CHARACTERIZATION OF THE HUMORAL AND CELLULAR IMMUNE RESPONSES TO THYROGLOBULIN IN RODENT MODELS OF AUTOIMMUNE THYROIDITIS

by

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To my MOTHER who taught me to fight today for tomorrow, knowing the cost of living like this.

To DORIS ROSENTHAL who opened the horizon of science to me, showing with her example that one should live for the whole.

Chaque jour nous laissons une partie de nous-mêmes en chemin.

AMIEL.

ABSTRACT

In order to understand the mechanisms underlying autoimmune thyroiditis, spontaneous (SAT) and induced (EAT) autoimmune responses to thyroglobulin were investigated in various inbred strains of rats. The humoral response was assessed using a solid-phase immunoradiometric method and the cell-mediated immunity was evaluated by histology and immunofluorescence analysis.

Anti-Tg autoantibodies, spontaneously arising in BB rats (S-TgAb) and induced in various inbred strains of rats (I-TgAb), differed in their fine specificity. With a panel of thyroglobulins from different animal species it was demonstrated that I-TgAb bound to all species of Tg, whereas S-TgAb either recognized a restricted number of Tg-species or all thyroglobulins. Moreover, it was shown that the iodination site of thyroglobulin is the epitope recognized by both spontaneous and induced TgAb. Taken together these data are consistent with the view of restricted epitope recognition by Tg autoantibodies in the rat.

Comparing the induction of thyroiditis in a variety of intra-RT.1 recombinant rat strains, it was demonstrated that on identical genetic backgrounds the $RT.1^{c}$ haplotype was directly associated with high and the $RT.1^{u}$ haplotype with low responses to EAT induction. The severity of the induced thyroiditis did not correlate with Tg-autoantibody titres in either strain.

The inflammatory infiltrate was essentially composed of macrophages and T lymphocytes in both spontaneous and induced rat thyroiditis models. However, in the SAT of the BB/E rat a

substantial number of B lymphocytes was also present in the infiltrates. Immunoglobulin deposition was seen in the thyroid interstitium and in damaged follicles in both EAT and SAT.

MHC Class II was expressed on the thyroid epithelium in EAT but not SAT and was observed only in areas of massive lymphocytic infiltration. Furthermore, interferon-gamma induced class II expression on a rat thyroid epithelial cell line (FRTL-5) in vitro.

In the non obese diabetic mouse protocols for induction of thyroiditis and tolerization were standardized. Preliminary data indicate that induction of thyroiditis by immunization with Tg can be abrogated by prior i. v. injection of a high dose of Tg.

These findings strongly suggest a central pathogenic role for T cell-dependent mechanisms, although antibody-mediated cytotoxicity cannot be ruled out. They support the notion that thyroid epithelial class II MHC antigen expression is a secondary event in the pathogenesis of rat EAT, mediated through cytokines released by activated cells in the inflammatory infiltrate.

CONTENT

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LIST OF FIGURES AND TABLES

LIST OF ABBREVIATIONS

ADCC	Antibody dependent cellular cytotoxicity
AO	Albino Oxford
APC	Antigen presenting cell
AUG	August
BB	Bio Breeding
BSA	Bovine serum albumin
BSS	Balanced salt solution
BUF	Buffalo
CDF	Cesarian Derived Fisher
CFA	Complete Freund's adjuvant
CMI	Cell-mediated immunity
ConA	Concanavalin A
DABCO	Diazabicyclo-octano
DIT	Di-iodotyrosine
DNP	Dinitrophenol
EAE	Experimental autoimmune encephalomyelitis
EAT	Experimental autoimmune thyroiditis
EDTA	Ethylenediaminetetra-acetic acid
FITC	Fluorescein isothiocyanate
FRTL-5	Fisher-rat thyroid line-5
HBSS	Hank's balanced salt solution
H/E	Haematoxylin/Eosin
HEL	Hen egg lysozyme
HTg	Human thyroglobulin
IDDM	Insulin-dependent diabetes mellitus
IFN-γ	Interferon-gamma
I^-	Iodide
Ia	I region antigen
Ig	Immunoglobulin
IIF	Indirect immunofluorescence
IL-1	Interleukin-1
IL-2	Interleukin-2
IRA	Immunoradiometric assay
Ir-Tg	Immune-response gene to thyroglobulin
I-Tg	Iodinated thyroglobulin
I-TgAb	Induced thyroglobulin autoantibodies
LATS	Long acting thyroid stimulator
LEW	Lewis
LGFL	Log green fluorescence
LNC	Lymph node cell

LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
MIT	Mono-iodotyrosine
MoAb	Monoclonal antibody
MTg	Mouse thyroglobulin
NMS	Normal mouse serum
NOD	Non-obese diabetic
NRbS	Normal rabbit serum
NRtS	Normal rat serum
NZB	New Zealand Black
NZW	New Zealand White
OS	Obese strain
PBF	Phosphate-buffered formalin
PBL	Peripheral blood lymphocyte
PBS	Phosphate-buffered saline
PBS/T	PBS/tween 20
PBS/BT	PBS/T+BSA
PHA	Phytohaemagglutinin
PMA	Phorbol myristate acetate
PPD	Purified protein derivative of mycobacterium
rIFN-γ	Recombinant interferon-gamma
SAS	Saturated ammonium sulphate
SAT	Spontaneous autoimmune thyroiditis
SLE	Systemic lupus erythematosus
S-TgAb	Spontaneous thyroglobulin autoantibodies
T3	Tri-iodothyronine
T4	Tetra-iodothyronine (Thyroxine)
TCR	T cell receptor
TEC	Thyroid epithelial cells
TFEC	Thyroid follicular epithelial cells
Tg	Thyroglobulin
TgAb	Thyroglobulinautoantibody
TNF	Tumor necrosis factor
TSH	Thyroid stimulating hormone
X±SE	Mean ± standard error

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CHAPTER 1

GENERAL INTRODUCTION

1.1 SELF REACTIVITY VERSUS SELF TOLERANCE

To protect the body from foreign aggression but avoid the danger of self-destruction the immune system has to discriminate self from foreign antigens. The theoretical basis of this concept goes back to 1900, when Ehrlich proposed that autoimmunity is pathological per se and normally prevented by the "horror autotoxicus", an innate mechanism of vigilance. Consequently, autoimmunity received the connotation of disease, namely a state in which the physiological tolerance to self is broken (Dameshek, 1965).

