10^{-5} to 1 ug/ml (Fig. 8.33 b).

Effect of IL-1 antibodies and rhIL-1 upon dendritic cell-T lymphocyte clustering in the dendritic cell induced oxidative mitogenesis reaction and auto MLR. The HIL1190 and S76 IL-1 alpha antibodies and the ANOC205, OCT204 and S77 IL-1 beta antibodies, all of which had no effect upon proliferation in DC induced oxidative mitogenesis, also had no effect upon DC-T lymphocyte clustering in this response. Similarly, the HIl1190 IL-1 alpha antibody and the ANOC205 and OCT204 IL-1 beta antibodies, each of which inhibited proliferation in the DC induced auto MLR, had no effect upon DC-T lymphocyte clustering in this response either (not shown).

rhIL-1 alpha and rhIL-1 beta were also were also tested for their effects upon DC-T lymphocyte clustering in the DC induced oxidative mitogenesis reaction and auto MLR. In the former response, where both of these cytokines enhanced proliferation, no effect upon DC-T lymphocyte clustering was observed. Likewise, in the latter response, where neither of these cytokines influenced proliferation, there was also no effect upon clustering (not shown).

Fig. 8.1

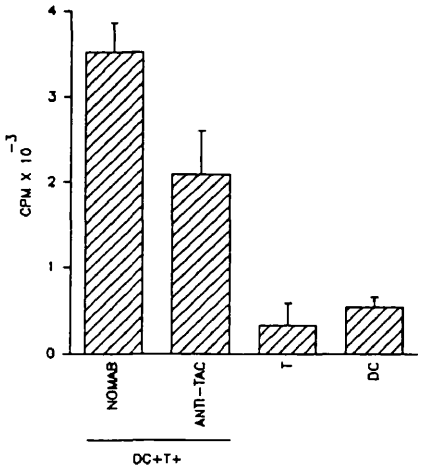


Fig. 8.2

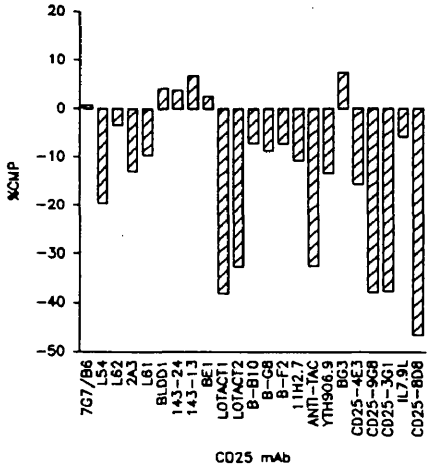


Fig. 8.3

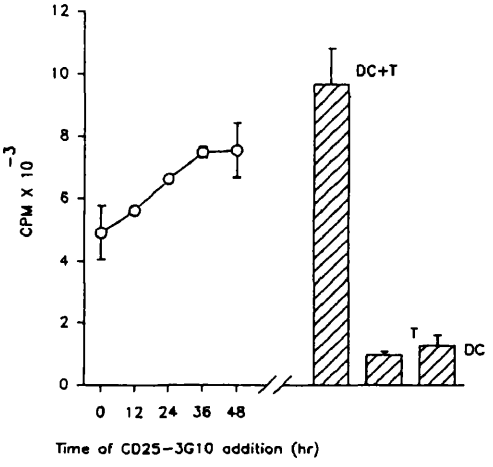


Fig. 8.4

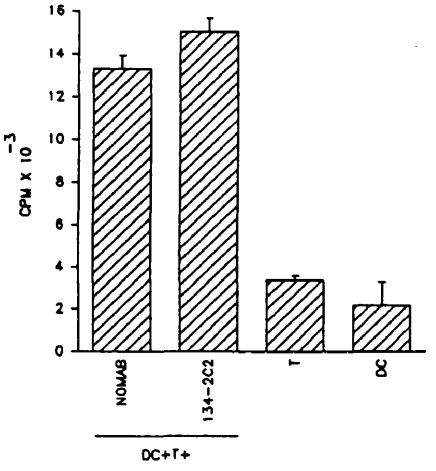


Fig. 8.5

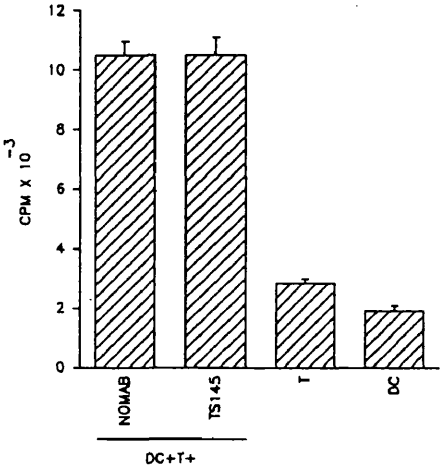


Fig. 8.6

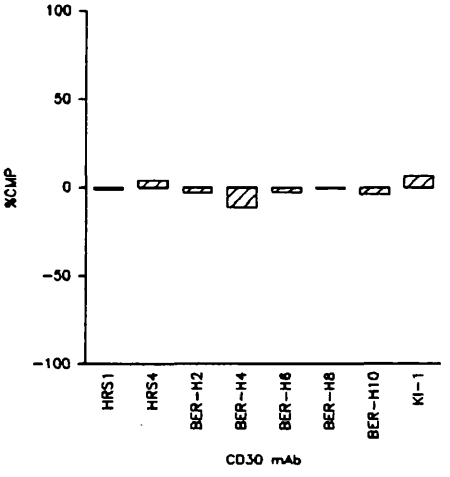


Fig. 8.7

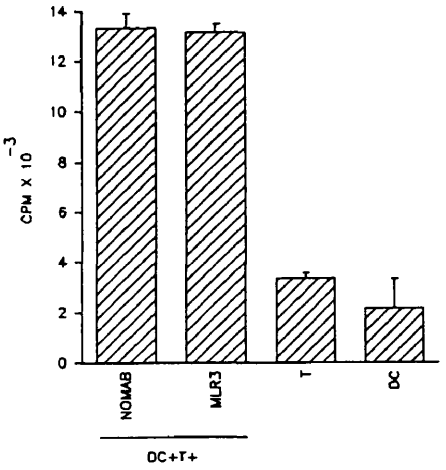


Fig. 8.8

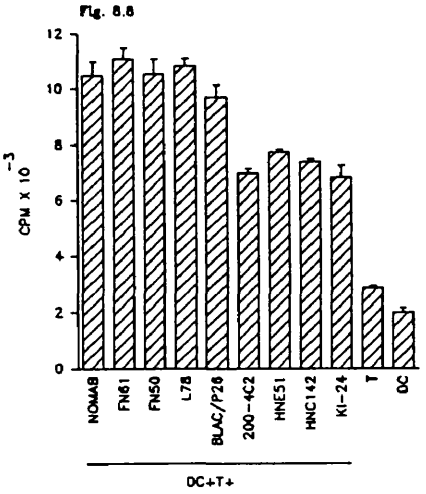


Fig. 8.9

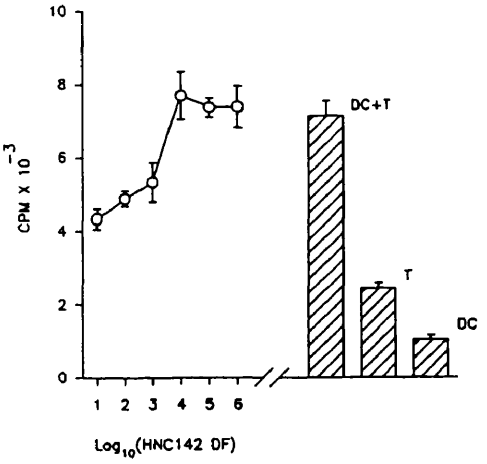


Fig. 8.10

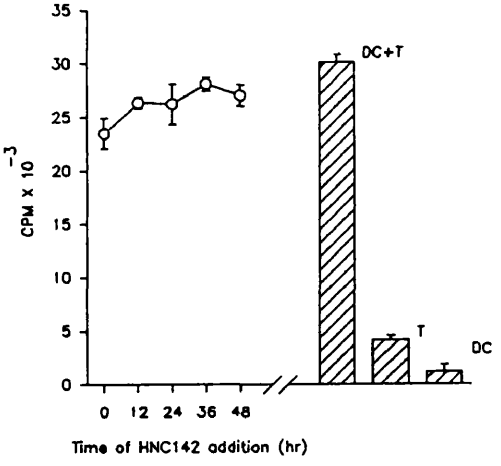


Fig. 8.11

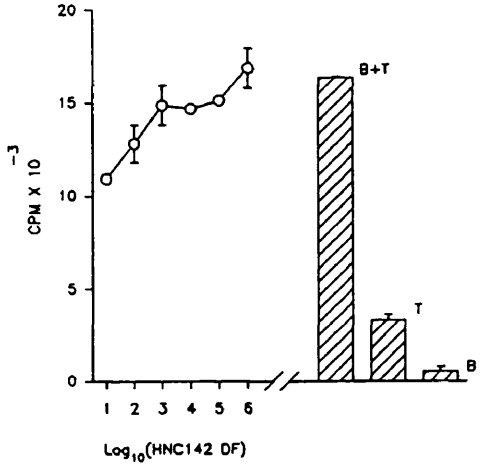


Fig. 8.12

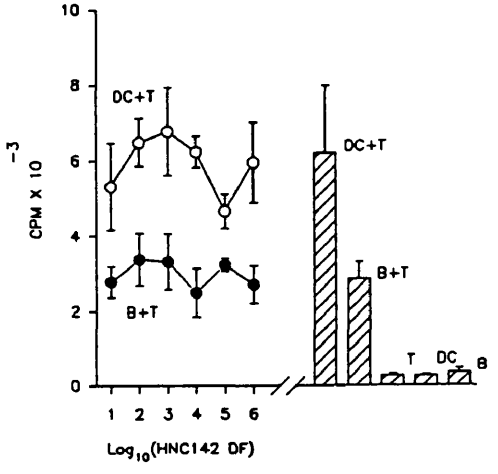
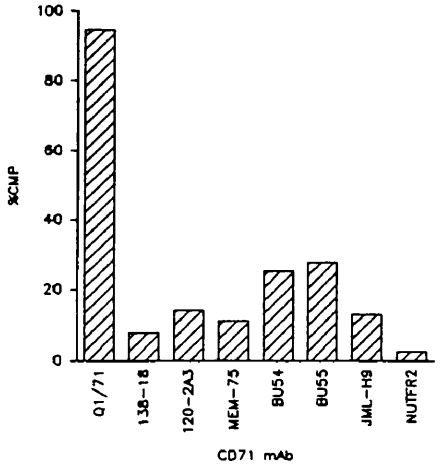


Fig. 8.13



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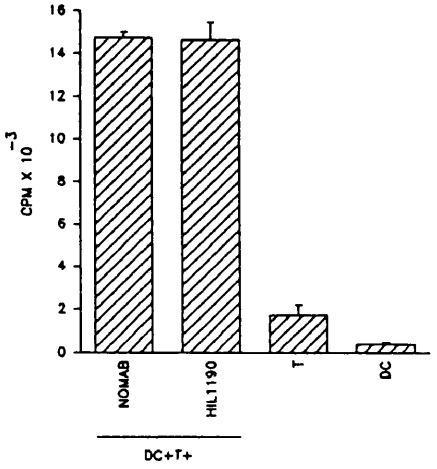

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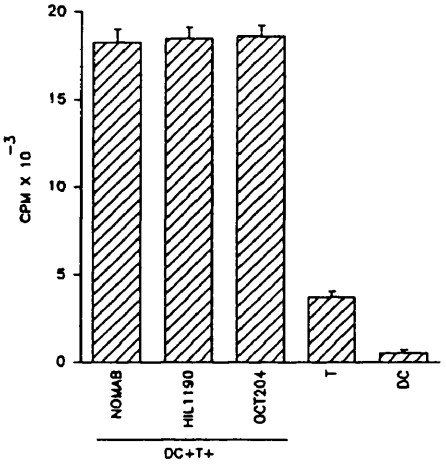

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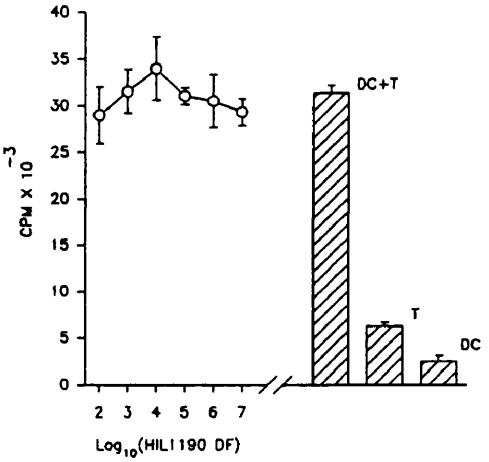

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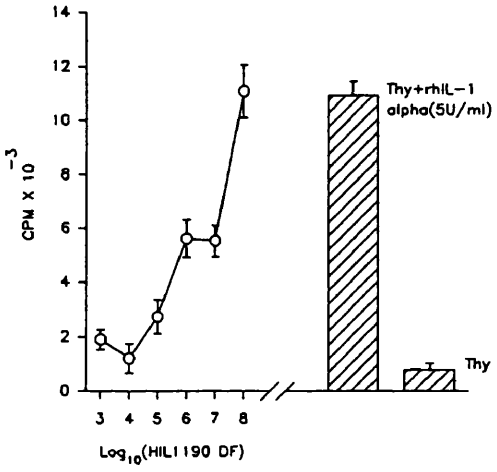

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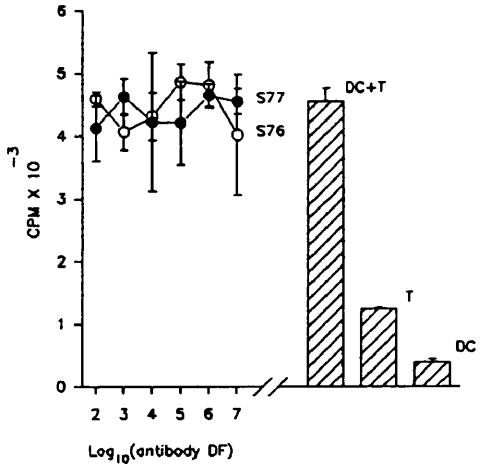

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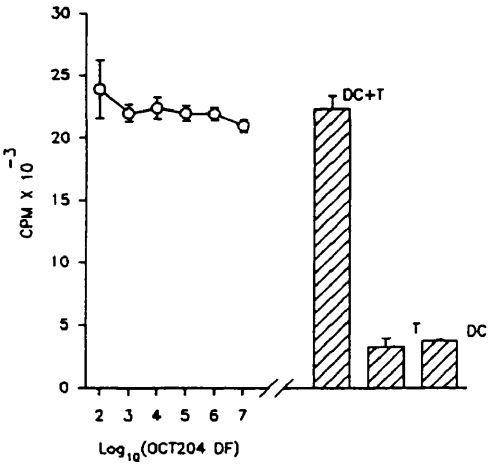

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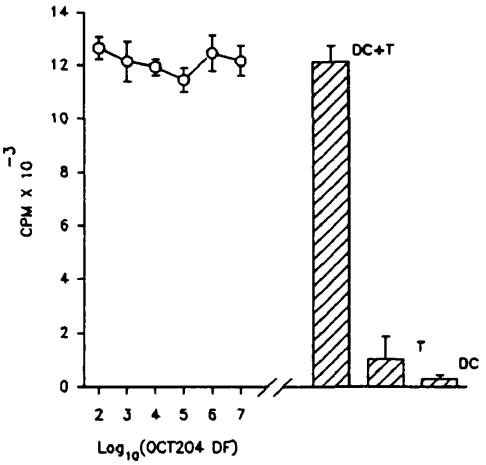

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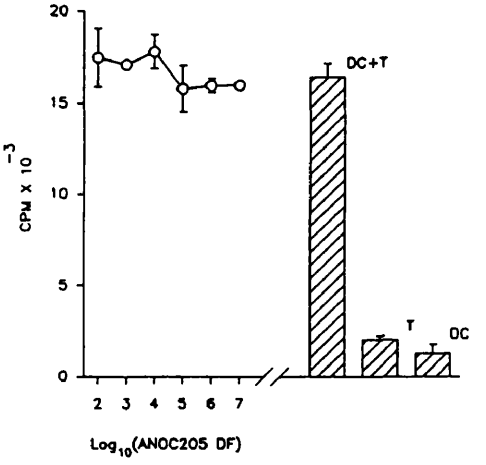

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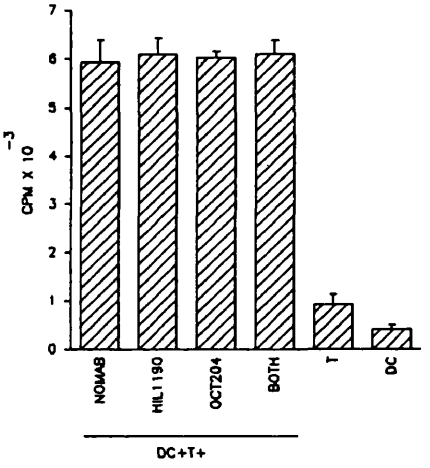

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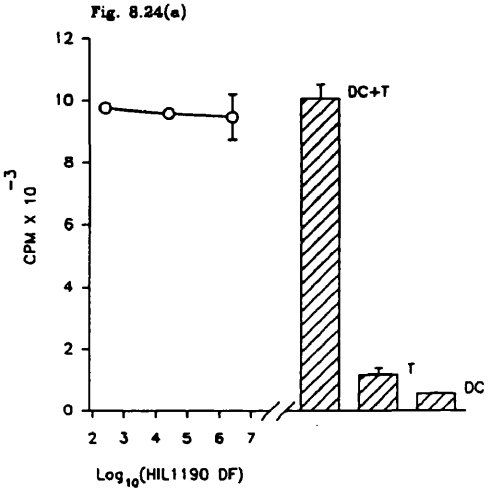

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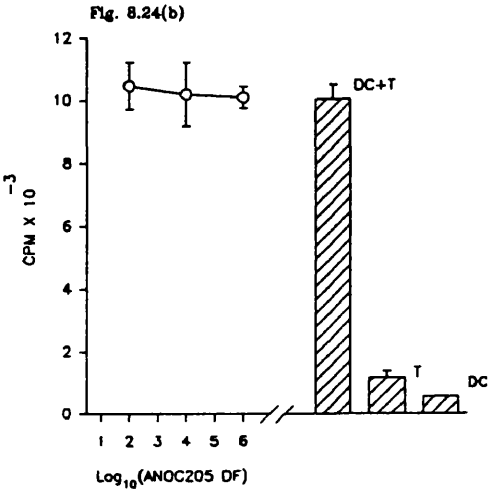

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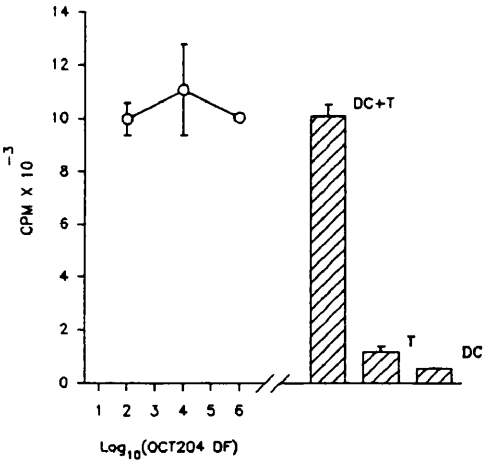

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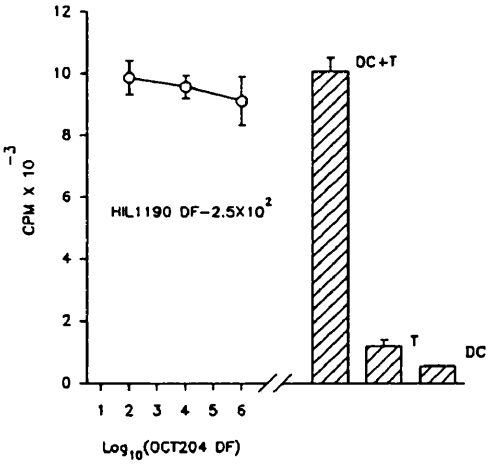