To explain tolerance, it was first proposed that autoreactive immunocytes are deleted during ontogeny (Burnet, 1957). This theory of clonal abortion predicts that lymphocytes recognizing unaltered self antigens do not persist in normal animals. However, lymphocytes potentially able to recognize and react with autologous thyroglobulin have been detected in healthy individuals (Bankhurst, Torrigiani and Allison, 1973). Furthermore, autoimmune thyroiditis has been induced in animals by immunization with thyroid extracts either alone (Weigle, 1965) or in combination with complete Freund's adjuvant (Witebsky and Rose, 1956). These observations lead to the concept that tolerance to self antigens must rely on

active regulatory mechanisms operating throughout the entire life (Jerne, 1974). These might involve suppressor cells, anti-idiotypic antibodies and anti-idiotypic T cells. In this context, breaking of tolerance and subsequent autoimmune phenomena are understood as the result of an imbalance between effector and suppressor mechanisms favouring the former (Rose et al., 1981). In summary, rather than forbid and abort autoreactive clones, the immune system regulates their function by suppressive mechanisms.

1.1.1 Thymic ontogeny and tolerance

It is now well established that after entering the thymus, T cell precursors rearrange and express their T cell receptor in conjunction with the CD3 complex (Kronenberg et al., 1986). At that stage they are still negative for both "classical" accessory molecules CD4 and CD8 (double negative). Possibly after progression through a CD4-/CD8+ stage (MacDonald, Budd and Howe 1988a), thymocytes express both CD4 and CD8 simultanously. The majority of cortical thymocytes are of this double positive phenotype. Most of them probably die, but a minority is believed to develop into mature single positive ($CD4^+/8^-$ or $CD4^-/8^+$) thymocytes (Fowlkes, Schwartz and Pardoll, 1988, and Kisielow et al., 1988b) which ultimately enter the circulation as functional T cells (von Boehmer, 1986).

Thymic education, consisting of both a positive selection for T cell precursors recognizing self-MHC (Zinkernagel and Doherty, 1979) and a negative selection inactivating autoreactive cells,

must be associated with this phenotypic differentiation. In the context of this thesis the negative selection of autoreactive T cell precursors is particularly important as a mechanism of generating and maintaining self tolerance. Clonal deletion and clonal anergy have both been implicated in the process.

Recent major technical advances have shed some light on this problem. The generation of T cell clones has permitted the sequencing of the T cell receptor (TCR) gene complex. The TCR$\alpha\beta$, carried by the majority of mature T cells, is a heterodimer. Transfection experiments have proved that α and β chain are sufficient to confer both antigen-specificity and MHC restriction to a T cell (Dembic et al., 1986). α and β chain genes are rearranged in a similar fashion to Ig chain genes (Davis and Bjorkman, 1988). Using monoclonal antibodies and gene sequencing, different variable region gene families could be identified, especially for the β chain (Vβ genes). By multicolour flow cytometric analysis of thymocytes or mature peripheral T cells, the fate of T cells of a certain Vβ family can be followed through ontogeny as defined by other surface markers.

In some cases Vβ elements alone seem to be sufficient for determination of a particular autoreactivity. Vβ17a$^+$ T cells recognize I-E^k (Kappler, Roehm and Marrack, 1987), and both Vβ8.1 (Kappler et al., 1988) and Vβ6 (MacDonald et al., 1988b) have been demonstrated to confer reactivity of T cells with mouse cells of Mls^a haplotype. Fascinatingly Vβ17a$^+$ T cells are virtually absent

from the periphery of mice expressing I-E, and neither mature $V\beta8^+$ nor mature $V\beta6^+$ T cells can be found in Mls^a mice, whilst in $I\text{-}E^-$ or Mls^b mice respectively a substantial fraction of the peripheral T lymphocyte pool expresses TCR of the related Vβ families (Kappler, Roehm and Marrack, 1987, Kappler et al., 1988, and MacDonald et al., 1988b). These observations strongly support clonal deletion as one model of T cell tolerance.

As a model for the fate of less dominant T cell specificities, which cannot normally be followed by flow cytometry, Kisielow and colleagues (1988a) have generated transgenic mice which express a TCR specific for the male H-Y gene product in the context of H-2 D^b . In female mice about 30% of all $CD8^+$ T cells respond to male cells, whilst cells from male transgenic mice are unresponsive. The thymus of the male animals is small, and the thymocyte population severely depleted, again arguing for clonal deletion as a major factor leading to tolerance in these mice. However, some thymocytes and a considerable proportion of T cells from the peripheral pool in male mice expressed the transgene. All these cells were $CD4^-$ and $CD8^-$ or CD8-dull. The authors interpret this as evidence for a role of accessory molecules in the process of clonal deletion in the thymus, so that $CD4^-/8^-$ cells can possibly escape. These, however, are functionally silent so that they do not threaten tolerance. They can only be observed in transgenic mice in which the vast majority of T cells expresses the transgene and the peripheral pool therefore cannot be easily replenished by T cells of other specificities (Kisielow et al, 1988a).

In animals which eliminate certain TCR specificities, these are found in normal numbers in the $CD4^-/8^-$ and the $CD4^+/8^+$ thymocyte population, however they are absent from the mature thymocyte pool (Kappler, Roehm and Marrack, 1987, Kappler et al., 1988, and MacDonald et al., 1988b). The fact that neither $CD4^+$ nor $CD8^+$ Vβ6 bearing T cells are detectable in the periphery of Mls^a mice, even though only $CD4^+$ cells react with Mls^a cells in lymphocyte cocultures (MacDonald et al., 1988b), indicates that negative selection operates on a common precursor of both T cell subpopulations. Accessory molecules must be critically involved in the deletion since $CD4^-/8^-$ cells can escape (Kisielow et al., 1988a) and anti-CD4 antibodies have been shown to interfere with the elimination (Fowlkes, Schwartz, and Pardoll, 1988, and McCarthy et al., 1988). Taken together the observations point to the $CD4^+/8^+$ double positive thymocyte as the subject of negative selection (Kisielow et al., 1988a, and Fowlkes, Schwartz, and Pardoll, 1988) .

Much less information is available on B cell tolerance. In one elegant experiment Goodnow and colleagues (1988) have generated mice transgenic for both a predefined antigen (HEL) and a high affinity antibody against it. They observed anergy of the B cells expressing the transgene even though these were not deleted. They expressed however very low amounts of IgM on their membrane in the presence of relatively high levels of IgD. While this may indicate an important role for IgD in B cell tolerance, the physiological significance of this phenomenon remains an open question, since IgM-dull IgD-bright cells are not commonly observed in normal animals.

1.1.2 Autoimmunity and autoimmune disease

Self reactivity is now understood as part of the normal functions of the immune system involving the removal of metabolic products by macrophages (Elson and Weir, 1967) and, particularly, interactions of cells of the immne system with each other. Recognition of self components such as histocompatibility antigens, idiotypes and immunoglobulin constant regions are of crucial importance in the regulation of the immune response to foreign organisms. This self-reactivity does not normally result in autoimmune disease, because it is under the control of immune regulatory circuits. If these fail, however, it may give rise to cellular and humoral manifestations of self-aggression with destruction of autologous structures (Milgrom and Witebsky, 1962).