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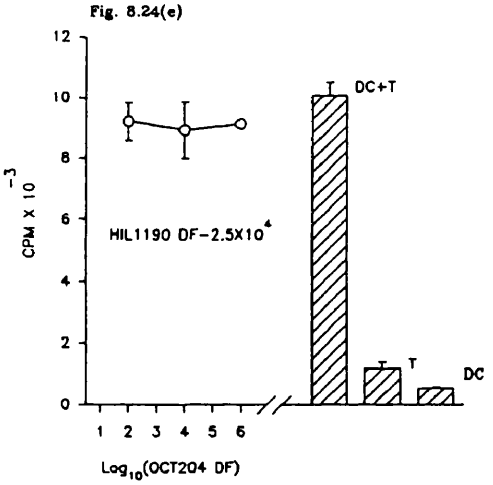

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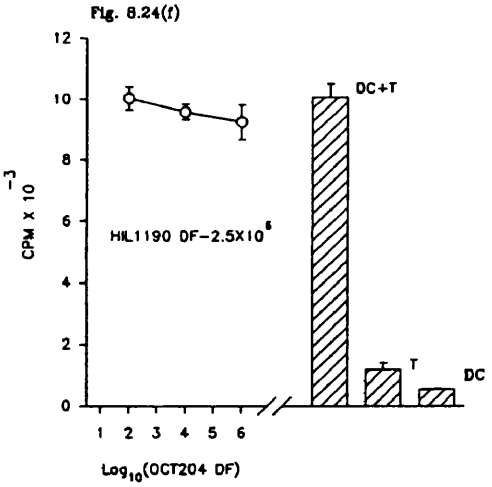

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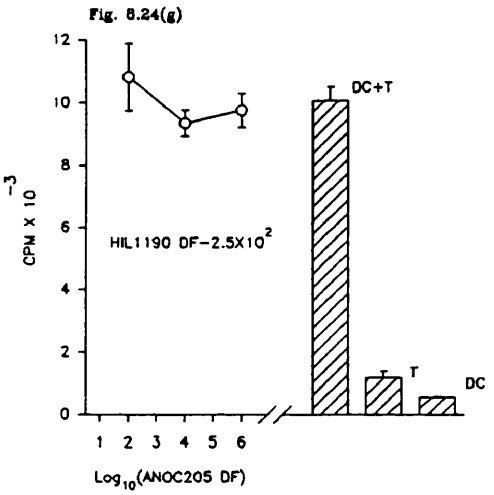

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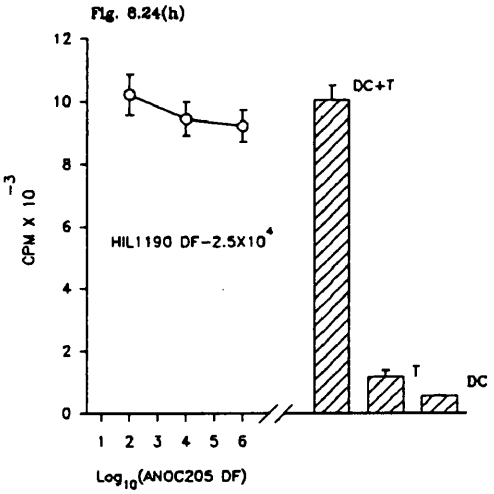

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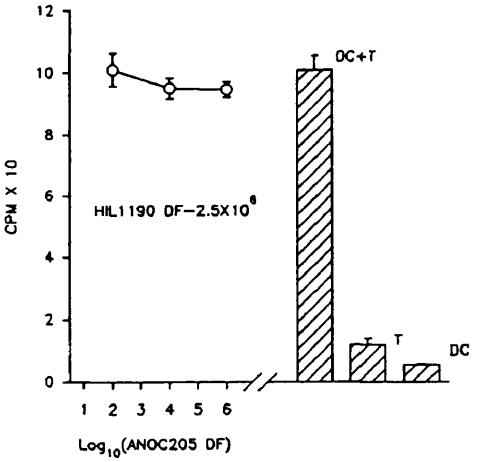
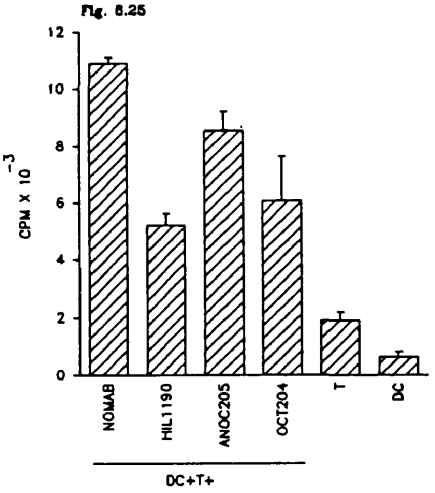


Fig. 8.25



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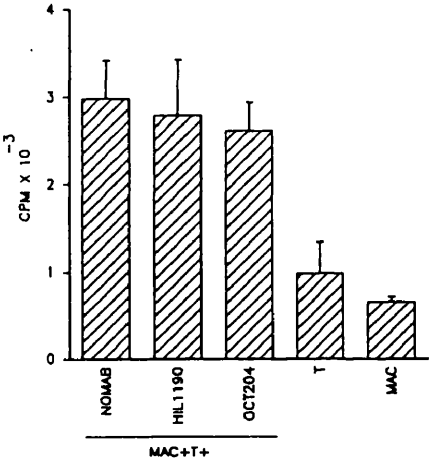

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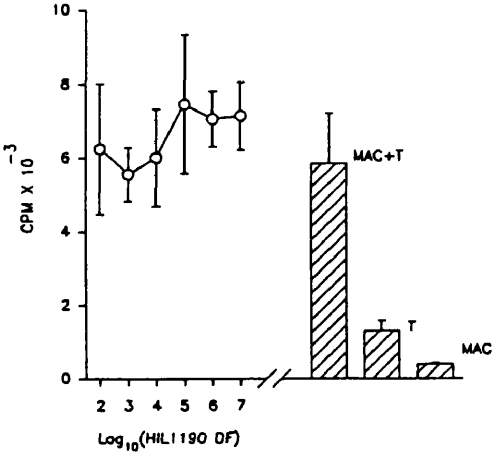

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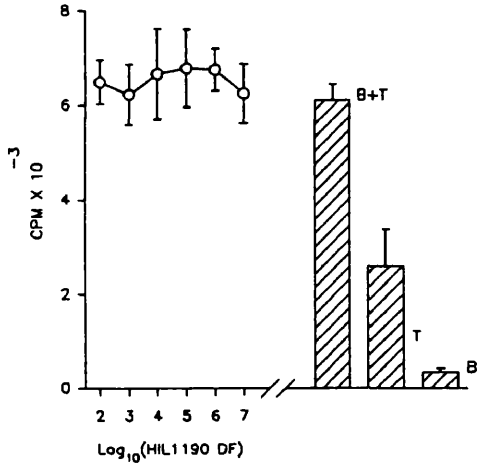

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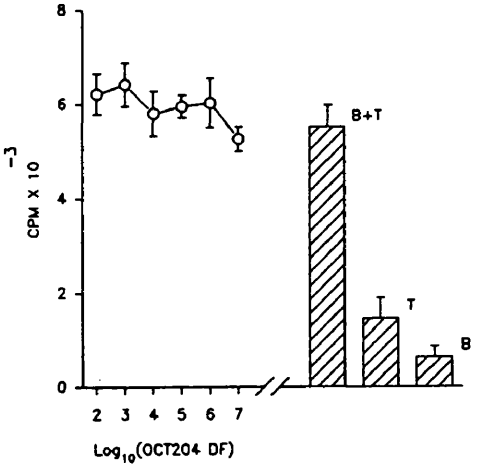
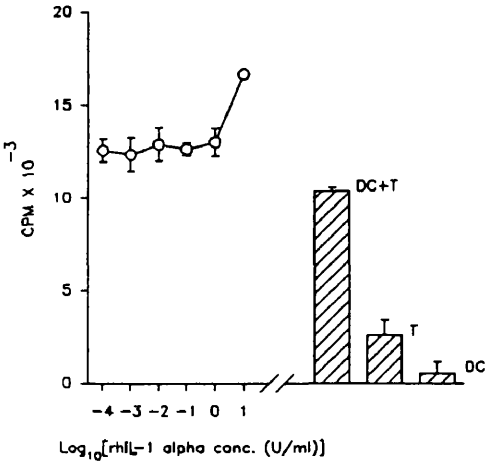


Fig. 8.30



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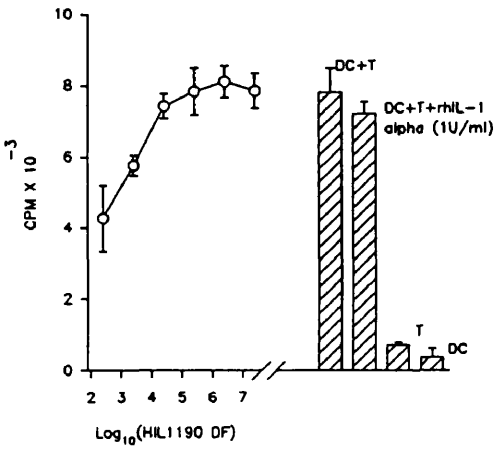

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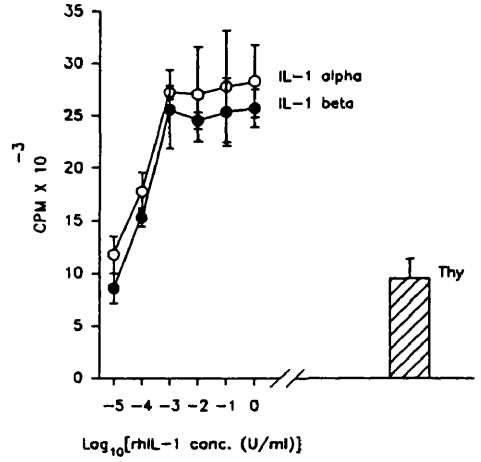
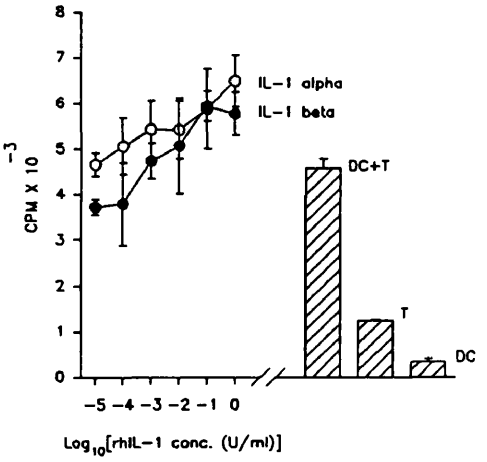


Fig. 8.33(a)



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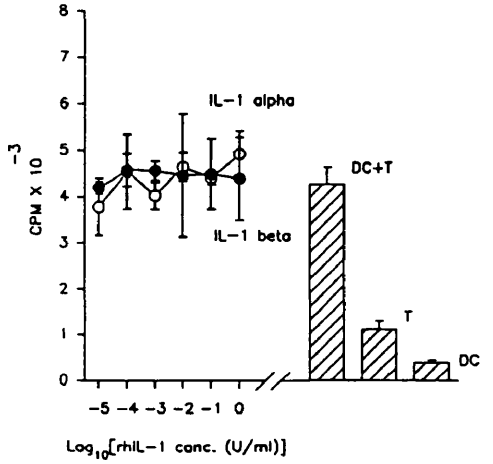


Fig. 8.14

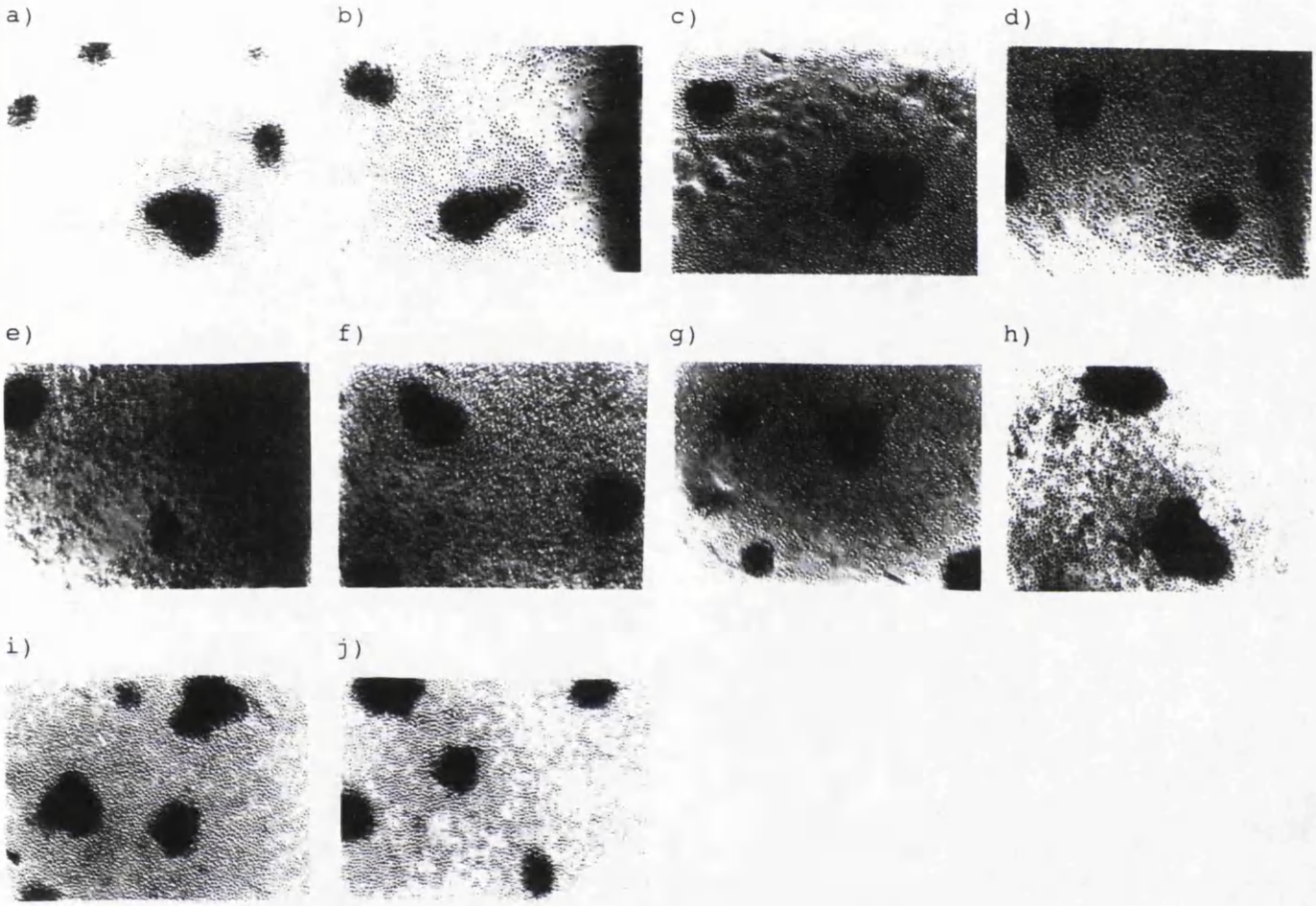


Fig. 8.15

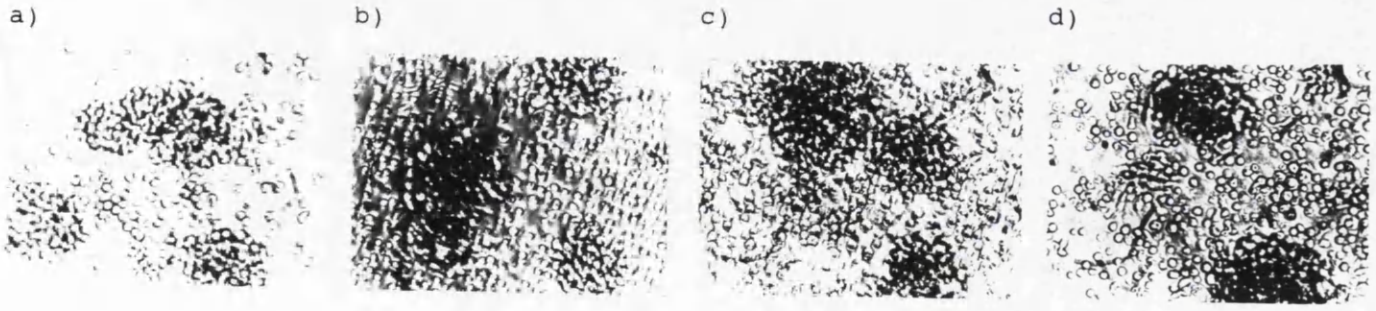


Figure Legends

See Materials and Methods plus:

Fig. 8.1. DC induced oxidative mitogenesis. DC number - 5 X 10^4/well, anti-Tac DF - 2 X 10^2.

Fig. 8.2. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well; all mAb DF 4 X 10^2; L54, 2A3, LOTact1, LOTact2, anti-Tac, YTH906.9, CD25-4E3, CD25-9G8, CD25-3G10 and CD25-8D8 inhibitions are SS; 7G7/B6, L62, L61, BL-DD1, 143-24, 143-13, B-E1, B-B10, B-G8, B-F2, 11H2.7, B-G3 and IL7.9L inhibitions or enhancements are not SS.

Fig. 8.3. DC induced oxidative mitogenesis. DC number - 5 X 10^4/well, CD25-3G10 DF - 6 X 10^2.

Fig. 8.4. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, 134-2C2 DF - 4 X 10^2.

Fig. 8.5. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, TS145 DF - 4 X 10^2.

Fig. 8.6. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, all mAb DF - 4 X 10^2 (Ki-1 - AF), all inhibitions and enhancements are not SS.

Fig. 8.7. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, MLR3 DF - 4 X 10^2.

Fig. 8.8. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well; all mAb DF - 4 X 10^2; 200-4C2, HNE51, HNC142 (AF) and Ki-24 inhibitions are SS; FN61, FN50, L78 and BL-Ac/p26 inhibitions or enhancements are not SS.

Fig. 8.9. DC induced oxidative mitogenesis. DC number - 4 X 10^4/well, HNC142 - CS.

Fig. 8.10. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, HNC142 (CS) DF - 10.

Fig. 8.11. B lymphocyte induced oxidative mitogenesis. B lymphocyte number - 1 X 10^5/well, HNC142 - CS.

Fig. 8.12. DC and B lymphocyte induced auto MLR. DC and B lymphocyte number - 1 X 10^5/well, HNC142 - CS.

Fig. 8.13. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well; all mAb DF - 4 X 10^2; Q1/71, 120-2A3, MEM-75, BU54, BU55 and JML-H9 enhancements are SS; 138-18 and NuTfR2 enhancements are not SS.

Fig. 8.16. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, HIL1190 DF - 2.5 X 10^2.

Fig. 8.17. DC induced oxidative mitogenesis. DC number - 5 X 10^4/well, HIL1190 DF - 2.5 X 10^2, OCT204 DF - 10^2.

Fig. 8.18. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well.

Fig. 8.19. Murine thymocyte co-stimulator assay. rhIL-1 alpha source - Dainippon Corporation.

Fig. 8.20. DC induced oxidative mitogenesis. DC number - 5 X 10^4/well.

Fig. 8.21(a). DC induced oxidative mitogenesis. DC number - 5 X 10^4/well.

Fig. 8.21(b). DC induced oxidative mitogenesis. DC number - 1 X 10^5/well.

Fig. 8.22. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well.

Fig. 8.23. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, HIL1190 DF - 2.5 X 10^2, OCT204 DF - 10^2.

Fig. 8.24(a-i). DC induced oxidative mitogenesis. DC number - 1 X 10^5/well.

Fig. 8.25. DC induced auto MLR. DC number - 1 X 10^5/well, HIL1190 DF - 2.5 X 10^2, ANOC205 and OCT204 DF - 10^2.

Fig. 8.26. Macrophage induced oxidative mitogenesis. Macrophage number - 3 X 10^4/well, HIL1190 DF - 2.5 X 10^2, OCT204 DF - 10^2.

Fig. 8.27. Macrophage induced oxidative mitogenesis. Macrophage number - 8 X 10^4/well.

Fig. 8.28. B lymphocyte induced oxidative mitogenesis. B lymphocyte number - 1 X 10^5/well.

Fig. 8.29. B lymphocyte induced oxidative mitogenesis. B lymphocyte number - 1 X 10^5/well.

Fig. 8.30. DC induced oxidative mitogenesis. DC number - 1 X 10^5/well, rhIL-1 alpha source - Dainippon Corporation.

Fig. 8.31. DC induced auto MLR. DC number - 1 X 10^5/well, rhIL-1 alpha source - Dainippon Corporation.

Fig. 8.32. Murine thymocyte co-stimulator assay. rhIL-1 alpha and beta source - Glaxo.

Fig. 8.33(a,b). DC induced oxidative mitogenesis (a) and auto MLR (b). DC number - 5 X 10^4/well. rhIL-1 alpha and beta source - Glaxo.

Figs. 8.14 and 8.15 - DC-T lymphocyte clustering

Fig. 8.14. Oxidative mitogenesis. All photographed at 12 hr, all DC+T plus (a) NomAb, (b) anti-Tac (CD25), (c) 7G7/B6 (CD25), (d) L54 (CD25), (e) L62 (CD25), (f) 2A3 (CD25), (g) L61 (CD25), (h) BL-DD1 (CD25), (i) L78 (CD69), (j) BL-Ac/p26 (CD69).

Fig. 8.15. Oxidative mitogenesis. All photographed at 12 hr, all DC+T plus (a) NomAb, (b) HNC142 (CDw70), (c) HNE51 (CDw70), (d) 120-2A3 (CD71).

The role of activation antigens in signal transduction in tonsillar dendritic cell induced oxidative mitogenesis. In the first section of this Chapter, to investigate any potential role of activation antigens in tonsillar DC induced oxidative mitogenesis, CD25, CD26, CD30, CD69, CDw70 and CD71 antibodies were each tested for their effects upon T lymphocyte proliferation and DC-T lymphocyte clustering in this response. In summary, CD25 and CDw70 antibodies inhibited, CD26 and CD71 antibodies enhanced and CD30 and CD69 antibodies did not affect proliferation. None of the antibodies had any effect upon DC-T lymphocyte clustering. These findings, therefore, suggest that the CD25, CD26, CDw70 and CD71 antigens, but not the CD30 and CD69 antigens are involved in signal transduction in tonsillar DC induced oxidative mitogenesis.

At least some T lymphocytes utilise the IL-2/IL-2R autocrine pathway to proliferate in tonsillar dendritic cell induced oxidative mitogenesis. The signal transduction role of the CD25 antigen in T lymphocyte proliferation induction is well established. Hence, the CD25 antigen (alpha chain), in combination with the p75 molecule (beta chain), forms a high affinity IL-2R upon T lymphocytes, which, in an end step, following binding of T lymphocyte released IL-2, instructs the these cells to divide (580-582). Presumably then, T lymphocyte reception of an IL-2 signal is the signal transduction role of the CD25 antigen defined here in tonsillar DC induced oxidative mitogenesis and in this context the ten CD25 antibodies which inhibit proliferation can be thought of as blocking an IL-2/IL-2R interaction whereas the thirteen CD25 antibodies which do not affect proliferation can be thought of as binding to non-functional epitopes upon the CD25 antigen.

On the question of whether or not all T lymphocytes use the IL-2/IL-2R

autocrine pathway in this response the finding that none of the inhibitory CD25 antibodies completely blocked T lymphocyte proliferation might be relevant. This could suggest that some T lymphocytes utilise different autocrine stimulatory pathways and in this regard a strong candidate is the IL-4/IL-4R pathway which has previously been implicated in the mediation of T lymphocyte proliferation (583-585). One reservation against this, however, is that the p75 molecule on it's own constitutes a functional intermediate affinity IL-2R (586-588). Hence, lack of complete inhibition caused by the inhibitory CD25 antibodies may reflect an involvement of the non-complexed p75 molecule in T lymphocyte IL-2 signal reception rather than an independence of some T lymphocytes from the IL-2/IL-2R pathway.

CD25 antibodies, although causing a diminishing degree of inhibition of proliferation with an increasing time delay between culture initiation and antibody addition, still significantly inhibited proliferation at all time points of antibody addition up to and including 48 hr. As discussed before (see Chapter 4), inhibition at all time points suggests that either the CD25 antigen is involved in a low affinity interaction at an early stage or alternatively is involved at a late stage in this response. However, the probability that the role of the CD25 antigen in tonsillar DC induced oxidative mitogenesis relates to high affinity IL-2 binding, the fact that the CD25 antigen is an activation antigen (not appearing upon PHA or PMA plus calcium ionophore stimulated T lymphocytes, for example, until 20 hr post-stimulation, 589) and the fact that IL-2/IL-2R interaction is conceived of as one of the final events in the induction of T lymphocyte proliferation in other systems all strongly favour the latter explanation. Also as discussed before (Chapter 4), a basis for the decreased level of inhibition seen at later time points of antibody addition might be that at these times some T lymphocytes have become committed to division.

Possible role of the CD26 antigen as a regulator of IL-2 activity. A clue as to the signal transduction role of the CD26 antigen in tonsillar DC induced oxidative mitogenesis comes from the recent finding of it's identity to an ectoenzyme known as dipeptidyl peptidase IV (DPPIV; 590,591). DPPIV is a serine type exopeptidase which cleaves X-Pro dipeptides from the N-terminal ends of polypeptides (592) and one suggestion has been that DPPIV serves to down regulate T lymphocyte proliferation by inactivating IL-2 (593) whose extreme N-terminal end contains the X-Pro dipeptide (594). A previous report has documented an ability of the 134-2C2 CD26 antibody to enhance the proliferation of T lymphocytes induced with PMA (595). In this situation and in tonsillar DC induced oxidative mitogenesis (where 134-2C2 also enhanced proliferation) this enhancing effect can be interpreted as blockade of the DPPIV active site, thus leading to increased local concentrations of IL-2. If this is correct, presumably the TS145 CD26 antibody does not affect proliferation in tonsillar DC induced oxidative mitogenesis because it does not block the enzyme activity of DPPIV.

Involvement of the CDw70 antigen in oxidative mitogenesis reactions but not in auto MLRs. Similar to the CD48 antigen (see Chapter 7), the finding that the CDw70 antigen is involved in signal transduction in tonsillar DC induced oxidative mitogenesis, is the first description of a functional role for this molecule. The CDw70 antigen appears to be very weakly expressed at cell surfaces (596) and thus in this interaction one possibility is that it functions as a T lymphocyte cytokine receptor.

A peculiar feature of CDw70 antibodies is their ability to inhibit T lymphocyte proliferation in the tonsillar DC and B lymphocyte induced oxidative mitogenesis reactions but not in auto MLRs stimulated by these cells. This might suggest that, in contrast to the view that the oxidative mitogenesis reaction is an accelerated and augmented auto MLR (see Chapter

3), there are more fundamental differences in the mechanism of these two responses. However, it is possible that expression of the CDw70 antigen upon T lymphocytes and it's involvement in oxidative mitogenesis is a result of quantitatively increased signalling to T lymphocytes, in turn a consequence of a Schiff base mediated increased T lymphocyte/accessory cell adhesion at the single T lymphocyte level; and in this view, because in oxidative mitogenesis hyper-stimulation of T lymphocytes would be a requirement for CDw70 antigen expression, the CDw70 antigen might act simply to increase the rate of this response.

Like CD25 antibodies, CDw70 antibodies, although causing less inhibition with an increasing time delay of antibody addition, still inhibited proliferation in tonsillar DC induced oxidative mitogenesis irrespective of their time of addition to culture. Once again the conclusion might be that the CDw70 antigen is involved at a late stage in this response and that decreasing levels of inhibition with time reflect a commitment of some T lymphocytes to division. Indeed, in other studies the CDw70 antigen has been described as a relatively late activation antigen which is not induced upon PBMC until 42 hr following stimulation with PHA or in an allo MLR (539,589).

Unique enhancing effect of CD71 antibodies related to T lymphocyte iron uptake or a novel signal transduction function of the transferrin receptor. As with the CD25 antigen the function of the CD71 antigen/transferrin receptor in T lymphocyte proliferation is well established. Following IL-2 mediated induction of the CD71 antigen upon T lymphocytes this molecule is thought to bind and transport iron, in the form of transferrin, into these cells, which is essential for their growth and division (597,598).

If delivery of iron to T lymphocytes is the signal transduction role of the CD71 antigen in tonsillar DC induced oxidative mitogenesis then, it

is not immediately apparent why six out of eight tested CD71 antibodies enhance rather than inhibit proliferation in this response. One explanation for this, however, may be that, to date, the only CD71 antibodies which inhibit cellular proliferation are multimeric (IgM or IgA) antibodies, which achieve their effects by cross-linking and then blocking an internalisation of the CD71 antigen/transferrin complex (598-600). In contrast, IgG CD71 antibodies do not affect cellular proliferation although do cause a rapid internalisation of the complex (598). Therefore, because all of the CD71 antibodies tested in this study are of the IgG isotype (excepting Q1/71 whose isotype is unknown) inhibition of proliferation would not be expected. Further, enhancement of proliferation might result from an increased rate of delivery of transferrin to T lymphocytes. Presumably, in other systems, IgG CD71 antibodies do not enhance proliferation because the extracellular concentration of transferrin is not limiting. In tonsillar DC induced oxidative mitogenesis, however, due to hyper-proliferation the extracellular concentration of transferrin might indeed be limiting (much of the available transferrin would be tied up intracellularly).

Of course, another explanation for enhancement is that the CD71 antigen functions as a receptor for an alternative ligand in a separate positive signalling pathway and in this view the CD71 antibodies might mimic the function of this alternative ligand. In fact there is previous evidence (albeit suggestive) that the CD71 antigen performs such an additional signalling role. Thus although with some cell lines inhibition of proliferation mediated by multimeric CD71 antibodies can be overcome by addition of soluble iron, with other cell lines and with peripheral blood lymphocytes, soluble iron only partially or does not at all overcome this type of inhibition (601-603).

Endogenous and exogenous roles for IL-1 in tonsillar dendritic cell induced responses: Distinction between the tonsillar dendritic cell induced oxidative mitogenesis reaction and auto MLR. To examine if IL-1 is involved in tonsillar DC induced oxidative mitogenesis, in the second section of this Chapter, IL-1 antibodies were tested for their effects upon proliferation and clustering in this response. Also, for comparison, IL-1 antibodies were tested for their effects upon in the tonsillar DC induced auto MLR. Interestingly, IL-1 alpha and beta antibodies did not affect proliferation or clustering in the oxidative mitogenesis reaction but both types of antibody inhibited proliferation in the auto MLR (although did not affect clustering). Thus, these findings suggest that in the tonsillar DC induced auto MLR, but not in the tonsillar DC induced oxidative mitogenesis reaction, IL-1 is involved in signal transduction, probably functioning as a "second signal".

Why IL-1 is involved in the tonsillar DC induced auto MLR but not in the oxidative mitogenesis reaction induced by these cells is unclear but there are three distinct possibilities each of which is consistent with the additional finding that IL-1 alpha and beta antibodies also have no effect upon T lymphocyte proliferation in the tonsillar macrophage and B lymphocyte induced oxidative mitogenesis reactions. Firstly, one explanation for non-involvement in oxidative mitogenesis reactions might be that by 64 hr significant induction of IL-1 from DC (or other accessory cell types) has not occurred. Secondly, IL-1R might not be induced upon T lymphocytes in oxidative mitogenesis. Thirdly, it is possible that IL-1 is indeed involved in oxidative mitogenesis reactions, simply that IL-1 antibodies fail to block these responses because Schiff base bridges between T lymphocytes and accessory cells prevent antibody access to the intercellular spaces in which IL-1 might be expected to act.

Also in the second section of this Chapter, to confirm that, exogenous IL-1 can boost human DC induced T lymphocyte responses by enhancing DC-T lymphocyte clustering, rhIL-1 alpha and rhIL-1 beta were, too, tested for their effects upon proliferation and clustering in the tonsillar DC induced oxidative mitogenesis reaction and auto MLR. Opposite to the effects upon proliferation of IL-1 antibodies both cytokines boosted proliferation in the tonsillar DC induced oxidative mitogenesis reaction but neither cytokine had any effect upon proliferation in the tonsillar DC induced auto MLR. However, in the tonsillar DC induced oxidative mitogenesis reaction, IL-1 boosting of proliferation was not paralleled by an enhancement of clustering (and neither did rhIL-1 affect clustering in the tonsillar DC induced auto MLR).

The most simple interpretation of the specific enhancing effect of IL-1 upon the tonsillar DC induced oxidative mitogenesis reaction draws upon the first model that was invoked to explain the different effects of IL-1 antibodies (see above). In this regard, IL-1 might boost tonsillar DC induced oxidative mitogenesis by acting at the T lymphocyte level, and in this view boosting of the tonsillar DC induced oxidative mitogenesis reaction and not the tonsillar DC induced auto MLR would be a consequence of a saturation of T lymphocyte IL-1R in the latter response but not in the former. It is also possible though, that as reported previously for mouse DC induced T lymphocyte responses, IL-1 boosts tonsillar DC induced oxidative mitogenesis by acting at the DC level instead. However, in this hypothesis, it is necessary to envisage that DC express IL-1R in an oxidative mitogenesis reaction but not in an auto MLR. Further, if IL-1 does act at the DC level in tonsillar DC induced oxidative mitogenesis this must be associated with an augmented signalling rather than an enhanced clustering capacity of these cells.

Summary

To summarise, in this Chapter, it has been shown that CD25, CD26, CDw70 and CD71 antigens play a role in signal transduction in tonsillar DC induced oxidative mitogenesis. In contrast, CD30 and CD69 antigens are not involved in proliferation induction in this response. The signal transduction roles played by the CD25 and CD71 antigens are probably high affinity IL-2 binding and transferrin binding by T lymphocytes respectively and for the CD25 antigen this would be consistent with its apparent late involvement in the response. The signal transduction roles of the CD26 and CDw70 antigens are less clear. The CD26 antigen may play a negative role by inactivating IL-2 and the CDw70 antigen might function as a T lymphocyte cytokine receptor. Whatever the role of the CDw70 antigen the function of this molecule appears to be peculiar to oxidative mitogenesis reactions where it is involved at a late stage in the response.

Also in this Chapter it has been demonstrated that IL-1 does not play an endogenous role in any of the tonsillar DC, B lymphocyte or macrophage induced oxidative mitogenesis reactions but does play an endogenous role in the tonsillar DC induced auto MLR, probably functioning as a T lymphocyte "second signal". Also, it has been demonstrated that exogenous IL-1 boosts T lymphocyte proliferation in the tonsillar DC induced oxidative mitogenesis reaction but not in the tonsillar DC induced auto MLR. The most obvious interpretation of these findings is that non-involvement of IL-1 in oxidative mitogenesis reactions is a consequence of their short time course and, further, that IL-1 boosts oxidative mitogenesis by acting at the T lymphocyte rather than the DC level.

CHAPTER 9

QUANTITATIVE ASSAYS OF ACCESSORY CELL-T LYMPHOCYTE ADHESION

INTRODUCTION

Although visual assessment of DC-T lymphocyte clusters provides a quick and reliable method for determining the effects of antibodies upon clustering, to confirm such effects a quantitative assay of clustering is desirable. As outlined in "Aim of studies", however, the fact that tonsillar DC are non-adherent presents difficulties, precluding, for example, the employment of simple quantitative assays in which T lymphocyte binding to substratum bound DC could otherwise be monitored. In this Chapter, therefore, a novel quantitative clustering assay is described which has been used to examine the effects of antibodies upon tonsillar DC-T lymphocyte clustering. In this assay, DC-T lymphocyte clusters are separated from free cells by sieving and consequently a requirement for substratum adherence is circumvented.

For comparative purposes, tonsillar T lymphocyte binding to the human U937 promonocytic and HL-60 promyelomonocytic cell lines (both induced to differentiate along the mononuclear phagocytic pathway by pre-treatment with PMA) and the molecular mechanisms involved therein have, too, been examined in this quantitative sieving assay. Both of these differentiated cell types, however, are firmly adherent to plastic and, hence, in parallel, a second quantitative clustering assay, which exploits this feature of these cells, has also been used. This adherent cell-T lymphocyte binding assay has proven particularly useful for the investigation of the molecular events which occur during clustering.

MATERIALS AND METHODS

Cells. DC, low and high density T lymphocytes were isolated from human tonsils as described in Chapter 2. HL-60 and U937 cells (ATCC, Rockville, USA) were kept in continuous culture in the laboratory in 10% complete medium in a 5% CO_2/37^{o}C humidified incubator and were passaged every 2-3 d.

PMA differentiation of HL-60 and U937 cells. HL-60 and U937 cells were induced to differentiate by culture in 10% complete medium containing 5 ng/ml PMA (Sigma). For sieving assays 10^7 U937 or HL-60 cells were seeded into 102 mm diameter plastic petri dishes in 10 ml volumes of medium. For well assays 2.5 X 10^5 U937 or HL-60 cells were seeded into the the wells of 24 well plastic plates in 0.6 ml volumes of medium. Culture of dishes or plates was for 72 hr in a 5% CO_2/37^{o}C humidified incubator.

^{51}Cr labelling of T lymphocytes. High and low density T lymphocytes, resuspended at 5 X 10^7 cells/ml in 10% complete medium, were ^{51}Cr labelled labelled by incubation with 50 uCi/ml of $Na_2{}^{51}CrO_4$ (Amersham) for 45 min at 37^{o}C. Following this time, lymphocytes were, washed twice with RPMI 1640, resuspended in RPMI 1640 and incubated for a further 30 min at 0-5^{o}C. After a final three washes in RPMI 1640 T lymphocytes were used in cluster assays.

Sieving assay of dendritic cell-T lymphocyte clustering. For the assessment of DC-T lymphocyte clustering by sieving 5 X 10^6 ^{51}Cr labelled high density T lymphocytes (0.5 ml in 10% human AB serum) were added to 1 X 10^6 DC (also 0.5 ml in 10% human AB serum) in 10 ml centrifuge tubes. Antibodies were added to some tubes (in small volumes) and additional control tubes contained T lymphocytes alone (i.e. T lymphocytes were added to 0.5 ml 10% human AB serum rather than DC). To assist cluster formation tubes were spun at 170 X g for 5 min and cells were then incubated as pellets for 4 hr at

37°C. After this time, cells were resuspended in their own supernatants and applied to 15 um pore size nylon meshes (Cadisch) stretched across the openings of scintillation counting vials (LKB, Croydon, Surrey, UK), using a 1 ml syringe (Sterlin). Finally, meshes were washed once with RPMI 1640 (again using a 1 ml syringe) and radioactivity associated with clusters on the mesh and free cells collected in the vial (following removal of supernatants by spinning) was determined in a NE 1600 gamma counter.

Sieving assay of HL-60/U937-T lymphocyte clustering. For the assessment of PMA differentiated HL-60 and U937-T lymphocyte clustering by sieving HL-60 and U937 cells were harvested from petri dishes by scraping with the plunger of a 10 ml syringe. Cells were then aspirated from dishes and washed twice in RPMI 1640 (to remove residual PMA) before use in assays. Assays were similar to the sieving assay outlined above except that varying numbers of ^{51}Cr labelled high density T lymphocytes and 3.3 X 10^6 HL-60 or U937 cells were added to tubes; assays were performed in 10% complete medium rather than human AB serum; and following addition of T lymphocytes to HL-60 or U937 cells tubes were not spun but were gently vortexed and incubation before sieving was for 1 hr rather than 4 hr at 37°C.