Since the observation that both T and B cells can be tolerized to foreign antigens in a dose dependent fashion (Mitchison, 1969, Chiller et al., 1971) it has been postulated that autoantigens themselves play a part in the natural mechanisms of self-tolerance (Allison, 1971). T cells can be tolerized by very low antigen doses (low zone tolerance), whereas B cells are silenced at higher concentrations (high zone tolerance) (Mitchison, 1969). Since many self antigens circulate in minute amounts, it is expected that the antigen dependent mechanisms of self tolerance operate on the T cell level. If this is true, competent autoreactive B cells might be present under physiological circumstances, but they would be functionally silent because of the lack of T cell help. In this case a

bypass of the requirement for T cell help could lead to a break of self tolerance.

1.1.3 Induction of autoimmunity

Non-responsiveness to thymus dependent antigens could be terminated by three manoeuvres:

By-pass of tolerant T helper cells. Although tolerant to autologous antigens, T lymphocytes can recognize determinants on cross-reacting antigens and give help to B lymphocytes which then produce antibodies binding to the autologous antigen. This autoreactivity, which can be passively transferred with antibodies, leads to an inflammatory reaction the localization of which corresponds to the distribution of the autoantigen in the immunized animal like the thyroiditis observed in rabbits injected with bovine thyroglobulin (Nakamura and Weigle, 1969). Peripheral blood lymphocytes from these animals release macrophage inhibition factor when incubated with bovine thyroglobulin, but not with rabbit thyroglobulin (Clinton and Weigle, 1972). This indicates that the rabbit's T lymphocytes respond to antigenic determinants specific for bovine thyroglobulin but not rabbit thyroglobulin. Similar experiments in mice proved that also in this animal model the state of unresponsiveness to autologous thyroglobulin is due to a lack of active T cells, since competent B cells specific for both autologous and heterologous thyroglobulin are detected in spleen and bone marrow (Clagett and Weigle, 1974).

By-pass of the need for specific T cell help has also been demonstrated using degraded autoantigen. Thyroiditis and autoantibodies were induced in rabbits by immunization with homologous thyroglobulin partially degraded by proteolytic enzymes (Anderson and Rose, 1971). It has been argued that these findings suggest exposure of determinants by enzymatic digestion which are hidden in the native thyroglobulin molecule and that T cells not tolerized to these hidden epitopes can therefore give help to B cells crossreacting with the native molecule. Such a mechanism might also explain the spontaneous occurence of thyroiditis in conditions of thyroid damage (Wall et al., 1976). However, it is possible that the fragments more readily present T-cell epitopes than the native molecule and do not require complete Freund's adjuvant.

Non-specific polyclonal activation of B cells by bacterial products such as lipopolysaccharide (LPS) and purified protein derivative (PPD) might also account for the induction of autoantibodies. Injection of LPS with thyroglobulin into mice results in the production of thyroglobulin autoantibodies and thyroid lesions (Esquivel, Rose and Kong, 1977). Like other polyclonal B cell mitogens (Gery, Krüger and Spiesel, 1972) LPS can directly activate B cells to secrete their antibodies, although some controversy remains as to whether its adjuvant properties require T lymphocytes and macrophages as well. Activation of macrophages would render antigen presentation to T cells more efficient, either directly through IL-1 release or by increasing Ia expression on its surface (Ziegler, Staffileno and Wentworth, 1984).

Potentiation of antibody formation to unrelated antigen and induction of delayed type hypersensitivity reactions is also caused by administration of Bordetella pertussis vaccine alone or in combination with complete Freund's adjuvant (CFA) (Munoz and Sewell, 1984). This combination putatively leads to a rather complex mode of adjuvanticity, involving both enhancement of recirculation of lymphocytes (Tamura et al., 1985) and degradation of the antigen incorporated into CFA (Weigle, High and Nakamura, 1969). This could explain the efficiency of the antigen presentation and the severity of experimental thyroiditis induced using this protocol (Twarog and Rose, 1969).

Many different bacterial and viral products have been shown to increase the susceptibility to breaking of self tolerance. Infections with Yersinia enterocolitica (Lidman et al., 1976) have been associated with an increased incidence of thyroid autoantibodies. Mice injected with the polysaccharide from Klebsiella pneumoniae developed autoimmune thyroiditis (Nakashima et al., 1977). Furthermore, in the mouse reovirus infection leads to the production of autoantibodies to a variety of endocrine organs (Onodera et al., 1982). Autoimmune manifestations have also been observed in mice infected with Trypanosoma brucei (Kobayakawa et al., 1979). To explain these experimental data it has been suggested that products released during infections with microorganisms may either act as mitogens or stimulators of cytokines such as IL-1 or IFN-γ or adjuvants or as epitopes which crossreact with autoantigens but present on new carriers. This would enable the T lymphocytes to recognize foreign determinants

and subsequently give help to B lymphocytes which recognize either auto- or heterologous determinants. The same concept of "altered self" has been used to explain manifestations of autoimmunity following administration of drugs like alpha-methyl dopa (Roitt, 1984) and hydantoin (Allison, 1977).

1.1.4 Suppressor cell dysfunction

Suppressor cell dysfunction on three different levels has been invoked in an attempt to explain autoaggression.

It has been proposed that delayed maturation of suppressor cells in the thymus would result in the emigration of relatively few competent suppressor cells into the periphery which, in conjunction with timely appearance of autoreactive cells, would lead to an imbalance. In accordance with this concept, the ability of thymus suspensions to inhibit thyroid specific cytotoxicity was found to be markedly reduced in O.S. chickens with SAT (Wick et al., 1982). A functional defect of suppressor cells has also been decribed in Hashimoto's thyroiditis and in Graves' disease in that peripheral blood lymphocytes from these patients failed to suppress the response of sensitized T lymphocytes to thyroid antigens as assessed by the release of migration inhibition factor (Okita, Row and Volpé, 1981).

Secondly, the observed deficiency of suppressor cell function in O.S. chickens with thyroiditis might result from insufficient production of a dialysable non-specific suppressor factor antagonizing IL-2 activity (Schauenstein et al., 1985). More

recently this factor has been identified as thymidine which clearly will have an effect in any proliferation assay and therefore the effect must be attributed to an in vitro artefact (Krömer et al., 1988). There is evidence that both T suppressor cells (Hardt et al., 1981) and macrophages (Krömer et al., 1987) can produce such a factor. Progress has been difficult in this field because of a lack of phenotypic markers which would precisely define suppressor cells and also because this subpopulation proves to be functionally very heterogenous (Dorf and Benacerraf, 1984).

Thirdly, an imbalance of cytokines acting on the suppressor cells rather than a primary defect in these lymphocytes themselves might also play a role. T lymphocyte suppressor function seems to be negatively affected by interferon-gamma (IFN-γ): thus in the mouse, injection of anti-IFN-γ monoclonal antibodies immediately before priming with horse red blood cells reduces the number of responding plaque-forming spleen cells by 50% (Frasca et al., 1988). Other signals implicated in secondary insufficiency of suppression are steroid mediators (Fässler et al., 1986) and antigen-specific factors released by T cells in response to an antigenic stimulus (Altman and Katz, 1980). The question of T suppression is still hotly debated mainly because the identity of the effectors of suppression has not been unequivocally determined (Möller, 1988, Tada, 1988, Pereira et al., 1988).

1.1.5 Inappropriate expression of MHC class II gene products

Antigen presentation in the context of class II major histocompatibility complex (MHC) molecules (Ia, HLA-DR) is a prerequisite for the activation of T helper/inducer cell subsets and therefore the initiation of an immune response (Erb and Feldman, 1975). Normally, Ia expression is restricted to lymphocytes, macrophages, dendritic cells and other specialized antigen presenting cells such as vascular endothelium and the epithelial lining of the small intestine and the respiratory tract (Natali et al., 1981). Recently it was shown that human thyroid epithelial cells, which are normally HLA-DR (= part of human MHC class II) - negative, could be induced by lectins such as phytohaemagglutinin to express these molecules in vitro (Pujol-Borrell et al., 1983). Later, thyroid glands from patients with autoimmune thyroid disease were found to spontaneously express class II (Hanafusa et al., 1983). An additional observation in these tissues was the presence of microsomal antigen, normally restricted to the apical pole of the thyroid epithelium, at the basal surface of the cells (Khoury, Bottazzo and Roitt, 1984). This reversal of polarity, seen only in follicles isolated from glands affected by autoimmune processes, could well make microsomal antigen accessible to the immune system (Hanafusa et al., 1984). These findings led Bottazzo Feldman and colleagues to propose the hypothesis that inappropriate HLA-DR expression confers the capacity to present surface autoantigens to autoreactive T lymphocytes to thyroid follicular

epithelial cells and thus trigger an autoimmune attack. If, in this case, specific suppressor mechanisms operate inadequately, full-blown autoimmune disease might evolve (Bottazzo et al., 1983).

Ia expression on thyroid epithelial cells can be induced by IFN-γ (Todd et al., 1985, Weetman et al., 1985, Rayner et al., 1987). Indeed the induction of class II MHC antigen expression on thyroid cells by PHA has been shown to be due to IFN-γ production by mitogen cultivated contaminating T cells raising the question, whether the altered phenotype of the gland really is the primary lesion leading to the development of autoimmunity (Iwatani et al., 1986). Local inflammation, triggered for example by viral infection, might result in the production of IFN-γ by activated lymphocytes and subsequent epithelial expression of class II (Bottazzo et al., 1983). This is consistent with the fact that spontaneous MHC class II expression on thyroid epithelium in obese strain chickens is only seen in severely infiltrated glands (Wick et al., 1984). Attempts to demonstrate antigen presentation by murine TFEC's bearing MHC class II molecules were unsuccessful (Ebner et al., 1987) until it was realized that pretreatment with phorbol ester was critical (Minami et al., 1987). In man, however, Londei et al. (1984) showed that class II positive TFEC's from patients with Graves' disease are capable of stimulating T cells derived from the patient's gland and are able to present viral peptides but not whole virus to appropriate human T cell clones. Additionally, de novo expression of class II gene products has been observed on thyroid monolayer cells

transfected with the virus SV 40 in vitro (Belfiori et al., 1986). This raises the possibility that viral infection leads to the de-repression of genes encoding these molecules, thus making the affected cells targets of an autoimmune attack. The fact that cornavirus-induced Ia expression on astrocytes is strain-specific and is directly correlated with the susceptibility to experimental autoimmune encephalomyelitis may support this idea (Massa, Brinkmann and Ter Meulen, 1987).

Whatever the mechanisms involved, in human disease inappropriate class II expression has been demonstrated both associated with massive lymphocytic infiltration - as on pancreatic islet β-cells in type I diabetes mellitus (Bottazzo et al., 1985) and on salivary duct cells in Sjögren's syndrome (Lindhal et al., 1985) - and in the absence of substantial lymphocytic infiltration - as in early stages of primary biliary cirrhosis (Ballardini et al., 1984).

1.2 AUTOIMMUNE THYROIDITIS

1.2.1 Autoantigens: molecular basis

Thyroglobulin is an iodinated glycoprotein with an average molecular weight of 660 Kilodaltons (kD) with a sedimentation coefficient of 19 S, although a 27 S dimer rich in thyroxine has also been described . It is a homodimer composed of two 330 kD subunits linked by disulfide bonds (Frati et al., 1974). The carbohydrate portion, a large part of which consists of sialic acid, contributes about 10% of the molecular weight, (Spiro, 1977).

Thyroglobulin is the main protein synthesized by thyroid follicular cells and its major function is the synthesis and storage of the thyroid hormones thyroxine (T4) and triiodothyronine (T3). This depends on the presence of tyrosine residues in its primary structure and on coupling reactions between the iodinated residues monoiodo- and diiodotyrosine (MIT and DIT). Of the 120 tyrosine residues contained in the molecule only 25 to 30 are iodinated in vivo and usually not more than 6 - 10 MIT and DIT units can be coupled to form thyroid hormone (Lissitzky, 1984).

Biosynthesis of thyroid hormones takes place at the apical border of the thyrocyte and the reactions are catalyzed by thyroid peroxidase. This involves a sequence of reactions starting with the uptake of iodine to terminate with the secretion of T4 and T3. For secretion iodinated thyroglobulin is trapped from the colloid stores by the follicular cells and digested by lyzosomal enzymes. This results in the release of thyroid hormones together with some partially digested thyroglobulin and iodinated aminoacids (Van Herle, Vassart and Dumont, 1979).