Well assay of HL-60/U937-T lymphocyte clustering. HL-60 and U937-T lymphocyte clustering was also quantified in a well assay which exploits the plastic adherent nature of PMA differentiated HL-60 and U937 cells. In standard assays (compare with modifications below) the wells of the 24 well plates in which the HL-60 or U937 cells had been differentiated (see above) and wells on the same plates which had contained 0.6 ml 10% complete medium/5 ng/ml PMA alone for 72 hr (for subsequent assessment of T lymphocyte binding to plastic) were all washed three times in RPMI 1640 (to remove non-adherent HL-60 or U937 and/or residual PMA). Each well then

received 0.2 ml of 10% complete medium, with or without an antibody (added to the medium in a small volume), followed by 0.2 ml of ^{51}Cr labelled high or low density T lymphocytes in varying concentrations again in 10% complete medium. Culture of plates was for 1 hr (or for varying time periods) at 37^{o}C in a shaking water bath (Grant Instruments Ltd., Cambridge, Cambridgeshire, UK) which was pre-set at 30 strokes/min. After this time, unbound T lymphocytes were harvested from wells in three washes of RPMI 1640 (the original aspiration plus two 0.4 ml washes), fractions from each well were pooled and associated radioactivity was measured as above (again supernatants were removed from T lymphocytes before counting). Similarly, radioactivity associated with bound T lymphocytes was measured following two rounds of solubilisation of these cells by incubation with agitation in 0.4 ml of 0.1M sodium hydroxide.

Modifications of the well assay of U937-T lymphocyte clustering. In certain experiments the standard well assay of U937-T lymphocyte clustering was modified as follows:

(i) Divalent cation assays - To examine the effect of divalent cation chelating agents upon upon clustering, assays were performed in 1% BSA medium [HBSS supplemented with 1% de-ionised BSA (Sigma) and 10 mM de-ionised Hepes] with or without varying concentrations of EGTA (Sigma) or EDTA (Sigma). Differentiated U937 cells were washed in HBSS rather than RPMI 1640 and were incubated in the above media for 10 min at 37^{o}C before culture with T lymphocytes. Likewise, after labelling, T lymphocytes were also washed in HBSS and incubated with the media for 10 min before resuspension and addition to U937 cells. Where the effects of antibodies were additionally examined these were added to the U937 cells immediately after the 10 min incubation period and before the addition of T lymphocytes. In some experiments metabolic inhibitors [5mM sodium azide (Sigma) and 25 mM 2-

deoxy-D-glucose (2DDG; Sigma)] were included with EDTA into 1% BSA medium.

In other divalent cation experiments Mg^{2+}/Ca^{2+} free 1% BSA medium was used [as 1% BSA medium only using HBSS free of Mg^{2+} and Ca^{2+} ions (Gibco)] to which was added (or was not added) differing concentrations of $MgCl_2$ (Sigma) and/or $CaCl_2$ (Sigma). In these experiments the U937 cells and T lymphocytes were washed with Mg^{2+}/Ca^{2+} free HBSS and neither cell type was pre-incubated with Mg^{2+}/Ca^{2+} free 1% BSA medium (with or without added Mg^{2+} and/or Ca^{2+} ions) before co-culture.

(ii) Fixation assays - To glutaraldehyde fix U937 cells, 300 ul of 0.05% glutaraldehyde (Agar Scientific, Stansted, Essex, UK; made up in PBS) was added to the wells after washing. After 30 s at RT 300 ul of 0.2 M l-lysine (Sigma; in RPMI 1640) was added to quench the glutaraldehyde. Wells that were not fixed received 600 ul of RPMI 1640 and before assay all wells were washed three times with this medium. A similar protocol was observed for the glutaraldehyde fixation of T lymphocytes only fixation was carried out in 10 ml centrifuge tubes. Again both fixed and unfixed T lymphocytes were washed three times in RPMI 1640 before experiments.

For the $ZnCl_2$ fixation of U937 cells, wells were washed three times in buffer A (50 mM N-morpholinoethansulphonic acid; Sigma), 5 mM $MgCl_2$ and 3 mM EGTA in PBS, pH - 6.0] before incubation in 400 ul of 1 mM $ZnCl_2$ (Sigma) supplemented buffer A for 2 min at RT. Wells that were not fixed were similarly treated but were incubated in buffer A for 2 min at RT rather $ZnCl_2$ supplemented buffer A. All wells were washed three times in PBS before experiments.

Paraformaldehyde fixation of cells was according to the method of Moreno and Lipsky (604). U937 cells were washed three times in PBS and were incubated in 400 ul of 1% solution (Sigma; in PBS) for 5 min at $37^{o}C$. 1 ml of 0.06% glycyl-glycine (Sigma; in PBS) was then added to wells to quench

the paraformaldehyde. As above, wells that were not fixed were similarly treated (only in this case were incubated in 400 ul of PBS for 5 min at 37^{o}C). All wells were washed three times (in PBS) before assay. Paraformaldehyde fixation of T lymphocytes was as for U937 cells with the difference that fixation was carried out in 10 ml centrifuge tubes. Again fixed and unfixed T lymphocytes were washed three times in PBS before assay.

In all fixation experiments, to measure radioactivity associated with bound T lymphocytes, wells were treated with 1 M rather than 0.1 M sodium hydroxide and for each round of solubilisation plates were placed into a shaking water bath (set as above) for 2 min.

(iii) Effect of other agents - Assays in which the effects of sodium orthovanadate (Na_3VO_4) were examined were performed in 1% BSA medium as indicated for EGTA/EDTA assays. Vanadate (Sigma) was included in the medium in varying concentrations and pre-incubation was for 20 min at 37^{o}C.

Finally, in some experiments (not concerned with the effects of EGTA/EDTA or vanadate), assays were performed in 10% human AB serum or in 1 or 5 % BSA medium.

Antibodies. Details of the antibodies used in clustering experiments are given in Tables 2.1, 2.2, 5.1, 6.2, 6.3 and 7.1. Additional quantities of some of these antibodies were obtained as AF preparations from F. Garrido (GRHL1 - CD44), G. Hale (YTH54.12 - CD45), V. Horejsi (MEM-30 and MEM-25 - CD11a), J. Ledbetter (LB-2 - CD54), T. Schulz (7F7 - CD54) and R. Villela (135-4C5 and 135-4H9 - CD45). A list of other antibodies used in clustering assays is given in Table 9.1. All antibodies are mAb and were AF preparations. Where appropriate antibodies were sterile filtered, dialysed and de-aggregated (see Chapter 4). In all assays CS antibodies were used at a final dilutions of <1:50 and AF preparations were used at dilutions of >1:100.

Preparation and SDS-PAGE analysis of an Fab fragment of the F10-44-2 CD44 antibody. F10-44-2 antibody concentration was determined at approximately 1 mg/ml in an Ultrospec 4050 spectrophotometer (LKB) and 1 ml of antibody was incubated with 50 ul of 1 M cysteine (Sigma; final concentration 50 mM), 50 ul of 20 mM EDTA (final concentration 1 mM) and papain that was immobilised to sepharose beads (Sigma; final concentration of papain 10 ug/ml). Digestion was allowed to proceed for 1, 2, 3, or 4 hr. At these time points papain was sedimented from the digestion mixture by centrifugation (650 x g for 5 min) and a 25 ul aliquot of the supernatant was removed for SDS-PAGE analysis (see below). The papain was then either resuspended (for further digestion at the 1, 2 and 3 hr time points) or was removed altogether from the digestion mixture (at the 4 hr time point).

To remove any undigested antibody and Fc pieces, 4 hr digests were passed over a protein A column (Pharmacia). The column was first equilibriated with 100 mM Tris buffer (pH 8.0), 1 ml of digest (i.e. 900 ul of original digest plus 100 ul of 1 M Tris) was then applied and the column was washed through, again with 100 mM Tris buffer. 1 ml fractions were collected from the column and those fractions containing protein (i.e. Fab fragments) were pooled and concentrated X 10 (final concentration approximately 1 mg/ml) on an Amicon membrane (Amicon, Stonehouse, Gloucestershire, UK).

SDS-PAGE was performed under reducing conditions on a Phastgel electrophoresis system (Pharmacia). Sample buffer was a solution of 10 mM Tris buffer (pH 8.0) containing 1 mM EDTA, 2.5% SDS (Sigma) and 0.01% bromophenol blue (Sigma). Samples were boiled with this solution for 5 min and 1 ul quantities were applied to gels. Gels were stained with Coomassie blue dye (Pharmacia).

Microscopy/photography. For microscopy of U937-T lymphocyte clusters, T

lymphocytes were left unlabelled and clusters were set up as indicated in standard well assays (see above). Phase contrast microscopy and photography of fields was as described in Chapter 4. Samples for electron microscopy were processed by a standard method and were viewed on a Zeiss EM109 electron microscope (Zeiss, Oberkacken, FDR). For photography TMAX black and white 100 ASA film (Kodak) was used. Finally, developed SDS gels were photographed using 50 ASA colour film (KODAK).

Statistics Assays were performed in duplicate or triplicate. For sieving assays results are expressed as mean percentage T sieved +/- 1 SD where percentage T sieved is as follows:

$$\%\ \text{T sieved} = \frac{\text{mesh CPM}}{\text{mesh CPM + filtrate CPM}} \times 100$$

For well assays results are expressed as mean percentage T bound +/- 1 SD where percentage T bound is as follows:

$$\%\ \text{T bound} = \frac{\text{bound CPM}}{\text{bound CPM + unbound CPM}} \times 100$$

Additionally, in both sieving and well assays the effect of an antibody upon clustering is expressed as a percentage change of mean DC/HL-60/U937-T binding as follows:

$$\%\ \text{change of mean DC/HL-60/U937-T binding} = \frac{\text{mean \% T sieved/bound in presence of antibody} - \text{mean \% T sieved/bound in absence of antibody}}{\text{mean \% T sieved/bound in absence of antibody} - \text{mean \% T sieved /bound in absence of DC/HL-60/U937 and antibody}} \times 100$$

Statistical analysis was performed as indicated in Chapter 4 comparing DC/HL-60/U937 plus T lymphocyte samples with DC/HL-60/U937 plus T lymphocyte

plus antibody samples. NSS - not SS.

Table 9.1 - Additional antibodies used in clustering experiments

Antibody	CD grouping/ specificity	Species of origin[a]	Isotype	Source
OKT11	CD2			P. Beverley
B-1y6	CD11c	M	G1	Workshop 4
L29	CD11c	M	G1	Workshop 4
BL-4H4	CD11c	M	G1	Workshop 4
MEM-59	CD43	M	G1	V. Horejsi
G10-2	CD43	M	G1	Workshop 4
MEM-85	CD44	M	G1	V. Horejsi
AA44	CD45	M	G1	Workshop 4
YTH24.5	CD45	R	G2b	Workshop 4/ G. Hale
124-2H12B	CD45	M	G2a	R. Villela
MEM-55	CD45-like	M	G1	Workshop 4
MEM-56	CD45R	M	G2b	Workshop 4
BRIC125	CD47	M	G2b	Workshop 4
HI36	CD53	M	G3	Workshop 4
HD77	CD53	M	M	Workshop 4
6.5B5	CD54			D. Haskard
F2B7.2	CD55	M	G1	Workshop 4
BRIC110	CD55	M	G1	Workshop 4
L185	CD56	M	M	Workshop 4
MEM-43	CD59	M	G2a	V. Horejsi
CRIS-4	CD76	M	M	Workshop 4

a) M - mouse, R - rat

Quantification of dendritic cell-T lymphocyte clustering by sieving. Fig. 9.1 shows the results of a typical experiment in which the extent of clustering between DC and autologous T lymphocytes (in the presence and absence of a CD11a antibody - MHM24) and between autologous T lymphocytes themselves (all after 4 hr of culture) was assessed by the sieving of clusters on 15um pore size nylon mesh. As evidenced by an approximate 2.5 fold increase in the percentage of T lymphocytes sieved (comparing DC plus T lymphocyte samples with T lymphocyte alone samples) DC efficiently clustered T lymphocytes. Also, MHM24 completely blocked this clustering.

In a series of similar experiments other antibodies were also tested for their effects upon DC-T lymphocyte clustering in this assay. The results of these experiments and the result obtained with MHM24 above are summarised in Table 9.2 which shows that a CD2 antibody (TS2/18.1.1) and a CD58 antibody (TS2/9) partially blocked clustering; a CD11a antibody (MHM24), a CD18 antibody (MHM23) and a CD54 antibody (84H10) either completely blocked or substantially blocked clustering; and a CD4 antibody (Leu3a), a class I MHC antibody (W6/32) and a class II MHC antibody (Hig-78) did not affect clustering.

Quantification of HL-60 and U937-T lymphocyte clustering by sieving. Fig. 9.2 shows that PMA differentiated HL-60 cells (a) and U937 cells (b) also clustered T lymphocytes (in this case allogeneic) as assessed in the quantitative sieving assay [in general in the sieving assay U937 and HL-60 cells were more efficient than DC at clustering T lymphocytes and in the sieving assay as well as in a well assay (see below) U937 cells were more efficient than HL-60 cells in this respect - see discussion]. With HL-60 cells the specific percentage of T lymphocytes sieved was constant over

Table 9.2 - Effect of antibodies upon dendritic cell-T lymphocyte clustering in a sieving assay

CD grouping/ specificity	Antibody	Experiment number	Antibody dilution	% change of mean DC-T binding	p
CD2	TS2/18.1.1	1	1:30	-17.9	<0.05
CD4	Leu3a	5	1:100	-9.0	NSS
CD11a	MHM24	3	1:1000	-116.1	<0.0005
CD18	MHM23	4	1:1000	-121.3	<0.0005
CD54	84H10	4	1:200	-80.5	<0.01
CD58	TS2/9	2	1:20	-26.0	<0.05
HLA-A,B,C	W6/32	1	1:20	-1.0	NSS
HLA-DP,DQ,DR	Hig-78	2	1:10	0.0	NSS

Table 9.3 - Effect of antibodies upon HL-60/U937-T lymphocyte clustering in a sieving assay[a)]

Cell type	CD grouping/ specificity	Antibody	Experiment number	Antibody dilution	% change of mean HL-60/U937-T binding	p
HL-60	CD2	TS2/18.1.1	2	1:50	+6.0	NSS
	CD58	TS2/9	1	1:20	-7.5	NSS
		TS2/9	2	1:20	-6.3	NSS
U937	CD8	UCHT4	4	1:10	-7.5	NSS
	CD11a	MHM24	3	1:1000	-88.5	<0.05
	CD11c	3.9	2	1:20	-1.9	NSS
	CD18	MHM23	2	1:2000	-87.0	<0.005
	CD58	TS2/9	3	1:20	+15.0	NSS
	HLA-A,B,C	W6/32	3	1:20	+8.8	NSS

a) T lymphocyte concentration - 4 X 10^6/tube in all assays

three different T lymphocyte concentrations. Likewise, with U937 cells the specific percentage of T lymphocytes sieved was too constant between a T lymphocyte concentration of 1 X 10^6 and 4 X 10^6/ml although dropped slightly at the highest T lymphocyte concentration of 1 X 10^7/ml.

A representative experiment in which different antibodies were tested for their effects upon HL-60 and U937-T lymphocyte clustering in the sieving assay is depicted in Fig. 9.3. As shown a CD2 antibody (TS2/18.1.1) and a CD58 antibody (TS2/9) did not affect HL-60-T lymphocyte clustering, a CD11c antibody (3.9) did not affect U937-T lymphocyte clustering and a CD18 antibody (MHM23) almost completely blocked U937-T lymphocyte clustering.

In other experiments additional antibodies were tested for their effects upon U937-T lymphocyte clustering. The results, together with the results from the above experiment, are summarised in Table 9.3 which shows that a CD11a antibody (MHM24) and a CD18 antibody (MHM23) almost completely blocked clustering whereas a CD8 antibody (UCHT4), a CD11c antibody (3.9), a CD58 antibody (TS2/9) and a class I MHC antibody (W6/32) did not influence U937-T lymphocyte clustering. Also shown in Table 9.3 is the result of another experiment in which the TS2/9 CD58 antibody was tested for it's effect upon HL-60-T lymphocyte clustering in the sieving assay. Again TS2/9 did not affect HL-60-T lymphocyte clustering.

A well assay for the quantitative assessment of HL-60 and U937-T lymphocyte clustering. As an alternative to the sieving assay a well assay was used for the quantitative assessment of HL-60 and U937-T lymphocyte clustering. This assay has the advantage that it requires far fewer HL-60 and U937 cells thus enabling a more extensive analysis of the effects of antibodies or of other agents. Fig. 9.4 depicts the results of a typical experiment using HL-60 cells in the well assay. As shown HL-60 cells efficiently clustered T lymphocytes. Also, a CD11a antibody (MHM24) almost completely inhibited

Table 9.4 - Effect of antibodies upon HL-60-T lymphocyte clustering in a well assay[a)]

CD grouping/ specificity	Antibody	Experiment number	Antibody dilution	% change of mean HL-60-T binding	p
CD2	TS2/18.1.1	3	1:10	+5.0	NSS
CD4	Leu3a	2	1:30	-12.1	NSS
CD8	UCHT4	5	1:10	+18.8	NSS
CD11a	MHM24	2	1:1000	-79.9	<0.005
CD11c	3.9	4	1:50	-7.2	NSS
CD18	MHM23	1	1:100	-111.7	<0.05
CD58	TS2/9	3	1:10	+15.3	NSS
		5	1:10	+3.0	NSS
HLA-A,B,C	W6/32	2	1:10	-3.2	NSS
HLA-DP,DQ,DR	Hig-78	2	1:10	+24.5	<0.05

a) T lymphocyte concentration - 3×10^6/well in all assays

Table 9.5 - Effect of MHC antibodies upon U937-T lymphocyte clustering in a well assay

Anti-body	Specificity	Allotype restriction	Experiment number	Antibody dilution	% change of mean U937-T binding	p
W6/32	HLA-A,B,C	None	53	1:10	-3.5	NSS
Hig-78	HLA-DP,DQ,DR	None	3	1:20	+13.4	NSS
			53	1:10	+12.9	NSS
Tu22	HLA-DQ,DR	DQ - None DR - ?	3	1:2500	+44.0	<0.025
Tu35	HLA-DP,DR	DP - ? DR - None	3	1:2500	+19.2	<0.025
Tu36	HLA-DR	?	3	1:20	+15.3	<0.005
Tu39	HLA-DP,DR,DY	None	3	1:2500	+37.3	<0.01
L243	HLA-DR	None	43	1:400	+24.2	<0.05
			47	1:400	+64.3	<0.005

See Footnote to Table 9.4

clustering, a class II MHC antibody (Hig-78) caused a small but significant enhancement of clustering and a CD4 antibody (Leu3a) and a class I MHC antibody (W6/32) did not affect HL-60-T lymphocyte clustering.

The results of other experiments in which additional antibodies were tested for their effects upon HL-60-T lymphocyte clustering in the well assay, along with the results from the above experiment, are summarised in Table 9.4. None of a CD2 antibody (TS2/18.1.1), a CD4 antibody (Leu3a), a CD8 antibody (UCHT4), a CD11c antibody (3.9), a CD58 antibody (TS2/9) and a class I MHC antibody (W6/32) affected clustering whereas a CD11a antibody (MHM24) almost completely blocked clustering, a CD18 antibody (MHM23) completely blocked clustering and a class II MHC antibody (Hig-78) significantly enhanced clustering.

U937 cells were investigated in more detail for their ability to cluster T lymphocytes in the quantitative well assay. Fig. 9.5 shows that U937 cells efficiently clustered both high (a) and low (b) density T lymphocytes over a range of T lymphocyte concentrations in this assay [see also Fig. 9.6 which shows phase contrast micrographs (a-d) and a transmission electron micrograph (e) of U937-high density T lymphocyte clusters set up in plastic wells]. In both cases the percentage of specific U937-T lymphocyte binding increased with an increasing number of added T lymphocytes to reach a plateau level of specific binding at an added T lymphocyte number of approximately 1 X 10^6/well (the plateau level of specific binding was slightly higher with low density T lymphocytes than with high density T lymphocytes). Probably, this increase and then levelling of specific adhesion reflects a tropism of T lymphocytes (whose adhesion to plastic steadily decreased with an increasing number of added T lymphocytes) for the well periphery (see discussion).

The effect of time upon U937-high density T lymphocyte clustering in

the well assay is shown in Fig. 9.7. In this experiment, which used 3 X 10^6 T lymphocytes/well, specific U937-T lymphocyte binding increased with time to reach a peak at 2 hr. Thereafter binding decreased slightly up to a time point of 4.5 hr.