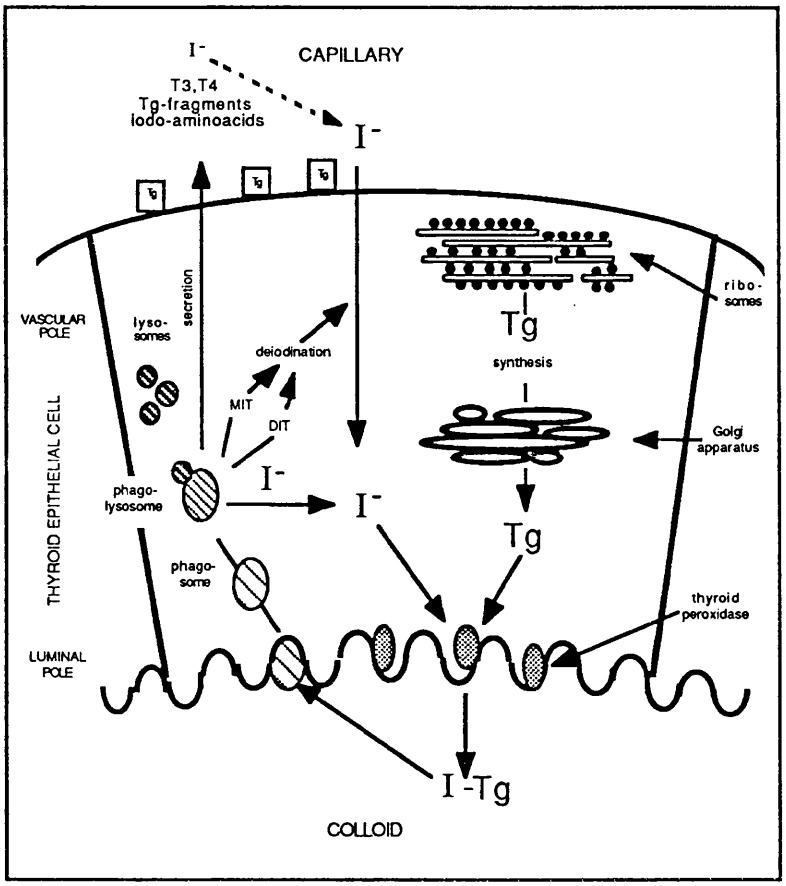

FIGURE 1.1 DIAGRAM OF THE BIOSYNTHESIS OF THYROID HORMONES. In the course of this process thyroglobulin or fragments are presented on the vascular surface of the epithelial cell and released into the circulation.

Although the primary structure of the protein is so far only available for the bovine (Mercken et al., 1985) and the human (Malthiéry and Lissitzky, 1987) thyroglobulins, it is well-known that only a few segments of the molecule are involved in the hormone formation. Such hormonogenic sites are mainly situated in the N-terminal region (Rawitch et al., 1983) where aminoacid sequences surrounding hormonogenic tyrosines show a remarkable level of evolutionary conservation (Vassart et al., 1985). However, the three-dimensional structure of this area, as inferred from

anti-thyroxine monoclonal antibody binding studies, shows differences between different animal species near the thyroxine-forming sites (Chan et al., 1986).

Evidence that enzymic degradation increases the affinity of the native thyroglobulin for thyroxine autoantibodies as measured by displacement studies (Byfield et al.,1982) suggests that hormonogenic sites are involved in the antigenicity of the molecule. It also implies the exposure of thyroxyl residues previously buried within the protein. Circular dichroism measurements indicate that conformation changes of the thyroglobulin structure only occur at high iodination rates (Zabel and Rawitch, 1984).

Thyroglobulin detected in the circulation is less iodinated than the protein stored in the colloid (Ikekubo et al., 1981). Also, the amount of iodine in the secreted thyroglobulin varies with disease states of the thyroid gland (Schneider, Ikekubo and Kuma, 1983). Although the form, in which thyroglobulin is presented to the immune system, is not known, the fact that highly iodinated thyroglobulin is more immunogenic than the less iodinated molecule (Sundick et al., 1986) suggest that thyroxine residues play a part in its antigenicity. However, this interpretation has been disputed by other authors, claiming that the observed differences in reactivity might be ascribed to structural differences rather than to iodine content (Kim, Dunn and Dunn, 1988).

The other thyroid antigen often associated with thyroid autoimmunity is the 'microsomal antigen', more recently identified as the enzyme thyroid peroxidase, which is involved in the hormone biosynthesis (Czarnocka et al., 1986). This is consistent with its

cellular distribution, which is normally restricted to the cytoplasmic and luminal membranes of the thyroid follicular epithelial cells. The role of the thyroid microsome in thyroiditis is a neglected issue, despite the fact that anti-microsomal antibodies are a common finding in human thyroiditis. In this respect rabbits (Mangkornkanok, Marcowitz and Battifora, 1972) and monkeys (Kite, Argue and Rose, 1966) are among the few species in which experimental induction of thyroiditis has been reported using the microsomal antigen.

1.2.2 Genetics of susceptibility

One of the first observations pointing to a role of genetic influences in the predisposition to the development of autoimmune thyroiditis was the higher prevalence of antibodies to thyrogloblulin in relatives of patients with Hashimoto's thyroiditis as compared with the normal population (Hall, Owen and Smart, 1960). The fact that females were affected three times more frequently than males indicated that genes mapping to the X chromosome or preponderance of female hormones might be responsible for this predisposition. However, the finding of a significant overrepresentation of HLA-DR3 and B8 haplotypes, which are in linkage disequilibrium, in Hashimoto's thyroiditis (Weetman and McGregor, 1984) suggested that other genetic locations might be involved. The first experimental demonstration of an association of genes from the major histocompatibility complex (MHC) with susceptibility for thyroiditis came from

experiments in mice. Immunizing mice with thyroglobulin emulsified in CFA, Vladutiu and Rose (1971) found that thyroid lesions and the appearance of autoantibodies were strictly dependent on the H-2 (MHC) haplotype of the animals. They also observed that mice bearing the H-2^k haplotype were high responders whereas those from the H-2^b or H-2^d were poor responder strains. Recombinant strains derived from high and low responder EAT haplotypes enabled Tomazic, Rose and Shreffler (1974) to localize the gene controlling the susceptibility to EAT between the K-end and the I-A subregions of the H-2 complex. Furthermore, in high responder strains, D-end genes were shown to influence the severity of the EAT (Kong et al., 1979). Since the genes encoding TNFα and TNFβ are located close to the D subregion (Müller et al., 1987) this effect might be due to products of these genes and not an MHC antigen itself. Taking into account the dissociation between antibody response and the thyroid damage (Tomazic and Rose, 1977), it seems likely that Tg-autoantibody production is under other genetic influences.

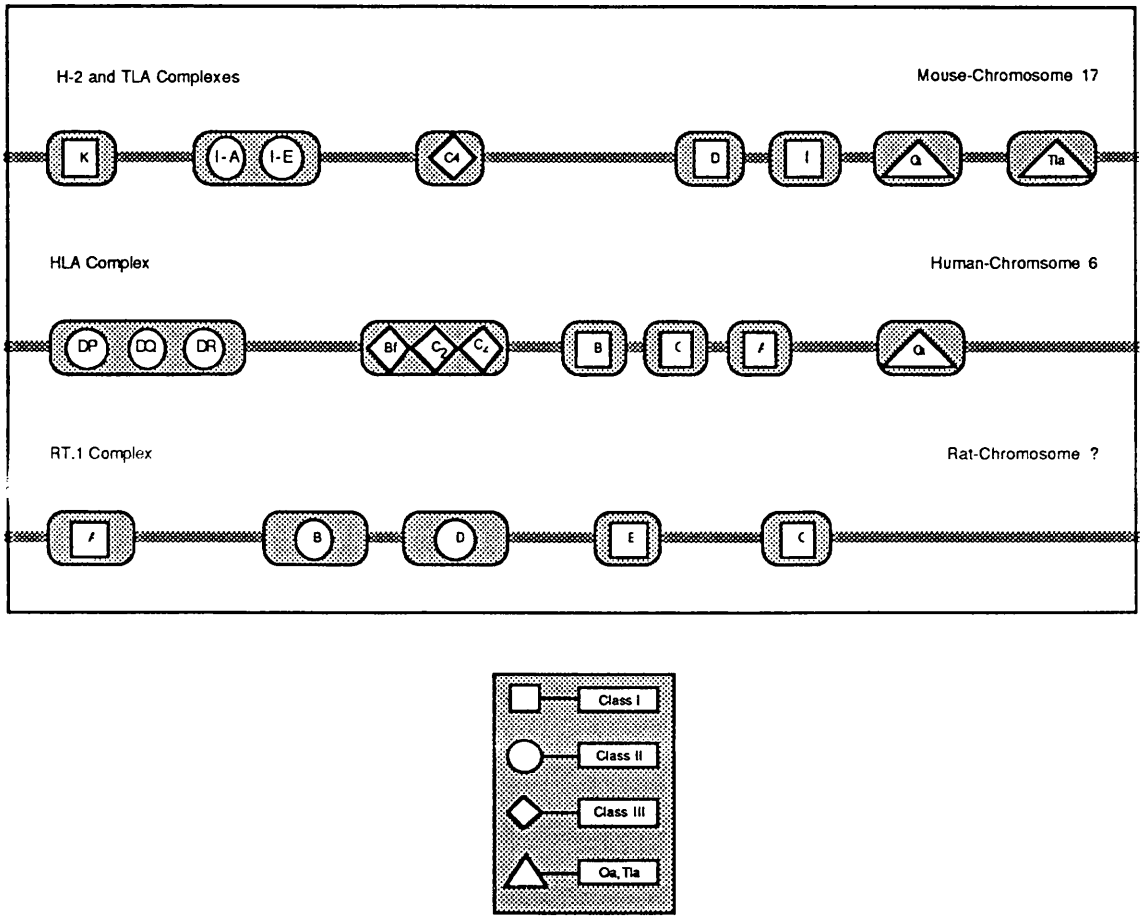

FIGURE 1.2: SCHEMATIC REPRESENTATION OF MOUSE, HUMAN AND RAT MHC'S. These complexes are subdivided into regions which encode three classes of proten molecules. Regions and subregions specifying class I, I, III (complement) and Qa, Tla genes are indicated by squares, circles, lozenges and triangles respectively (according to Degos, 1988).

An "immune response gene to thyroglobulin" (Ir-Tg) within the MHC region has been postulated to account for susceptibility to mouse EAT, although genes outside the H-2 complex are likely to exert a modulatory influence in the EAT response (Beisel et al., 1982). This is particularly true for the OS chickens in which the heritability of autoimmune thyroiditis shows great variation within the same B haplotype (MHC). Thus, in some colonies the homozygous

haplotypes $B^{13}B^{13}$ and $B^{5}B^{5}$ conferred high and low susceptibility, whereas in other colonies $B^{13}B^{13}$ and $B^{5}B^{5}$ both conditioned high susceptibility to the disease in contrast to the haplotype $B^{15}B^{15}$ (Wick et al., 1982). Additionally, an intermediate susceptibility was identified in $B^{5}B^{15}$ heterozygous animals (Bacon, Kite and Rose, 1974). Another indication of polygenic influences is the inherited primary defect in their thyroid function, expressed as high iodine uptake, which could not be suppressed by using either thyroxine or cyclosphosphamide (Sundick et al., 1979).

Attempts to locate the Ir-Tg in the rat MHC (RT.1) have failed so far, although inbred strains respond differently to Tg immunization according to their RT.1 haplotypes (Rose,1975). In this respect, Penhale et al. (1975) demonstrated that strains with haplotypes $RT.1^{c}$ and $RT.1^{u}$ were associated with high and low susceptibility, respectively. However, a marked lack of concordance in responsiveness among rat strains bearing the same RT.1 haplotype indicates that the so-called Ir-Tg may lie outside the MHC (Lillehoj and Rose, 1982). Examining the thyroiditis response to Tg in rats of high and low responder strains Lillehoj, Beisel and Rose (1981) observed that F1 hybrids showed intermediate susceptibility as compared to the parental strains. F2 and backcross progenies demonstrate a wide variation of disease susceptibility, implying that the immune response to rat Tg is under polygenic control. An additional genetic factor controlling that response is linked to the X chromosome. Thus backcross animals carrying the X chromosome from the high responder strain both in homozygous and heterozygous pattern had a higher incidence of disease than those

with the X chromosome of the poor responder strain. The fact that females of both high and poor responder strains developed higher antibody titres than males suggests that the humoral response to Tg is linked either to the X chromosome or to hormonal factors. Although the data seem to favour a role of non-MHC genes in the determination of the immune response to Tg, the question of the localization of the rat Ir-Tg will remain open until studies on intra-RT.1 recombinant strains are accomplished.

1.2.3 Thyroid epithelium as a target of autoimmune attack

Several pieces of evidence indicate that humoral and cell-mediated immunity cooperate in the pathogenesis of thyroiditis, although the mechanisms leading to the thyroid damage are still a matter of controversy. The main question concerns the primary event in the initiation of the autoaggression against the thyroid follicle, namely whether there is a defect in the immune system, or whether normal effector immunocytes are reacting against cell surface components efficiently presented to them. Whatever the initiating mechanism implicated, both autoantibodies and leukocyte infiltration seem to contribute to the full expression of thyroid damage (Romball and Weigle, 1983).

The precise role of autoantibodies as an effector mechanism of the humoral attack against the thyroid epithelium is not yet clear. One possibility is that autoantibodies to Tg can become cytotoxic by complexing with thyroglobulin expressed on the surface of the thyroid epithelium, similar to observations made in

culture (Ebner et al., 1987). Supporting this supposition is the fact that immune complexes are deposited between the follicular basement membrane and the vascular pole of the follicular cell in the development of mouse EAT (Clagett, Wilson and Weigle, 1974). Since these Tg autoantibodies are predominantly of the IgG1 isotype (Pontes de Carvalho and Roitt, 1982) and therefore able to fix complement, it is possible that complement mediated cytotoxicity represents an important step in the generation of thyroid lesions.