The results of experiments in which large panels of MHC antibodies, LeuCAM and ICAM-1 antibodies, T and B lymphocyte and myeloid antibodies, and non-lineage restricted antibodies were tested for their effects upon U937-T lymphocyte clustering in the well assay summarised in Tables 9.5-9.8 respectively. Representative experiments from each of these Tables are depicted in Figs. 9.8-9.11 respectively. In all of these antibody experiments high density T lymphocytes were used at a concentration of 3 X 10^6/well and assay duration was kept at 1 hr. Most experiments were performed in 10% complete medium (i.e. 10% FCS) although some experiments were performed in 1% BSA medium. Fig. 9.12 shows that U937 cells were equally efficient at clustering T lymphocytes in these two media.

Firstly (Table 9.5 and Fig. 9.8), a class I MHC antibody (W6/32) did not affect U937-T lymphocyte clustering. In contrast to this, however, five out of six tested class II MHC antibodies (i.e. Tu22, Tu35, Tu36, Tu39 and L243) caused a small but statistically significant enhancement of clustering. Indeed, even the class II MHC antibody which did not significantly affect clustering (Hig-78) in fact caused a small degree of enhancement ($p<0.1$ in experiment 3 and $p<0.15$ in experiment 53).

With respect to LeuCAM and ICAM-1 antibodies (Table 9.6 and Fig. 9.9), CD11a, CD18 and CD54 antibodies inhibited clustering. Not all of the antibodies within these three CD groups, however, achieved this effect. Thus, of the eleven CD11a antibodies that were tested five (MEM-25, MEM-95, 25.3.1, MHM24 and 2F12) moderately or strongly inhibited clustering whereas six (122-2A5, MEM-30, MEM-83, YTH81.5, BU17 and 459) did not affect

Table 9.6 - Effect of LeuCAM and ICAM-1 antibodies upon U937-T lymphocyte clustering in a well assay

CD grouping	Antibody	Experiment number	Antibody dilution	% change of mean U937-T binding	p
CD11a	122-2A5	1	1:400	+4.5	NSS
	MEM-30	1	1:400	-18.3	NSS
		31	1:400	-4.4	NSS
	MEM-83	1	1:400	-14.2	NSS
	YTH81.5	1	1:400	-0.5	NSS
	BU17	14	1:200	-0.4	NSS
	MEM-25	1	1:400	-30.9	<0.025
		31	1:400	-80.5	<0.0005
	MEM-95	1	1:400	-53.0	<0.005
	25.3.1	1	1:400	-36.5	<0.025
	459	1	1:400	-11.1	NSS
	MHM24	1	1:400	-60.3	<0.025
		35	1:400	-81.5	<0.0005
		46	1:400	-64.7	<0.0005
		53	1:100	-65.3	<0.005
	2F12	35	1:400	-75.7	<0.0005
		36	1:400	-73.5	<0.01
		46	1:400	-61.6	<0.0005
CD11b	44	6	1:100	+5.6	NSS
		15	1:400	-3.1	NSS
	LPM19C	12	1:100	+1.6	NSS
		13	1:200	-3.5	NSS
	14B6.E2	13	1:200	+2.0	NSS
	5A4.C5	13	1:200	-14.3	NSS
		44	1:400	-10.7	NSS
	MN41	14	1:200	+6.7	NSS
CD11c	B-ly6	35	1:400	-6.5	NSS
	L29	40	1:400	-13.4	NSS
	BL-4H4	45	1:400	+14.6	NSS
	SHCL3	46	1:400	-5.3	NSS
	3.9	39	1:100	-0.6	NSS
CD18	11H6	1	1:400	-7.3	NSS
	M232	1	1:400	-54.6	<0.005
		44	1:400	-101.0	<0.005
		46	1:400	-82.1	<0.0005
	MHM23	1	1:400	-59.9	<0.005
		12	1:100	-71.5	<0.05
		24	1:400	-82.8	<0.0005
		41	1:400	-92.1	<0.005
		46	1:400	-77.9	<0.0005
		52	1:100	-100.8	<0.0005
	CLB-54	1	1:400	-46.3	<0.01
	YFC51.1	1	1:400	-47.9	<0.005
	GRF1	13	1:200	-94.7	<0.05
		17	1:400	-96.8	<0.05
CD54	6.5B5	3	1:400	-59.2	<0.01
	RR1/1.1.1.	4	1:360	-46.1	<0.025
	LB-2	4	1:360	-45.7	<0.05

CD grouping	Antibody	Experiment number	Antibody dilution	% change of mean U937-T binding	p
		21	1:400	-62.7	<0.001
		22	1:400	-82.1	<0.01
	MY13	4	1:360	-7.3	NSS
	WEHICAM-1	21	1:400	-62.0	<0.001
		22	1:400	-49.6	<0.01
		45	1:400	-55.1	<0.05
		46	1:400	-44.2	<0.005
	7F7	21	1:400	-10.4	NSS
		23	1:400	+3.2	NSS
		43	1:400	-0.6	NSS

See Footnote to Table 9.4

clustering; of the six CD18 antibodies that were tested five (M232, MHM23, CLB-54, YFC51.1 and GRF1) strongly or completely inhibited clustering but one (11H6) did not affect clustering; and of the six CD54 antibodies that were tested four (6.5B5, RR1/1.1.1., LB-2 and WEHICAM-1) moderately or strongly inhibited clustering whereas two (MY13 and 7F7) did not affect clustering. In contrast to the effects of CD11a, CD18 and CD54 antibodies, none of five CD11b antibodies (44, LPM19C, 14B6.E2, 5A4.C5 and MN41) and none of five CD11c antibodies (B-ly6, L29, BL4H4, SHCL3 and 3.9) had any significant influence upon U937-T lymphocyte clustering.

Included in the T and B lymphocyte and myeloid panel (Table 9.7 and Fig. 9.10) were two CD2 antibodies (M-T910 and TS2/18.1.1), one CD3 antibody (T3), two CD4 antibodies (T4 and Leu3a), two CD8 antibodies (T8 and UCHT4), one CD14 antibody (MEM-15), three CD16 antibodies (BW209/2, HUNK2 and CLBFcRGranI), one CD20 antibody (B1), two CD31 antibodies (SG134 and LAK-1), five CDw32 antibodies (CIKM5, 41H16, 2E1, IV.3 and KB61), one CD38 antibody (NOE2/6/C6) and one CD76 antibody (CRIS-4). None of these antibodies affected U937-T lymphocyte clustering.

Finally, a large number of non-lineage restricted antibodies (Table 9.8 and Fig. 9.11) enhanced U937-T lymphocyte clustering. Most notable amongst these were antibodies belonging to the CD44 and CD45 clusters. All three CD44 antibodies that were tested (F10-44-2, GRHL1 and MEM-85) strongly enhanced clustering in a consistent fashion. Also, of the twenty seven CD45 antibodies that were tested twenty two (U87, AA44, AA14, X16, GRT3, HI30, TL-1, F10-89-4, RP1/10, D3/9, 135-4C5, 135-4H9, 136-4B5, 144-2, 155-2, 138-3, YTH24.5, BMAC2, GRT2, T29/33, BMAC3 and BMAC1) strongly and consistently enhanced clustering (see also Fig. 9.13 which shows that enhancement of clustering with the 136-4B5 CD45 antibody is dose dependent) although five (124-2H12B, HI73, YTH54.12, AB187 and NULPAN) did not significantly affect

Table 9.7 - Effect of T and B lymphocyte and myeloid antibodies upon U937-T lymphocyte clustering in a well assay

CD grouping	Antibody	Experiment number	Antibody dilution	% change of mean U937-T binding	p
CD2	M-T190	45	1:400	+20.3	NSS
		47	1:400	+15.0	NSS
		48	1:400	+3.0	NSS
		49	1:400	-1.0	NSS
		50	1:400	-7.2	NSS
	TS2/18.1.1	53	1:10	+2.0	NSS
CD3	T3	43	1:400	-2.7	NSS
CD4	T4	43	1:400	+13.6	NSS
	Leu3a	53	1:300	-5.6	NSS
CD8	T8	41	1:400	-3.6	NSS
	UCHT4	39	1:10	+2.1	NSS
CD14	MEM-15	44	1:400	-9.6	NSS
CD16	BW209/2	36	1:400	-6.5	NSS
		43	1:400	+2.5	NSS
	HUNK2	40	1:400	-0.8	NSS
	CLBFcRGranI	44	1:400	+5.4	NSS
CD20	B1	40	1:400	+22.6	NSS
		46	1:400	+17.7	NSS
CD31	SG134	44	1:400	-10.9	NSS
	LAK-1	44	1:400	+6.3	NSS
CDw32	CIKM5	35	1:400	+0.5	NSS
	41H16	42	1:400	+3.9	NSS
	2E1	44	1:400	-1.3	NSS
		46	1:400	-7.8	NSS
	IV.3	44	1:400	-9.9	NSS
	KB61	45	1:400	-14.2	NSS
CD38	NOE2/6/C6	44	1:400	+11.8	NSS
CD76	CRIS-4	45	1:400	+1.6	NSS

See Footnote to Table 9.4

Table 9.8 - Effect of non-lineage restricted antibodies upon U937-T lymphocyte clustering in a well assay

CD grouping/ specificity	mAb	Experiment number	mAb dilution	% change of mean U937-T binding	p
CD43	MEM-59	31	1:400	+32.4	<0.01
	OTH71C5	37	1:400	-1.6	NSS
		46	1:400	+3.2	NSS
	G19-1	40	1:400	+15.2	NSS
	G10-2	47	1:400	+27.6	NSS
		49	1:400	+29.4	NSS
		50	1:400	+18.0	NSS
CD44	F10-44-2	8	1:200	+122.4	<0.005
		16	1:400	+105.2	<0.005
		17	1:400	+169.7	<0.025
		18	1:400	+114.1	<0.005
		25	1:400	+121.1	<0.005
		27	1:400	+63.8	<0.05
		29	1:400	+72.9	<0.01
		44	1:400	+81.5	<0.005
		47	1:400	+187.9	<0.005
	GRHL1	30	1:40	+36.7	<0.005
		44	1:400	+110.0	<0.005
		47	1:400	+197.9	<0.005
	MEM-85	31	1:400	+100.5	<0.0005
		32	1:400	+172.5	<0.005
		33	1:400	+117.1	<0.05
		34	1:400	+44.7	<0.0005
CD45	U87	2	1:400	+72.9	<0.01
	AA44	2	1:400	+76.4	<0.025
	AA14	2	1:400	+49.3	<0.01
	X16	2	1:400	+102.1	<0.05
	GRT3	2	1:400	+104.7	<0.005
	HI30	2	1:400	+125.7	<0.025
	TL-1	2	1:400	+180.7	<0.005
	F10-89-4	2	1:400	+52.9	<0.025
	RP1/10	2	1:400	+280.0	<0.001
	D3/9	2	1:400	+205.0	<0.005
	135-4C5	5	1:200	+141.5	<0.01
	135-4H9	5	1:200	+118.0	<0.005
	136-4B5	5	1:200	+136.3	<0.005
		7	1:1000	+122.9	<0.01
		9	1:400	+196.0	<0.05
		10	1:400	+105.0	<0.025
		11	1:400	+69.0	<0.05
		17	1:400	+140.9	<0.025
		19	1:400	+134.2	<0.05
		20	1:400	+87.6	<0.001
		32	1:400	+102.0	<0.005
		33	1:400	+85.5	<0.025
		34	1:400	+45.4	<0.05
	144-2	5	1:200	+144.7	<0.005

CD grouping/ specificity	mAb	Experiment number	mAb dilution	% change of mean T-U937 binding	p
		24	1:400	+79.1	<0.0005
		29	1:400	+93.8	<0.0005
	155-2	5	1:200	+183.2	<0.025
		24	1:400	+79.3	<0.0005
	124-2H12B	24	1:400	-3.6	NSS
	138-3	24	1:400	+71.8	<0.0005
	YTH24.5	19	1:400	+133.3	<0.05
	HI73	24	1:400	-1.5	NSS
	YTH54.12	23	1:400	-14.3	NSS
		24	1:400	+11.8	NSS
	GRT2	24	1:400	+32.1	<0.001
		47	1:400	+42.1	<0.025
	BMAC1	47	1:400	+66.4	<0.005
	AB187	44	1:400	+2.5	NSS
	NULPAN	46	1:400	+15.0	NSS
		47	1:400	-7.1	NSS
CD45-like	MEM-55	35	1:400	+29.9	<0.0005
		46	1:400	+24.5	<0.05
		47	1:400	+67.1	<0.005
	Tu150	35	1:400	+30.5	<0.001
		46	1:400	+129.0	<0.0005
	MEM-58	47	1:400	+13.6	NSS
		48	1:400	-3.0	NSS
		49	1:400	+22.5	NSS
		50	1:400	-4.0	NSS
CD45RA	G1-15	36	1:400	+6.0	NSS
CD45RO	UCHL1	10	1:400	+73.1	<0.05
		28	1:10	+30.5	<0.025
		46	1:400	+57.3	<0.001
CD45R	MEM-56	26	1:400	-4.8	NSS
	4KB5	38	1:400	-2.9	NSS
		49	1:400	+0.8	NSS
	X148	10	1:400	-6.9	NSS
CD46	HuLym5	38	1:400	+20.5	NSS
		39	1:400	0	NSS
	122-2	44	1:400	+51.4	<0.01
		47	1:400	+30.0	NSS
		48	1:400	+4.7	NSS
		50	1:400	+17.0	NSS
	J48	44	1:400	+27.5	NSS
		47	1:400	+52.9	<0.005
CD47	BRIC126	41	1:400	+4.0	NSS
	CIKM1	42	1:400	+44.1	<0.025
		46	1:400	+49.5	<0.01
		47	1:400	+51.8	<0.005
	BRIC125	45	1:400	-1.1	NSS
CD48	WM68	39	1:400	+10.5	NSS
	LO-MN25	40	1:400	-14.8	NSS
	J4-57	41	1:400	+5.2	NSS
	Tu145	43	1:400	+1.7	NSS
	WM63	47	1:400	+1.4	NSS
		49	1:400	+5.2	NSS

CD grouping/ specificity	mAb	Experiment number	mAb dilution	% change of mean T-U937 binding	p
	K31	45	1:400	+6.1	NSS
CDw52	097	36	1:400	+3.0	NSS
		47	1:400	-5.3	NSS
	YTH66.9	40	1:400	-1.6	NSS
	YTH361.10	43	1:400	+12.1	NSS
CD53	HI29	46	1:400	+6.8	NSS
	HI36	41	1:400	+3.8	NSS
	MEM-53	42	1:400	-0.4	NSS
	HD77	45	1:400	+15.3	NSS
		49	1:400	+0.3	NSS
CD55	F2B7.2	46	1:400	+44.2	<0.005
		47	1:400	+64.1	<0.005
	143-30	35	1:400	+6.2	NSS
	BRIC110	40	1:400	-6.2	NSS
	BRIC128	42	1:400	+39.1	NSS
		46	1:400	+11.4	NSS
		48	1:400	+87.0	<0.025
CD56	Leu19	35	1:400	+2.6	NSS
		44	1:400	-3.0	NSS
	NKH1	35	1:400	+0.2	NSS
	FP2-11-14	49	1:400	-7.2	NSS
		50	1:400	-7.0	NSS
	T-199	43	1:400	+11.3	NSS
	NKH1a	43	1:400	+12.1	NSS
	L185	46	1:400	+3.2	NSS
CD57	L186	41	1:400	+10.1	NSS
	Leu7	43	1:400	-5.4	NSS
	L183	43	1:400	+4.4	NSS
CD58	G26	48	1:400	-16.0	NSS
		49	1:400	-1.4	NSS
	BRIC5	43	1:400	-5.4	NSS
	TS2/9	45	1:400	-16.9	NSS
		51	1:20	-2.2	NSS
		53	1:10	+2.3	NSS
CD59	MEM-43	31	1:400	+41.5	<0.005
	YTH53.1	40	1:400	+28.5	NSS
		46	1:400	+17.3	NSS
		47	1:400	+29.4	<0.005
		48	1:400	+5.0	NSS

See Footnote to Table 9.4

clustering. Other antibodies which enhanced clustering included a CD43 antibody (MEM-59) which had a weak enhancing effect [three further CD43 antibodies (OTH71C5, G19-1 and G10-2) did not affect clustering], two CD45-like antibodies (MEM-55 and Tu150) which had a weak to strong enhancing effect [another CD45-like antibody (MEM-58) did not affect clustering], a CD45RO antibody (UCHL1) which had a weak to moderate enhancing effect, two CD46 antibodies (122-2 and J48) which had an inconsistent (i.e. one out of four and one out of two experiments respectively) moderate enhancing effect [a third CD46 antibody (HuLym5) did not affect clustering], a CD47 antibody (CIKM1) which had a moderate enhancing effect [two other CD47 antibodies (BRIC126 and BRIC125) did not affect clustering], two CD55 antibodies one of which (F2B7.2) had a consistent moderate enhancing effect and the other of which (BRIC128) had an inconsistent (one out of three experiments) strong enhancing effect [two other CD55 antibodies (143-30 and BRIC110) did not affect clustering] and two out of two tested CD59 antibodies (MEM-43 and YTH53.1) which had a weak to moderate enhancing effect (albeit YTH53.1 enhancing in only one out of four experiments). Of the remaining non lineage restricted antibodies that were tested for their effects upon U937-T lymphocyte clustering none of these which included a CD45RA antibody (G1-15), three CD45R antibodies (MEM-56, 4KB5 and X148), six CD48 antibodies (WM68, LO-MN25, J4-57, Tu145, WM63 and K31), three CDw52 antibodies (097, YTH66.9 and YTH361.10), four CD53 antibodies (HI29, HI36, MEM-53 and HD77), six CD56 antibodies (Leu19, NKH1, FP2-11-14, T-199, NKH1A and L185), three CD57 antibodies (L186, Leu-7 and L183) and three CD58 antibodies (G26, BRIC5 and TS2/9) had any significant effect.

Divalent cation requirements for U937-T lymphocyte clustering. Any requirement of divalent cations for U937-T lymphocyte clustering was first investigated by incorporating the chelating agents EDTA and EGTA into the

quantitative well assay. Fig. 9.14 shows that EDTA (which chelates both Ca^{2+} and Mg^{2+} ions) completely abolished clustering whereas EGTA (which chelates only Ca^{2+} ions) caused a small enhancement of clustering. This result suggested that U937-T lymphocyte clustering is dependent upon Mg^{2+} ions and that Ca^{2+} ions rather than promoting clustering might inhibit it. To explore this further U937-T lymphocyte clustering was examined in Mg^{2+}/Ca^{2+} free 1% BSA medium and in Mg^{2+}/Ca^{2+} free 1% BSA medium to which Mg^{2+} and/or Ca^{2+} ions were added in a variety of different concentrations. Table 9.9 confirms the dependency of U937-T lymphocyte clustering upon Mg^{2+} ions. In the absence of Mg^{2+} ions (and Ca^{2+} ions) little clustering between U937 cells and T lymphocytes occured but addition of increasing concentrations of Mg^{2+} ions (from 0.1 to 4 mM) resulted in a dose dependent increase of U937-T lymphocyte clustering. The influence of Ca^{2+} ions, as shown in this experiment, was more complex. In agreement with the effects of EDTA and EGTA shown in Fig. 9.14 addition of increasing concentrations of Ca^{2+} ions, in the presence of constant concentrations of Mg^{2+} ions, resulted in a dose dependent decrease in the degree of clustering. This effect, however, was only apparent at Mg^{2+} ion concentrations of greater than 0.1 mM. Thus, in the absence of Mg^{2+} ions, Ca^{2+} ions in fact caused a small increase in clustering above background levels and in the presence of 0.1 mM Mg^{2+} ions, Ca^{2+} ions did not affect clustering.

Divalent cation requirements for U937-T lymphocyte clustering in the presence of CD44 and CD45 antibodies. Next, any requirement of divalent cations for U937-T lymphocyte clustering in the presence of CD44 and CD45 antibodies was tested. Fig. 9.15 shows that, in contrast to clustering in the absence of an antibody, clustering in the presence of a CD44 antibody, F10-44-2, is not completely abolished in Mg^{2+}/Ca^{2+} free 1% BSA medium (indeed specific clustering is reduced by only 46%). Additionally, Fig. 9.16

Table 9.9 - Influence of divalent cations upon U937-T lymphocyte clustering

U937	Mg^{2+} (mM)	Ca^{2+} (mM)	% T bound
+	0	0	6.7+/-1.8
+	0	0.1	7.9+/-1.7
+	0	1	9.9+/-0.02
+	0	2	10.4+/-1.0
+	0	4	10.5+/-0.5
+	0.1	0	19.6+/-3.3
+	0.1	0.1	21.2+/-0.9
+	0.1	1	20.9+/-0.6
+	0.1	2	17.6+/-0.3
+	0.1	4	17.9+/-0.8
+	1	0	37.9+/-2.6
+	1	0.1	33.0+/-0.7
+	1	1	32.1+/-1.4
+	1	2	31.9+/-1.4
+	1	4	30.4+/-1.2
+	2	0	42.0+/-3.3
+	2	0.1	34.8+/-0.8
+	2	1	39.7+/-3.2
+	2	2	35.5+/-0.9
+	2	4	31.9+/-0.6
+	4	0	48.3+/-2.1
+	4	0.1	40.4+/-0.5
+	4	1	38.8+/-2.1
+	4	2	38.0+/-0.04
+	4	4	30.2+/-2.0
-	0	0	0.7+/-0.1
-	0	1	0.6+/-0.1
-	1	0	1.4+/-0.3
-	1	1	1.8+/-0.4

See footnote to Table 9.4

shows that, in contrast to clustering in the absence of an antibody, clustering in the presence of F10-44-2 is not ablated by EDTA (in this case specific clustering is reduced by only 72%). Unlike clustering in the presence of a CD44 antibody, clustering in the presence of a CD45 antibody, 136-4B5, is completely abolished in Mg^{2+}/Ca^{2+} free 1% BSA medium (Fig. 9.17).

Effect of EDTA, metabolic inhibitors, LFA-1 plus ICAM-1 and CD2 plus LFA-3 antibodies upon U937-T lymphocyte clustering in the absence and presence of CD44 and CD45 antibodies. A qualitative difference in the molecular mechanism of U937-T lymphocyte clustering in the absence of CD44 and CD45 antibodies and in the presence of CD45 antibodies as compared to U937-T lymphocyte clustering in the presence of CD44 antibodies was further demonstrated by incorporating metabolic inhibitors [sodium azide and 2-deoxy-D-glucose (2DDG)] and CD11a (2F12) and CD54 (LB-2) antibodies in addition to EDTA into the well assay. As shown in Fig. 9.18 these reagents completely blocked clustering in the absence of CD44 and CD45 antibodies and in the presence of a CD45 antibody, 144-2, but only partially blocked clustering in the presence of the F10-44-2 CD44 antibody (a 35% reduction of specific clustering resulted).

The effect of a CD11a antibody (2F12) in combination with a CD54 antibody (LB-2) and/or a CD2 antibody (OKT11) in combination with a CD58 antibody (TS2/9) upon U937-T lymphocyte clustering in the absence and presence of the F10-44-2 CD44 antibody is shown in Fig. 9.19. In this experiment 2F12 and LB-2 reduced specific adhesion by 48% in the absence of F10-44-2 but by only 18% in the presence of F10-44-2 [given that some CD18 antibodies have the ability to completely block normal U937-T lymphocyte clustering (see Table 9.6) an inability of 2F12 and LB-2 to completely block clustering in the absence of F10-44-2 here is probably due to non-saturating

concentrations of antibody rather than to LFA-1/ICAM independent adhesion]. OKT11 and TS2/9, by contrast, did not affect clustering in the absence or presence of F10-44-2 and neither did these antibodies augment inhibition of clustering caused by 2F12 and LB-2 antibodies again in the absence or presence of F10-44-2. In a similar experiment the 25.3.1 CD11a antibody was used in place of 2F12, a CD18 antibody (CLB-54) was included as part of an LFA-1/ICAM-1 antibody cocktail and EDTA was added to all wells. The results are depicted in Fig. 9.20 which shows that in the absence of F10-44-2 little or no U937-T lymphocyte clustering occurs whereas in the presence of F10-44-2 significant U937-T lymphocyte clustering does occur. Also, in both cases, 25.3.1 plus CLB-54 plus LB-2, OKT11 plus TS2/9 or a combination of all of these antibodies had little or no effect upon clustering.

Effect of an Fab fragment of the F10-44-2 CD44 antibody upon U937-T lymphocyte clustering. One possible way in which CD44 and CD45 antibodies might enhance clustering is by antibody mediated cellular cross linking. Such cross linking, which could lead to enhancement as detected in the well assay, could result from the binding by an antibody of FcR upon U937 cells and antigen upon T lymphocytes (U937-T cross linking) and/or owing to the bivalent or multivalent nature of antibodies from the binding by an antibody of antigen upon U937 cells and antigen upon T lymphocytes (U937-T cross linking) or even of antigen upon different individual T lymphocytes (T-T cross linking).

This explanation would seem more credible for CD44 antibodies than for CD45 antibodies because as indicated above in the absence of Mg^{2+} and Ca^{2+} ions or in the presence of metabolic inhibitors, CD11a/CD54 antibodies and EDTA, CD45 antibodies do not induce any clustering. Moreover, against the idea that CD45 antibodies might enhance clustering via an interaction with U937 FcR the 136-4B5 CD45 antibody enhanced clustering to a similar degree

in the presence or absence of a CD16 antibody (BW209/2) and a CDw32 antibody (2E1) as in the absence of these last two antibodies (albeit BW209/2 and 2E1 themselves caused an unexplained small enhancement of clustering in this experiment - see Fig. 9.21). Further, both the 136-4B5 and 144-2 CD45 antibodies were found to enhanced clustering to a similar degree in 10% human AB serum (which would block FcR) as in 10% complete medium (Fig. 9.22 - note also that the MT2 and X148 CD45R antibodies failed to enhance clustering in both types of medium).

To exclude the possibility that CD44 antibodies act by cross linking the effect of an Fab fragment of the F10-44-2 CD44 antibody upon clustering was investigated. F10-44-2 was chosen for it is a mouse IgG2a antibody and as such is both relatively resistant to secondary papain digestion (used for the generation of an Fab fragment) and has a high affinity for protein A (used for the removal of undigested antibody molecules and Fc fragments of antibody molecules from digestion mixtures). Fig. 9.23 shows the high purity of F10-44-2 Fab fragments obtained after 4 hr exposure of F10-44-2 to papain and subsequent passage of the digestion mixture over a protein A column. In fact as early as 1 hr after exposure of F10-44-2 to papain all antibody molecules were effectively converted to Fab fragments and Fc fragments and thus the effect of papain upon 4 hr digests was only to remove Fc pieces. Fig. 9.24 compares this Fab fragment of F10-44-2 with F10-44-2 whole antibody for it's effect upon U937-T lymphocyte clustering. Over a range of concentrations the Fab fragment was as effective as whole antibody in enhancing clustering showing that F10-44-2 enhancement does not depend upon cellular cross-linking.

Effect of cellular fixation upon U937-T lymphocyte clustering in the absence and presence of a CD45 antibody. Fig. 9.25 shows the effect of fixing U937 cells and/or T lymphocytes (with glutaraldehyde) upon U937-T lymphocyte

clustering in the well assay. Fixation of the U937 had little effect upon clustering. In contrast, fixation of the T lymphocytes greatly reduced clustering as did fixation of both cell types.

Fig. 9.26 confirms that fixation of U937 cells (in this case using zinc chloride) does not affect U937-T lymphocyte clustering and shows further that U937 fixation abrogates the ability of the 136-4B5 antibody to enhance clustering. Similarly, Fig. 9.27 confirms that fixation of U937 (in this case using paraformaldehyde) does not affect clustering but that fixation of T lymphocytes or U937 cells plus T lymphocytes virtually abolishes clustering. Also, confirming and extending the finding of Fig. 9.26, Fig. 9.27 shows that the 136-4B5 CD45 antibody fails to enhance clustering when the U937 or U937 and T lymphocytes are fixed but does enhance clustering when the T lymphocytes alone are fixed.

Effect of sodium orthovanadate upon U937-T lymphocyte clustering in the absence and presence of CD45 antibodies. Figs. 9.28 shows the effect of sodium orthovanadate, an inhibitor of the CD45 antigens PTPase activity, upon U937-T lymphocyte clustering in the absence and presence of the 136-4B5 CD45 antibody. Vanadate had little or no effect upon clustering in the absence of the antibody and this was true over a range of vanadate concentrations. In contrast, however, vanadate blocked the ability of 136-4B5 to enhance U937-T lymphocyte clustering in a dose dependent manner.

Fig. 9.1

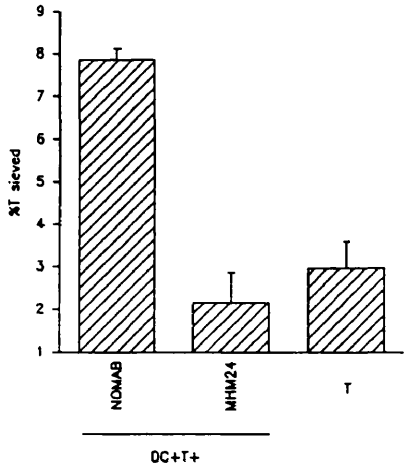


Fig. 9.2(a)

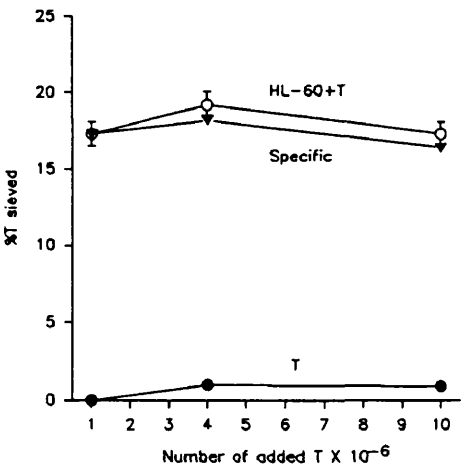


Fig. 9.2(b)

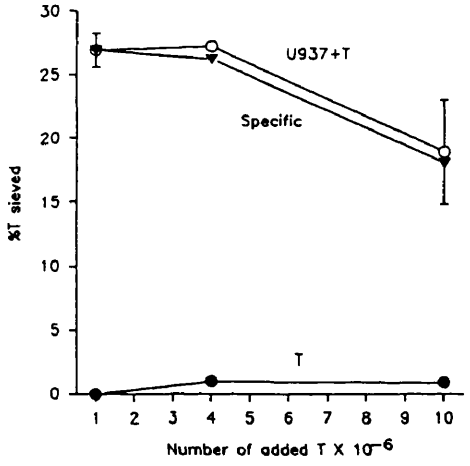


Fig. 9.3

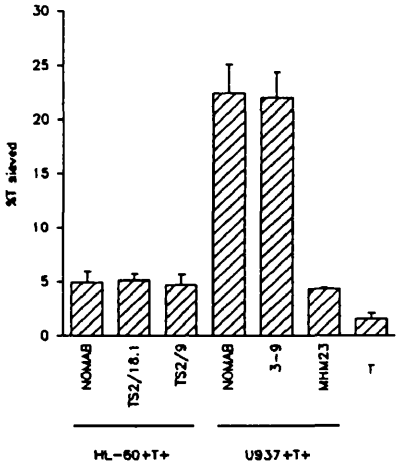


Fig. 9.4

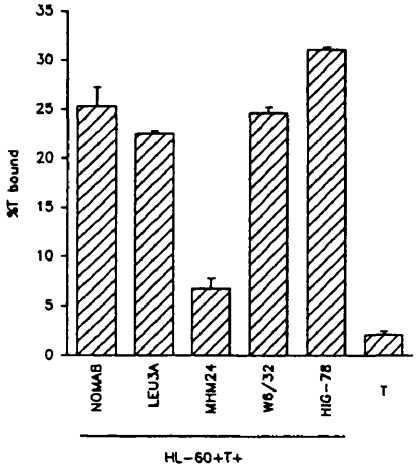


Fig. 9.5(a)

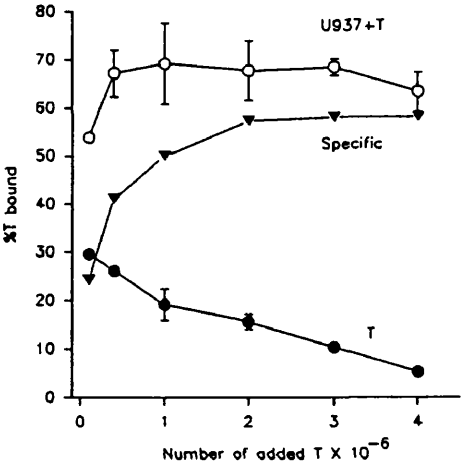


Fig. 9.6(b)

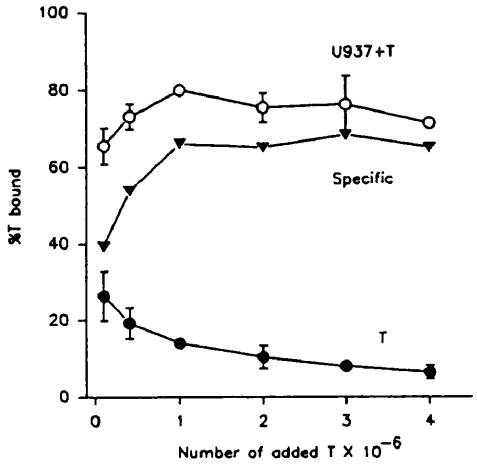


Fig. 9.7

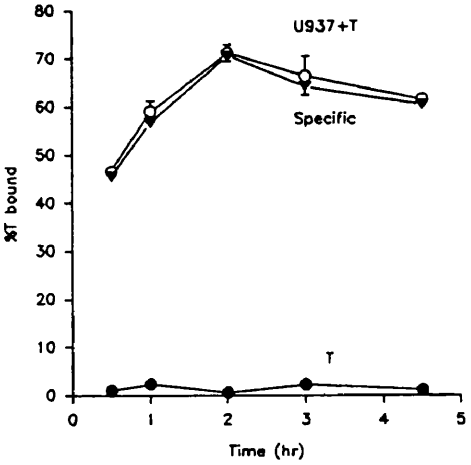


Fig. 9.8

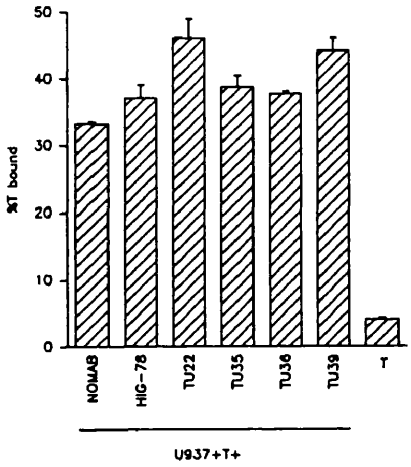


Fig. 9.9

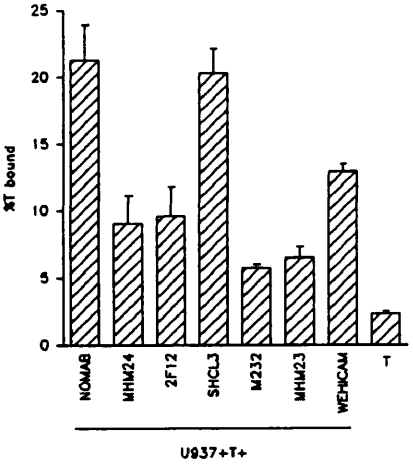


Fig. 9.10

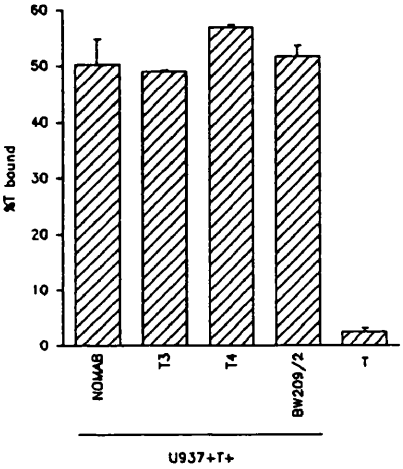


Fig. 9.11(a)

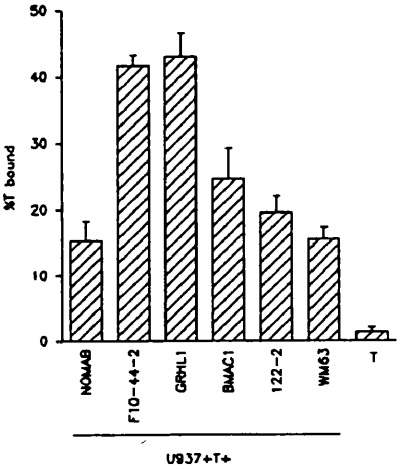


Fig. 9.11(b)

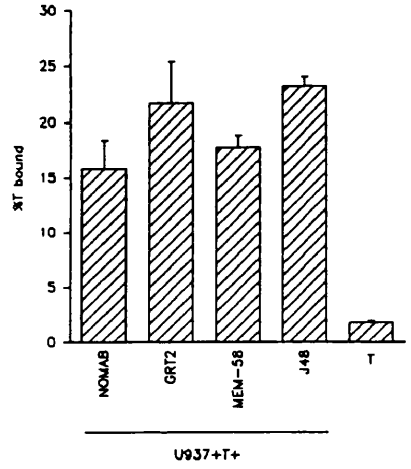


Fig. 9.11(c)