Alternatively, Tg-autoantibodies could induce cytotoxicity through Fc-receptor bearing cells as antibody dependent cellular cytotoxicity (ADCC). This mechanism has been demonstrated both in the spontaneous autoimmune thyroiditis of the O.S. chicken (Wick et al., 1982) and in Hashimoto's thyroiditis (Calder and Irvine, 1975). Whether or not autoantibodies are directly cytotoxic to the thyroid epithelium, severe lesions have developed in mice (Vladutiu and Rose, 1971) and in chickens (Jaroszewsky et al., 1978) after transfer of high titer EAT antisera. Moreover, sera of thyroidectomized mice were the most effective of these pathogenic autoantibodies, suggesting that the thyroid might deplete the serum of such cytophilic antibodies (Vladutiu and Rose, 1971). In another study mice injected with two monoclonal IgG1 and IgM Tg-autoantibodies developed thyroiditis which was more severe when CFA was added to the immunization protocol (Guarnota et al., 1982).

Although in most species thyroid autoantibodies are specifically directed towards thyroglobulin, evidence of complement-fixing antibodies in man, monkey and rabbit binding the

thyroidal microsomal fraction indicate that the antibody attack might involve various antibody populations. Although the sources of these microsomal antibodies are not yet defined, the fact that Tg-antibodies are produced outside the thyroid in the EAT (Weetman et al., 1982) makes less likely that antigen-antibody immune complexes are a mechanism operative in thyroid damage. In fact, autoantibody titres and the severity of lesions are not correlated. This does not rule out, however, a participation of ADCC mechanisms in the generation of thyroid damage.

Fundamental differences are seen between induced and spontaneous thyroid lesions in relation to the involvement of cell-mediated immunity. For example, germinal centres are found in the spontaneous but not in the induced inflammatory lesion (Silverman and Rose, 1978). Although it is possible that B lymphocytes and antibody-forming cells (plasma cells) play a dominant role in the pathogenesis of the thyroid damage, the presence of other cell phenotypes suggest that additional mechanisms may operate simultaneously. Voorbij, Kabel and Drexhage (1986) have shown an accumulation of dendritic cells in the BB/W thyroid long before the autoimmune reaction takes place. They proposed that these cells present the thyroid antigen to the immune system at the draining lymph node, initiating a traffic of sensitized cells to the gland which would then cause the lesions seen in later stages. The thyroid infiltrate, in their observation, showed a good correlation with the cell populations in the draining lymph node and comprised CD4, CD8, macrophages, B lymphocytes and plasma cells.

Looking at the thyroid infiltrate in EAT of mice Creemers et al. (1984) observed that the first leukocytes invading the thyroid were predominantly T cells which were gradually replaced by non-T/non-B cells as the disease evolved. Additionally, they noticed an initial predominance of Lyt-1+ cells followed by a relative increase in Lyt-2+ cells which they interpreted as an emergence of T cytotoxic/suppressor cells with evolution of the disease.

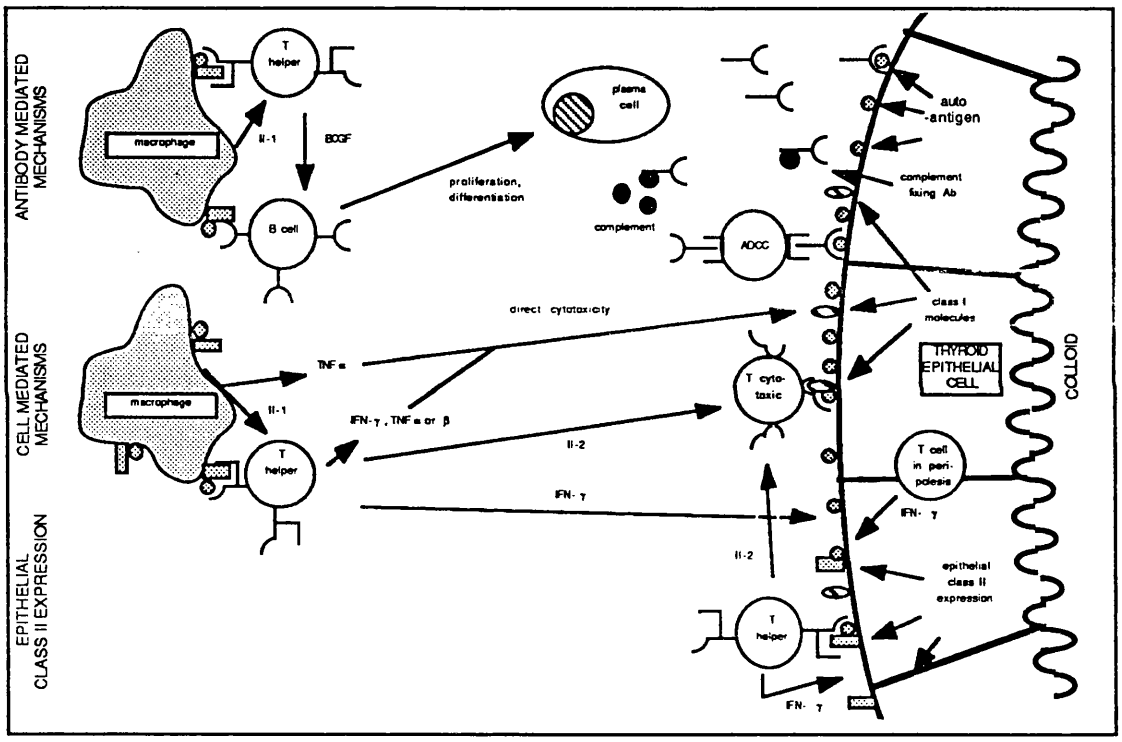

FIGURE 1.3 DIAGRAM OF POTENTIAL MECHANISMS IMPLICATED IN THE DESTRUCTION OF THYROID FOLLICLE IN AUTOIMMUNE THYROIDITIS. Antibody mediated mechanisms involving complement lysis and antibody dependent cell cytotoxicity (ADCC) are depicted in the top. Cell mediated mechanisms (CMI) involving direct cell cytotoxicity and cytokines mediated cell killing are illustrated in the middle. The bottom shows induction of inappropiate class II expression on thyroid epithelial cells.

According to the protocol adopted for the induction of EAT qualitative differences in the inflammatory infiltrate were observed in the rat. Thus, thymectomized animals developed a thyroid infiltrate mainly composed of T cells, comprising equal number of CD4 and CD8 positive cells, and of B cells to a lesser extent. In contrast, infiltrates of Tg + CFA immunized animals mainly consisted of macrophages or dendritic cells and B lymphocytes (Cohen and Weetman, 1987). Infiltrates of Hashimoto's thyroiditis characteristically show a predominance of $CD4^+$ cells (Jansson, Karlsson and Forsum, 1984) a pattern shared with the SAT infiltrates of the OS chickens (Krömer et al., 1985). Londei, Bottazzo, and Feldmann (1985) have cloned activated T cells from thyroid infiltrates from patients with Graves' disease and demonstrated that some of them are indeed autoreactive, proliferating either in response to autologous thyroid epithelial cells or, more generally, to autologous thyroid and peripheral blood cells. In accordance with the reported histological findings, all the autoreactive clones were $CD4^+$. B lymphocytes, although present, constituted a minor population in these infiltrates only. A low number of NK cells were also detected in the Hashimoto's infiltrate (Aichinger, Fill and Wick, 1985).