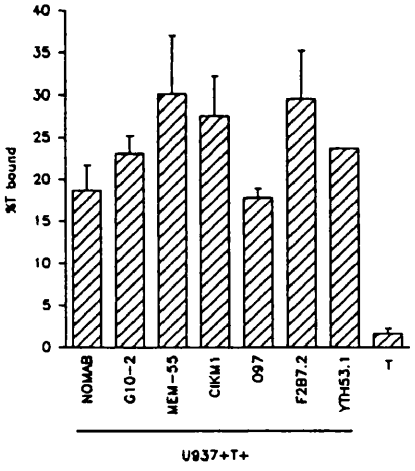


Fig. 9.12

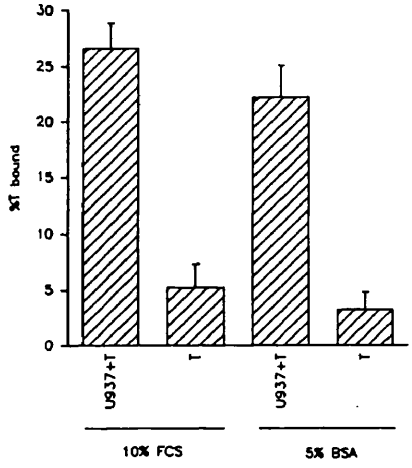


Fig. 9.13

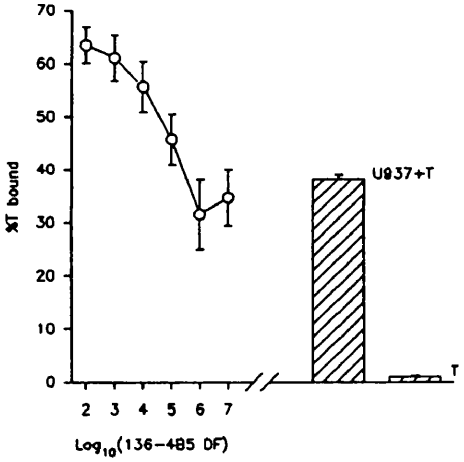


Fig. 9.14

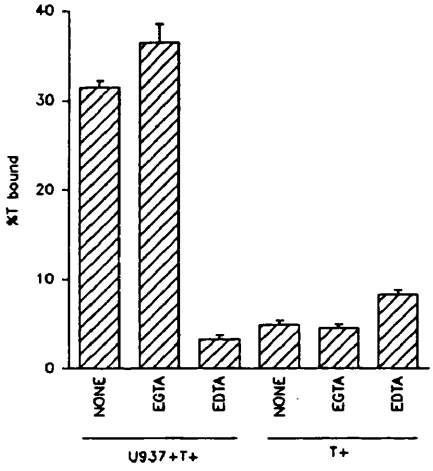


Fig. 9.15

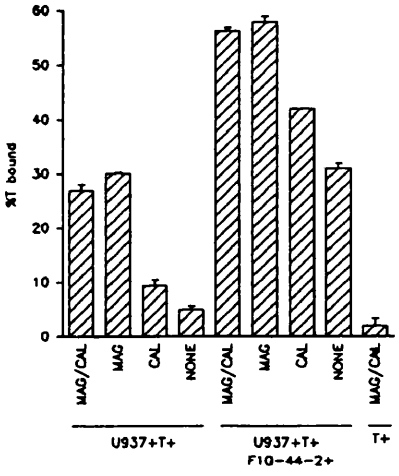


Fig. 9.16

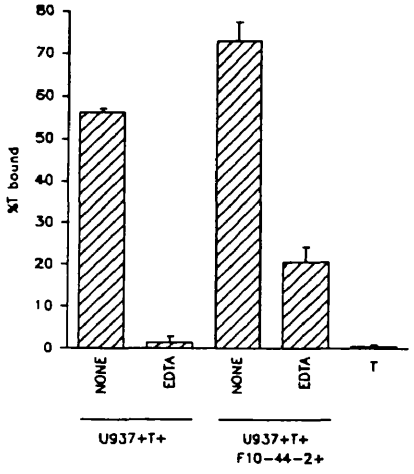


Fig. 9.17

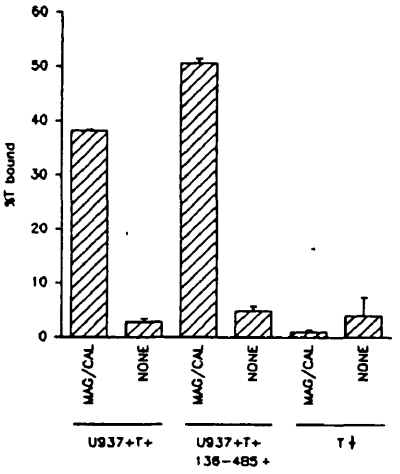


Fig. 9.18 Inhibitors (INHIB) = NaN_3/2DDG/EDTA/2F12/LB-2

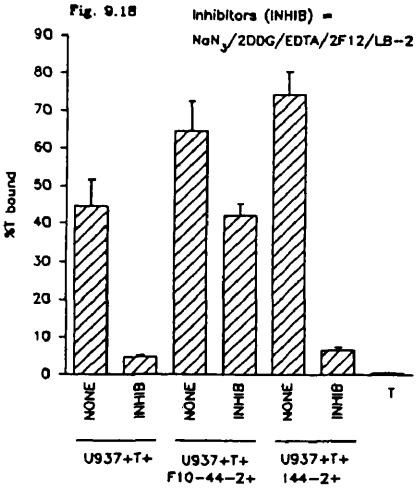


Fig. 9.19

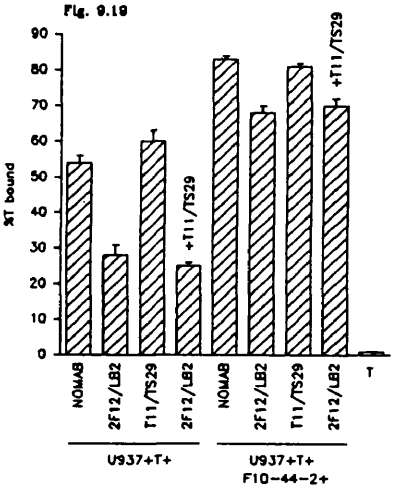


Fig. 9.20

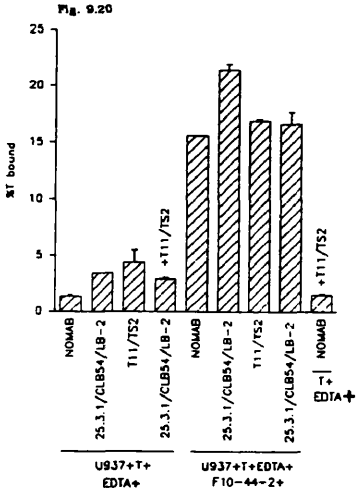


Fig. 9.21

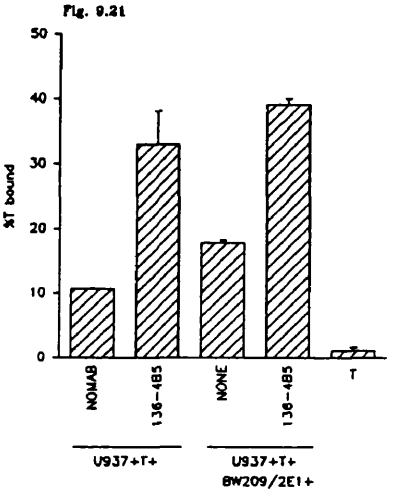


Fig. 9.22

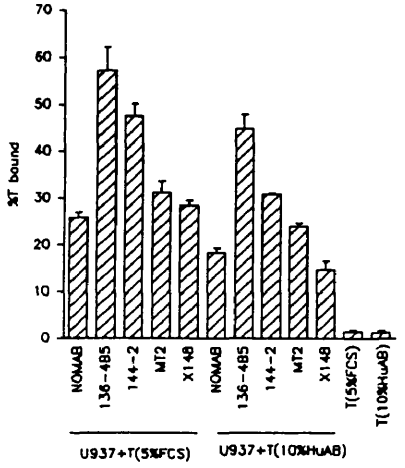


Fig. 9.24

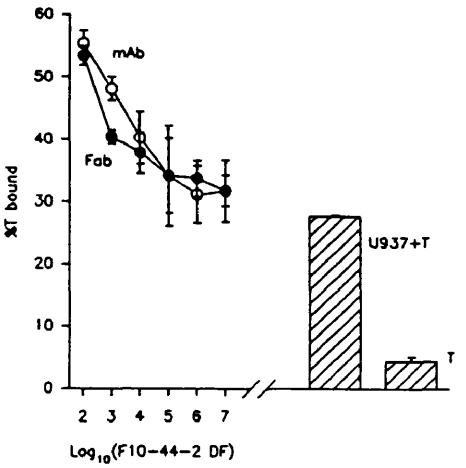


Fig. 9.25

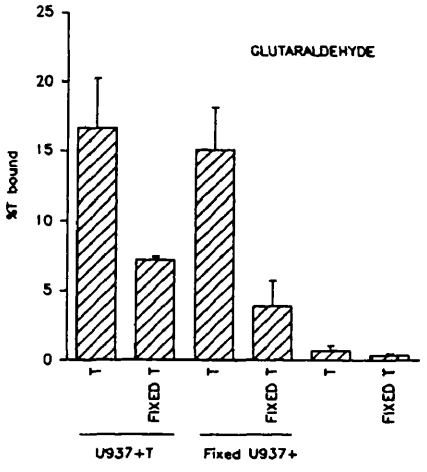


Fig. 9.26

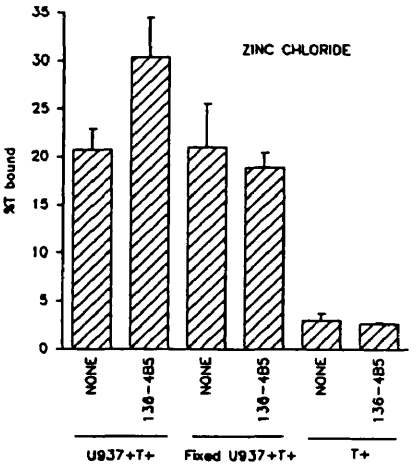


Fig. 9.27

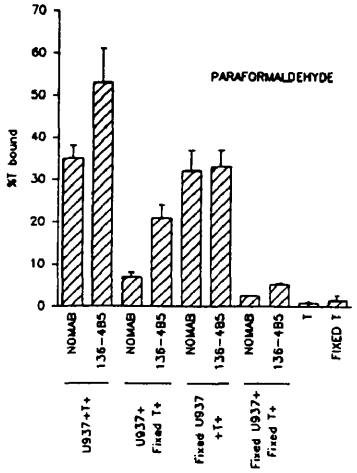


Fig. 9.28

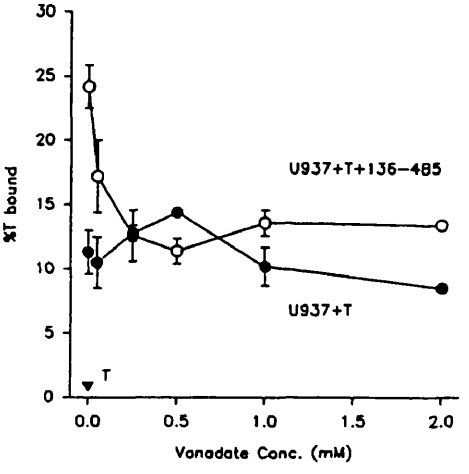
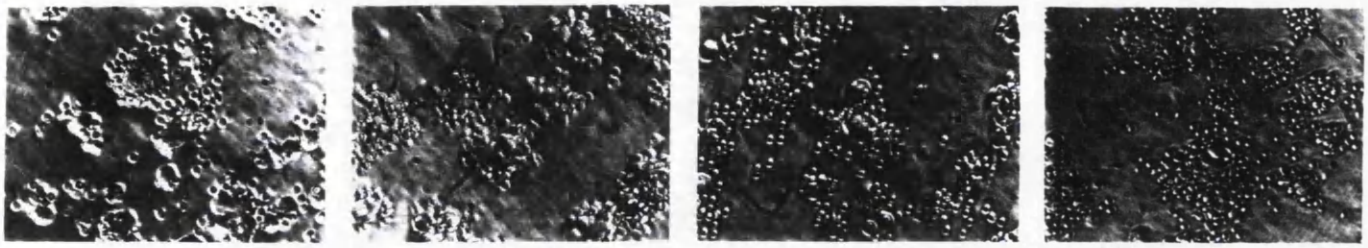


Fig. 9.6

a) b) c) d)



e)

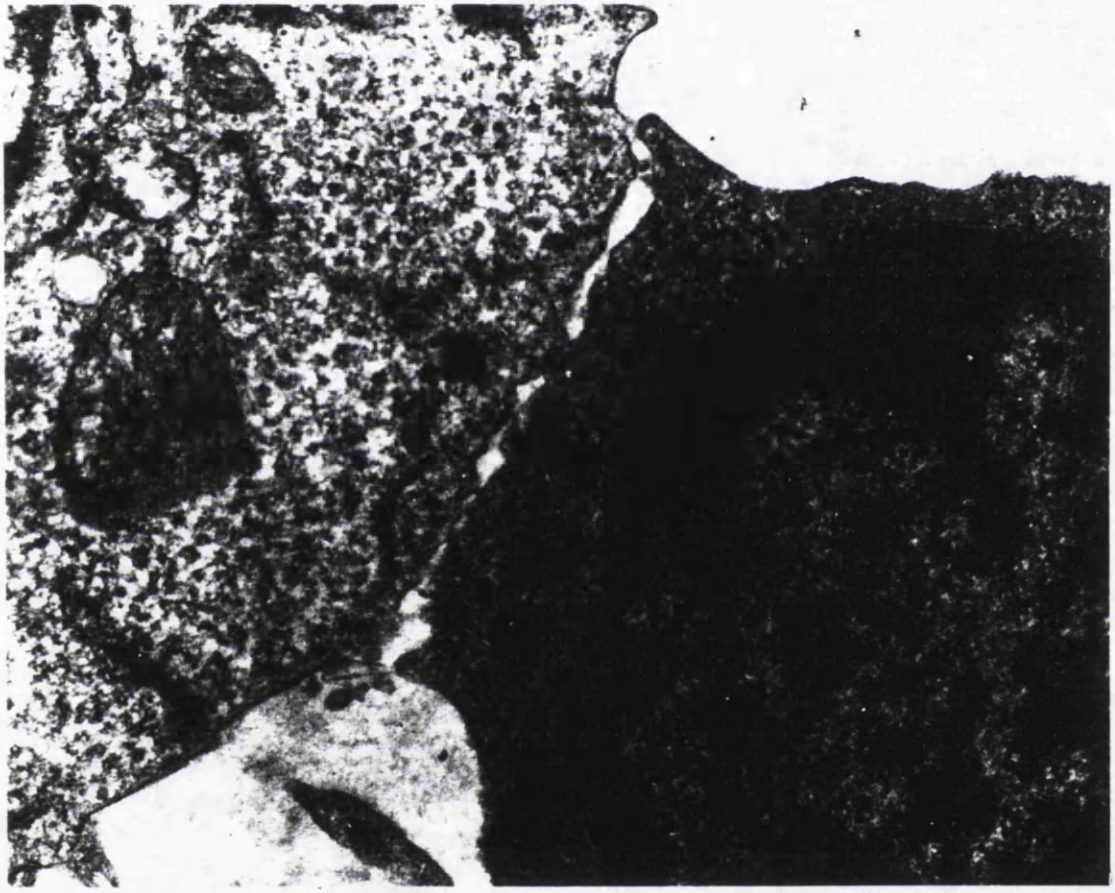


Fig. 9.23

a b c d e f g

Legends to Figures

See Materials and Methods plus:

Fig. 9.1. Sieving assay of DC-T lymphocyte clustering. See Table 9.2 (experiment 3).

Fig. 9.2(a)-9.3. Sieving assay of U937/HL-60-T lymphocyte clustering. For Fig. 9.3 see Table 9.3 (experiment 2).

Fig. 9.4. Well assay of HL-60-T lymphocyte clustering. See Table 9.4 (experiment 2).

Figs. 9.5(a,b), 9.7-9.22 and 9.24-9.28. Well assay of U937-T lymphocyte clustering. Figs. 9.7, 9.12-9.22 and 9.24-9.28 - T lymphocyte number - 3 X 10^6/well; Fig. 9.8 - see Table 9.5 (experiment 3); Fig. 9.9 - see Table 9.6 (experiment 46); Fig. 9.10 - see Table 9.7 (experiment 43); Figs 9.11 (a-c) - see Table 9.8 (experiment 47; each Fig. depicts the results from different plates); Fig. 9.14 - EDTA conc. - 3.3 mM, EGTA conc. - 6.7 mM; Fig. 9.15 - Mg^{2+} and Ca^{2+} concs. - 1 mM each, F10-44-2 DF - 4 X 10^2; Fig. 9.16 - EDTA conc. - 0.1 mM, F10-44-2 DF - 4 X 10^2; Fig. 9.17 - Mg^{2+} and Ca^{2+} concs. - 1 mM each, 136-4B5 DF - 4 X 10^2; Fig. 9.18 - EDTA conc. - 0.1 mM, all mAb DF - 4 X 10^2; Fig. 9.19 - all mAb DF - 4 X 10^2; Fig. 9.20 - EDTA conc. - 1 mM, all mAb DF - 4 X 10^2; Fig. 9.21 - 136-4B5 DF - 4 X 10^2, BW209 and 2E1 DF - 2 X 10^2; Fig. 9.22. - all mAb DF - 4 X 10^2; Fig. 9.24 - Fab and mAb at approximately equal concs., i.e. neat = 1 mg/ml; Figs. 9.26-9.28 - 136-4B5 DF - 4 X 10^2.

Fig. 9.6 (a-d) Phase contrast micrographs of U937-high density T lymphocyte clusters (X 50; U937 are the larger central cells); (e) Transmission electron micrograph of a U937-high density T lymphocyte cluster (X 9000; U937 cell - top left hand corner, T lymphocyte - bottom right hand corner).

Fig. 9.23. SDS-PAGE analysis of papain digested F10-44-2. (a) undigested, (b-e) 1, 2, 3 and 4 hr digested respectively, (f,g) 4 hr digested protein A column passed; (a-e and g) 250 ng protein, (f) 330 ng protein.

DISCUSSION

Confirmation by quantitative sieving that LFA-1, ICAM-1, CD2 antigen and LFA-3 mediate tonsillar dendritic cell-T lymphocyte clustering. One aim of the studies presented in this Chapter has been to consolidate some previous conclusions concerning the involvement or non-involvement of different molecules in tonsillar DC-T lymphocyte clustering. For this purpose a novel quantitative sieving assay of DC-T lymphocyte clustering was developed and the effects of selected antibodies upon clustering in this assay was examined. In summary, CD11a, CD18 and CD54 antibodies completely or almost completely blocked clustering; CD2 and CD58 antibodies caused a small reduction of clustering; and CD4, class I MHC and class II MHC antibodies did not affect clustering in this quantitative assay. These findings, therefore, confirm that DC-T lymphocyte clustering is mediated by a major LFA-1/ICAM-1 interaction and a minor CD2 antigen/LFA-3 interaction and also confirm that the CD4 antigen, class I MHC and class II MHC antigens are not involved in this process.

HL-60/U937-T lymphocyte clustering assessed by quantitative sieving. For comparison with DC, T lymphocyte binding to macrophages was also assessed in the quantitative sieving assay. Rather than tonsillar macrophages, however, HL-60 promyelomonocytic cells and U937 promonocytic cells, both induced to differentiate along the mononuclear phagocyte pathway by treatment with PMA as previously described (605-607), were used as a more convenient cell source. Surprisingly, in the assay, both HL-60 and U937 cells bound a greater percentage of T lymphocytes than DC. This is not necessarily, however, to contradict the notion that that DC are the most effective physiological cell type with regards a capacity to cluster T lymphocytes. One reason for the higher percentage binding by HL-60 and U937 cells might

be that PMA induces certain cell surface phenotypic changes in these cells that are atypical of mature macrophages. Examples include conversion of the LFA-1 molecule to a permanent high avidity state and loss of membrane sialic acid both of which have been reported for other cell types following PMA treatment (385,608) and both of which might be expected to confer upon these cells a potent clustering capacity (see below). Another likely reason for the higher percentage binding is that PMA treatment of HL-60 and U937 induces homotypic aggregation of these cells. Thus, HL-60 and U937 cells might in fact be less efficient at clustering T lymphocytes than DC merely that homotypic aggregation results in larger sized clusters which are more readily trapped upon the mesh.

An additional finding in the sieving assay using HL-60 and U937 cells was that the specific percentage of T lymphocytes clustered to these cells was constant over a range of T lymphocyte concentrations. At least this was true with the exception of the highest T lymphocyte concentration used in the case of U937 cells where specific binding dropped somewhat. A constant percentage of clustering was expected. As increased numbers of T lymphocytes are added an increased number adhering to HL-60 or U937 would be balanced by an increased number that are unable to adhere. Also, "tailing off" of specific clustering at higher T lymphocyte concentrations with U937 cells is consistent with the finding that U937 were more potent than HL-60 at clustering and can be interpreted as a saturation of binding spaces upon the former cell type.

With regards the effects of antibodies upon HL-60 and U937-T lymphocyte clustering as assessed in the sieving assay only a limited number were tested. Of these, CD11a and CD18 antibodies nearly completely blocked U937-T lymphocyte clustering suggesting that, as with DC-T lymphocyte clustering, this adhesive event is mediated by LFA-1. CD8, CD11c, CD58 and

class I MHC antibodies, however, did not affect U937-T lymphocyte clustering and CD2 and CD58 antibodies did not affect HL-60-T lymphocyte clustering either suggesting that the antigens recognised by these antibodies are not involved in the two respective types of adhesion. Non-involvement of the CD2 antigen and LFA-3 molecule in clustering is in agreement with a previously discussed study of monocyte-T lymphocyte adhesion (see Chapter 6). Again, at least with U937, such non-involvement cannot be explained in terms of a low level of expression of LFA-3 for these cells express as much LFA-3 as human tonsillar DC (899) and, therefore, this leaves the possibility that a CD2/LFA-3 interaction might be prevented by like-like charge repulsion between U937 cells and T lymphocytes (see Chapter 6).

Use of a well assay for an extended analysis of the effects of antibodies upon HL-60 and U937-T lymphocyte clustering. PMA differentiated HL-60 and U937 cells are plastic adherent and hence a well assay, which requires far fewer HL-60 and U937 cells and is less labour intensive than the sieving assay, was used for an extended analysis of the effects of antibodies upon HL-60 and U937-T lymphocyte clustering. With HL-60 cells, CD11a and CD18 antibodies completely or almost completely blocked clustering showing that the LFA-1 molecule mediates HL-60-T lymphocyte clustering too. CD2, CD4, CD8, CD11c, CD58 and class I MHC antibodies, however, did not influence clustering thus confirming that a CD2/LFA-3 interaction is not involved and showing further that the CD4, CD8, CD11c and class I MHC antigens also do not play a role. One other interesting finding concerning HL-60-T lymphocyte clustering in the well assay was that the Hig-78 class II MHC antibody enhanced clustering. This was in contrast to DC-T lymphocyte clustering and is discussed more detail below.

U937 cells were investigated more thoroughly. On several features of the assay itself, firstly, in contrast to the sieving assay, the specific

percentage of T lymphocytes clustered to U937 increased with an increasing number of T lymphocytes added to reach a plateau level at approximately 1 X 10^6 added T lymphocytes. This phenomenon can be explained almost solely in terms of a decreasing percentage of T lymphocytes that adhere to plastic, in turn most likely a consequence of the fact that such adherence takes place exclusively at the well periphery, i.e. at some point the well periphery would become saturated with T lymphocytes. Secondly, at the plateau level, the specific percentage of T lymphocytes clustered to U937 was slightly higher with low density T lymphocytes than with high density T lymphocytes. This would be consistent with the idea that low density T lymphocytes (as a population of blast cells) express higher levels of adhesion molecules than high density T lymphocytes (a mixed population of virgin and memory cells). Thirdly, T lymphocyte clustering to U937 cells in the well assay increased up to a time point of 2 hr and then dropped slightly up to the last examined time point of 4.5 hr. The lag period to the 2 hr time point is probably a reflection of the time that is required for all T lymphocytes to encounter U937 cells and the subsequent slight drop in clustering is probably due to non-specific factors such as cell death, rather than to any active de-adhesion process.

Of the large number of antibodies that were screened for their effects upon U937-T lymphocyte clustering in the well assay, the results of these studies revealed further similarities and differences between U937 cells and tonsillar DC. Similarities of the effects of LeuCAM and ICAM-1 antibodies were striking (see Chapter 5). Thus, the same LFA-1 and ICAM-1 antibodies that inhibited DC-T lymphocyte clustering also inhibited U937-T lymphocyte clustering. Also, CD11b and CD11c antibodies did not affect the adhesion. These results suggest that the same epitope of the LFA-1 molecule is involved in the mediation of DC and U937-T lymphocyte clustering, that ICAM-

1 is involved in U937-T lymphocyte clustering (again probably the same epitope that mediates DC-T lymphocyte clustering) and that the MAC-1 and p150,95 molecules are not involved (confirming for p150,95 results obtained in the sieving assay).

Complete concordance was also observed with respect to the effects of CD45 antigen family antibodies. Hence, the same CD45, CD45-like and CD45RO antibodies enhanced both types of clustering whereas CD45RA and CD45R antibodies had no effect. These findings indicate that the CD45 antigen is involved in U937-T lymphocyte clustering as well as DC-T lymphocyte clustering and and further that whatever the adhesive role of the CD45 antigen (see below) this role is the same in the two different types of adhesive event.

Other similarities between U937 cells and DC were that CD3, CD4, CD8, CD14, CD16, CD20, CD31, CDw32, CD38, CD48, CDw52, CD53, CD56, CD57, CD76 and class I MHC antibodies did not affect U937-T lymphocyte clustering (see Chapters 4, 6 and 7) confirming the finding in the sieving assay that the CD8 and class I MHC antigens are not involved in clustering and showing further that none of the antigens recognised by the other antibodies play a role either. In addition, as well as with DC-T lymphocyte clustering (see Chapter 7) the CIKM1 CD47 antibody enhanced U937-T lymphocyte clustering whereas other CD47 antibodies had no effect. This suggests an adhesive role for the CD47 antigen in U937-T lymphocyte clustering too which is the same as the adhesive role played by the same molecule in DC-T lymphocyte clustering.

Differences between U937 cells and DC included the fact that CD2 and CD58 antibodies had no effect upon U937-T lymphocyte clustering in the well assay confirming findings in the sieving assay (see above). More intriguing, however, was that CD43, CD44, CD46, CD55, CD59 and class II MHC antibodies

(like CD45 antigen family and CD47 antibodies) enhanced U937-T lymphocyte clustering. Enhancement with CD46, CD55 and CD59 antibodies though was inconsistent and thus it is unclear whether this effect represents a true difference between U937 and DC or whether further experimentation with DC would have revealed a similar inconsistency. Also, enhancement with CD43 antibodies was inconsistent and in fact the only CD43 antibody which enhanced U937-T lymphocyte clustering was the MEM-59 antibody (which was not tested for it,s effects upon DC-T lymphocyte clustering) suggesting the possibility that this apparent difference between U937 and DC can be explained in terms of a difference in CD43 antibody epitope specificity.

These explanations, however, cannot be put forward to explain the enhancing effects of CD44 and class II MHC antibodies for the same CD44 and class II MHC antibodies which had no effect upon DC-T lymphocyte clustering enhanced U937-T lymphocyte clustering in a consistent fashion. The most obvious interpretation of this then is that the CD44 antigen and class II MHC function as passive anti-adhesion molecules in U937-T lymphocyte interaction by virtue of a high degree of associated negative charge. High negative charge itself would result from the high content of oligosaccharide modifications and the attachment of chondroitin sulphate for the CD44 antigen (see Chapter 7 and 551,609,610) and the attachment of chondroitin sulphate (to the invariant chain) for class II MHC (611). However, upon DC these modifications of the CD44 antigen and class II MHC might be absent and this would be in line with the concept that DC have a low net cell surface negative charge. Thus, in DC-T lymphocyte clustering the CD44 antigen and class II MHC might not function as passive anti-adhesion molecules explaining the lack of enhancement with CD44 and class II MHC antibodies. Indeed, the CD43, CD46, CD55 and CD59 antigens might also function in passive anti-adhesion in U937-T lymphocyte clustering for each of these

molecules are too heavily glycosylated (612-617). Hence, apart from other factors, de-glycosylation of these molecules upon DC or their absence altogether (CD43, CD46 and CD55 antigens could not be detected upon tonsillar DC - see Chapter 2) could provide another basis for the differential enhancing effects of CD43, CD46, CD55 and CD59 antibodies.

Further investigation of the mechanism of U937-T lymphocyte clustering and the mechanism of enhancement of U937-T lymphocyte clustering caused by CD44 and CD45 antibodies. Studies which examined the effect of EDTA/EGTA and varying concentrations of magnesium and calcium upon U937-T lymphocyte clustering demonstrated a dependency of clustering upon divalent cations. This dependency was expected for LFA-1/ICAM-1 mediated adhesion has been shown to require divalent cations before (378). Not previously reported, however, was an inhibitory effect of calcium upon clustering in the presence of magnesium. In light of the fact that calcium was much less effective than magnesium in promoting clustering this finding can be interpreted as competitive inhibition by calcium of magnesium binding to the putative divalent cation binding site upon the LFA-1 molecule.

Divalent cation studies also revealed an important difference in the mechanism by which CD44 and CD45 antibodies enhance U937-T lymphocyte clustering. Firstly on CD44 antibody enhancement, in contrast to clustering in the absence of an antibody, clustering in the presence of a CD44 antibody (F10-44-2) was not completely abolished by exclusion of divalent cations from the medium or by exclusion of divalent cations and incorporation of metabolic inhibitors and LFA-1/ICAM-1 antibodies into the medium. These findings, along with the finding that an Fab fragment of the F10-44-2 antibody can enhance clustering (ruling out the possibility of cellular cross-linking), suggest that CD44 antibodies enhance U937-T lymphocyte clustering by permitting the involvement of an additional adhesion molecule

pair (apart from LFA-1 and ICAM-1), whose interaction is both independent of divalent cations and metabolic energy. Furthermore, however the CD44 antigen might be linked to these additional adhesion molecules, this linkage is too not dependent upon metabolic energy. Following on from previous discussions (see Chapters 6, and above) this is most consistent with the idea that the CD44 antigen functions as a passive anti-adhesion molecule in U937-T lymphocyte clustering, that CD44 antibodies negate this anti-adhesion by sterically hindering the CD44 antigen rather than causing it's cell surface redistribution or down regulation, and that the additional adhesion molecules that are involved are the CD2 antigen and and LFA-3. Surprisingly, however, a combination of the OKT11 CD2 antibody and the TS2/9 CD58 antibody (both of which are known to bind the "adhesotopes" of the respective antigens, 618,619) did not block U937-T lymphocyte clustering in the presence of the F10-44-2 antibody, either in the presence or absence of divalent cations, and thus the additional adhesion molecule pair remains to be identified.

Secondly, on CD45 antibody enhancement, in stark contrast to clustering in the presence of a CD44 antibody (but like clustering in the absence of an antibody), clustering in the presence of CD45 antibodies (136-4B5 and 144-2) was completely abolished by exclusion of divalent cations from the medium (and was also abolished by inclusion of metabolic inhibitors and LFA-1/ICAM-1 antibodies into divalent cation free medium). Unlike CD44 antibody enhancement therefore, CD45 antibody enhancement of U937-T lymphocyte clustering must be mediated exclusively through LFA-1.

Further resolution of the mechanism of U937-T lymphocyte clustering and the mechanism by which CD45 antibodies enhance clustering was provided by fixation studies. In this regard, fixation of U937 cells with glutaraldehyde, zinc chloride or paraformaldehyde had little or no effect

upon clustering but fixation of T lymphocytes significantly reduced clustering and fixation of both U937 cells and T lymphocytes reduced clustering still further. Given the dependency of the LFA-1 molecule upon metabolic energy for adhesion (378,384,385,396) these findings argue in favour of the notion that LFA-1 functions mainly at the T lymphocyte level and that ICAM-1 functions mainly at the U937 level in the mediation of U937-T lymphocyte clustering (relative levels of expression of LFA-1 and ICAM-1 upon the two cell types could explain this). Moreover, when the U937 cells were fixed or when both the U937 cells and T lymphocytes were fixed the 136-4B5 CD45 antibody failed to enhance clustering but the same antibody did enhance clustering when the T lymphocytes alone were fixed. Of all the possible ways in which CD45 antibodies might enhance clustering (discussed in the context of DC-T lymphocyte clustering - see Chapter 7), therefore, these findings strongly suggest that such enhancement is mediated at the accessory cell (U937) level and is an active process depending upon signal transduction. Certainly, in the case of the 136-4B5 antibody the PTPase of the CD45 antigen appears to be involved because an inhibitor of the CD45 antigens PTPase activity, sodium orthovanadate (571), blocked enhancement of clustering.

Summary

In this Chapter a quantitative assay of accessory cell-T lymphocyte clustering has been used to confirm that tonsillar DC-T lymphocyte clustering is mediated by a major LFA-1/ICAM-1 interaction and a minor CD2 antigen/LFA-3 interaction. In addition, quantitative assays have been used to examine the molecular mechanisms of T lymphocyte clustering to macrophages, particularly U937 cells differentiated with PMA. U937-T lymphocyte clustering is also mediated by an LFA-1/ICAM-1 interaction and the CD45 antigen, too, plays an indirect role. Unlike DC-T lymphocyte clustering, however, a CD2 antigen/LFA-3 interaction is not involved in U937-T lymphocyte clustering and in this form of adhesion the CD44 antigen and class II MHC additionally play indirect roles. Studies which examined the indirect roles of the CD44 and CD45 antigens in U937-T lymphocyte clustering revealed these roles to be distinct. Thus, the CD44 antigen appears to function as a passive anti-adhesion molecule in this system. In contrast, the CD45 antigen functions as an active adhesion molecule which is linked to the LFA-1/ICAM-1 pathway via the PTPase activity of its intracellular domains.

CONCLUSIONS AND FUTURE DIRECTIONS

Hitherto, human peripheral lymphoid DC have neither been isolated nor tested for their accessory activity in vitro. Two aims of the present studies, therefore, have been to isolate DC from human tonsils and compare these DC with other isolated tonsillar cell populations as inducers of T lymphocyte proliferation. DC were isolated by a process of negative selection and were highly enriched as assessed by morphology, strong expression of class II MHC and an absence of markers known to be associated with all other possible contaminating cell types. Other putative accessory cells, i.e. macrophages, B and T lymphocyte blasts, and resting responder T lymphocytes were isolated as by-products of the DC purification process and were also highly enriched as assessed by the expression of specific markers. In each of the periodate oxidative mitogenesis reaction, the auto MLR and the allo MLR, tonsillar DC were able to induce T lymphocyte proliferation. Moreover, tonsillar DC were more effective inducers of T lymphocyte proliferation in these reactions than the other tonsillar accessory cells. These findings thus uphold the concept that in humans, as in experimental laboratory animals, peripheral lymphoid DC are the principal physiological accessory cells involved in the induction of T lymphocyte immunity.

Despite the fact that DC are potent accessory cells, the molecular events that occur during DC-T lymphocyte interaction remain largely unexplored. Furthermore, although DC-T lymphocyte clustering has been recognised as an essential early event in response induction there is controversy over the molecules involved. In light of this a third and main aim of the present studies has been to examine the molecular mechanisms of tonsillar DC induced T lymphocyte proliferation using the oxidative mitogenesis system as a convenient model. For this purpose, large panels of antibodies were screened for their effects upon proliferation in this reaction in order to identify molecules that play a role in proliferation

induction. Also, antibodies were tested for their effects upon clustering in order to determine whether the role of an implicated molecule is in DC-T lymphocyte adhesion or in signal transduction. The results indicate that DC-T lymphocyte clustering is mediated by a major LFA-1/ICAM-1 interaction and a minor CD2 antigen/LFA-3 interaction and at this stage the CD45 antigen is also involved, indirectly. A variety of different molecules are involved in signal transduction including each of the molecules implicated in clustering and in addition the CD3, CD4, CD5, CD25, CD26, CD28, CD39, CD44, CD48, CDw70, CD71 and CDw78 antigens and class I and class II MHC.

One problem with the assessment of DC-T lymphocyte clustering is the lack of a sensitive quantitative clustering assay. Hence, a fourth aim of these studies has been to examine the molecular mechanisms of T lymphocyte clustering to PMA differentiated U937 promonocytic cells, using antibodies and other agents in such an assay. The results of these experiments suggest that, like DC-T lymphocyte clustering, U937-T lymphocyte clustering is mediated by an LFA-1/ICAM-1 interaction and the CD45 antigen is indirectly involved. Unlike DC-T lymphocyte clustering, however, the CD2 antigen and LFA-3 are not involved and additionally the CD44 antigen and class II MHC also play indirect roles. Further, evidence was obtained that the roles of the CD44 and CD45 antigens in U937-T lymphocyte clustering are distinct. The CD44 antigen appears to function as an active anti-adhesion molecule which prevents the interaction of an unidentified adhesion molecule pair. By contrast, the CD45 antigen functions as an active adhesion molecule which is linked to the LFA-1/ICAM pathway via its PTPase activity at the U937 cell level.

An important question that is raised by these various studies is the molecular basis of the ability of DC to cluster increased numbers of T lymphocytes and thus in turn the molecular basis of the potency of DC

accessory activity. Equivalent levels of expression of LFA-1, ICAM-1 and LFA-3 upon DC and other accessory cells, the role of the CD2 antigen and LFA-3 as mediators of and the non-involvement of the CD44 antigen as a passive anti-adhesion molecule in DC-T lymphocyte clustering all argue that this basis is low DC surface negative charge. So far, however, only class II MHC molecules have been shown to be deficient in one negatively charged group, i.e. sialic acid, upon DC and therefore, these types of study should be extended to other DC surface molecules and negatively charged moieties (such as glycosaminoglycans) to gain support for the low negative charge hypothesis.

Another possible basis for the increased capacity of DC to cluster T lymphocytes might be hyper-PTPase activity associated with the CD45 antigen on DC. This, however, assumes that as upon U937 cells, upon DC the CD45 antigen functions as an active adhesion molecule, which is reasonable, but needs to be formally demonstrated, emphasising the requirement for a more sensitive quantitative DC-T lymphocyte clustering assay to examine this. Such an assay, which uses two colour FACS analysis of clusters as a read out system, is currently being developed in the laboratory.

Of course, clustering of T lymphocytes might not be the only the factor which controls potency. Hyper-signalling by DC might also play a role. In this context the identification of molecules that are involved in signal transduction in DC induced T lymphocyte responses is a first step to understanding mechanisms. Future work in this area should centre around identification of ligands or receptors (as appropriate), detailed comparative expression studies of implicated molecules upon different accessory cell types and more in depth analysis of precise signal transduction function.

APPENDIX 1 - FOOTNOTES TO INTRODUCTION

1) All CD antigens apply to humans only.

2) Excluded from this are the indeterminate cells of the skin. They are included in the "DC family" on the basis of morphological and cell surface marker characteristics but evidence, from in vivo or indeed in vitro experimental approaches, that they might act as accessory cells in the physiological immune system is lacking.

3) Mouse lymph node and splenic interdigitating DC are assumed to be identical with respect to phenotypic and functional criteria although in fact lymph node interdigitating DC have not been as extensively phenotyped as splenic interdigitating DC and also have not been directly compared with cultured Langerhans cells as accessory cells.

4) This argument relies on the assumption that expression of 2×10^3 copies of FcRII-IgG/cell is limiting for T lymphocyte activation in CD3 antibody mitogenesis - which is likely, given that mouse splenic DC, which express a similar but slightly reduced number of copies of FcRII-IgG/cell, i.e. 1×10^3, are inactive as accessory cells in this model.

APPENDIX 2 - ABBREVIATIONS

2DDG - 2-deoxy-D-glucose
AF - Ascites fluid
allo MLR - Allogeneic MLR
ATPase - Adenosine triphosphatase
auto MLR - Autologous MLR
BSA - Bovine serum albumin
CM - Conditioned medium
%CMP - Percentage change of mean proliferation
Con A - Concanavalin A
CPM - Counts per minute
CR - Complement receptor
CS - Culture supernatant
CTL - Cytotoxic T lymphocyte
DC - Dendritic cell
DF - Dilution factor
dGuO - Deoxyguanosine
DNFB - Dinitrofluorobenzene
DNP - Dinitrophenol
DPPIV - Dipeptidyl peptidase IV
DTH - Delayed type hypersensitivity
FACS - Fluorescence activated cell sorting
FcRIgG - Fc receptor for IgG
FCS - Foetal calf serum
FITC - Fluorescein isothiocyanate
GMCSF - Granulocyte macrophage colony stimulating factor
HBSS - Hanks balanced salt solution
HRBC - Horse red blood cells
HRP - Horse radish peroxidase
HSV - Herpes simplex virus
HuIg - Human Immunoglobulin
ICAM - Intercellular adhesion molecule
^{125}IdUrd - [5-^{125}I]-2-deoxyuridine
IFN - Interferon
Ig - Immunoglobulin
IL - Interleukin
%IMP - Percentage inhibition of mean proliferation
Ir gene - Immune reponse gene
KLH - Keyhole limpet haemocyanin
LCA - Leucocyte common antigen
LeuCAM - Leucocyte cell adhesion molecule
LeuOMe - Leucine methyl ester
LFA - Lymphocyte function associated antigen
LPS - Lipopolysaccharide
mAb - Monoclonal antibody
MCSF - Macrophage colony stimulating factor
MHC - Major histocompatability complex
MLR - Mixed leukocyte reaction
Mls - Minor lymphocyte stimulating
Na/GaO - Neuraminidase plus galactose oxidase
NHS - Normal horse serum
NK - Natural killer
NSS - Not statistically significant
ORBC - Ox red blood cells

OVA - Ovalbumin
PALS - Peri-arteriolar lymphatic sheath
PBMC - Peripheral blood mononuclear cells
PBS - Phosphate buffered saline
PHA - Phytohaemagglutinin
PMA - 4 beta phorbol 12 myristate 13 acetate
POL - Polymeric flagella protein of S. Typhimurium
Poly - Polyclonal antibody
PPD - Purified protein derivative of M. Tuberculosis
PTPase - Protein tyrosine phosphatase
RAMIg - Rabbit anti-mouse immunoglobulin
rhIL-1 - Recombinant human IL-1
RITC - Rhodamine isothiocyanate
RT - Room temperature
SD - Standard deviation
SRBC - Sheep red blood cells
SS - Statistically significant
SWM - Sperm whale myoglobin
TcR - T lymphocyte antigen receptor
TNF - Tumour necrosis factor
TNP - Trinitrophenol
Tris NH_4Cl - Tris ammonium chloride
UV - Ultraviolet
V-beta - Variable region beta

APPENDIX 3 - REFERENCES.

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