Evidence that the thyroid epithelium in humans (Hanafusa et al., 1984) and in OS chickens (Wick et al., 1984) expresses MHC class II gene products locally associated with the lymphocytic infiltration, point to multifactorial mechanisms involved in the pathogenesis of the thyroid lesions in both the spontaneous and induced disease. The fact that interferon-γ induces epithelial class

II expression in vitro (Weetman et al., 1985; Todd et al., 1985; Rayner et al., 1987) and that activated T lymphocytes are the main source of this lymphokine (Steeg, Moore and Oppenheim, 1980) suggest a primary role for T cells in the inappropriate epithelial class II expression. It remains a point of debate whether such expression is triggering the immune attack against the thyroid epithelium, an assumption suggested by observations in man (Bottazzo et al., 1983), since in the induced disease of the rat thyroid damage occurs without the simultaneous presence of Ia antigen (Cohen and Weetman, 1987). Similar observations have been reported in the spontaneous disease of the BB/W rat (Voorbij, Kabel and Drexhage, 1986). Since macrophages are the most frequent cell type in the thyroid infiltrates in these animals an alternative mechanism might be operating in the follicular damage. It is known that activated macrophages secrete tumor necrosis factor (TNFα) (Beutler, Milsark and Cerami, 1985) and also that the rat thyroid epithelial cell line FRTL-5 is sensitive to the cytotoxic action of this cytokine (Taverne et al., 1987). Furthermore, IL-1 released by infiltrating macrophages has been shown to inhibit directly thyroglobulin and cyclic AMP production by thyroid cells, suggesting that IL-1 may play a role in the molecular mechanisms leading to autoimmune thyroiditis (Rasmussen et al., 1987). In this in vitro model IFN-γ induced susceptibility to the cytotoxic effects of TNFα was manifest long before expression of class II antigens suggesting that TNFα may be an early mediator of the thyroid damage. In this case, TNFα would bypass the reported protective effect of IFN-γ

against cell-mediated cytotoxicity to human thyroid epithelial cells (Bogner, Sigle and Schleusener, 1988). The coexistence in the same infiltrate of the cell types able to release TNFα and IFN-γ, supports this hypothesis and suggests again that the epithelial class II expression may well have an enhancing rather than a causative effect in the initiation and propagation of the thyroid follicular attack.

Recently Sarvetnick and colleagues (1988) have coupled the gene for IFN–γ to the insulin promotor and introduced this construct into mice. In these transgenic animals, whose islet β-cells presumably secrete IFN-γ, pancreatitis with insulitis and class II expression of the β cells resulted in insulin dependent diabetes mellitus. It remains an open question whether the cause of the islet cell destruction was an autoimmune attack triggered by the local secretion of IFN–γ, whether it was due to disruption of insulin secretion following transgene over-expression by the β-cells or whether the authors observed a non-specific inflammation of the pancreas which finally also led to the destruction of the islets, similar to a "bystander effect".

IL-2 has also been invoked to play a role in the breaking of tolerance (Colizzi et al., 1985). It has been demonstrated that neonatal induction of tolerance by intravenous injection of allogeneic cells can be prevented and that established neonatally induced tolerance can be broken by the injection of interleukin-2 (Malkowsky et al., 1985). It is even sufficient to treat autologous lymphoid cells in the absence of antigen in vitro with rIL-2 to

induce precursors of alloreactive cytotoxic cells in vitro and rejection of long-term accepted allografts in vivo, indicating that in the repertoire of neonatally tolerized animals alloreactive T cells persist and can be non-specifically activated by IL-2 (Loveland et al., 1986). It seems likely, that T cells reactive with thyroglobulin are also not deleted from the normal repertoire, since it is relatively easy to induce an immune response to thyroglobulin. If this were the case, IL-2 released locally by activated T cells might recruit more autoreactive T cells, which were functionally silent before, and thus contribute to the chronicity of the thyroiditis. Furthermore, Malkovsky and colleagues (1987) have shown a direct augmentation of macrophage cytotoxicity by IL-2, suggesting, that this lymphokine, which may be released by T cells present in the infiltrates, might also enhance effector mechanisms leading to damage of thyroid follicles.

Studies on OS chickens have revealed primary abnormalities of the thyroid gland occurring long before the epithelium becomes a target of autoimmune aggression. These were characterized by abnormally high levels of thyroglobulin (Sanker, Sundick and Brown, 1983) as well as by degenerative changes such as vacuolization of the glandular epithelium (Wick and Graf, 1972) as early as during the first week after hatching. Furthermore, a decreased growth rate of OS thyroid epithelial cells in culture, reflected by decreased DNA synthesis has been observed (Truden et al., 1983). Considering also the functional defects of the thyroid leading to hypothyroidism (Sundick et al., 1979) this can be summed up in the statement that intrinsic alterations of the thyroid epithelium could render these

cells a target for the cellular and humoral aggression involved in the destruction of the gland.

1.3 EXPERIMENTAL ANIMAL MODELS

Experimental animal models have proved to be a useful tool in the investigation of autoimmune disease. For example by means of manipulations, which out of ethical considerations are not possible or desirable in man, they give insights into the disease process and its prevention or mitigation. However, the results obtained in animals may not always be directly relevant to the human disease. The immune system of each animal species has unique features which render comparisons with man sometimes difficult, and especially induced disease in animals may differ from the human homologue. Even though in clinical practice new therapeutic trials are usually only initiated after encouraging results have been obtained in animal models, only the successful treatment of the human disease can ultimately confirm that our understanding of human autoimmunity is correct.

1.3.1 Experimental autoimmune thyroiditis (EAT)

Since the first demonstration of Tg autoantibodies and thyroiditis in rabbits immunized with Tg + CFA (Rose and Witebsky, 1956), EAT has been induced in various species. Basically, three protocols have been used. The first involves direct injection of thyroglobulin either in conjunction with adjuvant or alone. This

procedure leads to an acute and self-limiting disease (Jones and Roitt, 1961). The second relies on the manipulation of immunoregulatory mechanisms of the animal, usually involving thymectomy and sublethal irradiation. This method induces a chronic disease (Penhale et al., 1973). The third involves adoptive transfer of serum (Vladutiu and Rose, 1971) or lymphoid cells (Twarog and Rose, 1970) from diseased animals.

For active immunization most protocols use adjuvants such as CFA (Jones and Roitt, 1961) or bacterial lipopolysaccharide - LPS (Esquivel, Rose and Kong, 1977). Either crude thyroid extract or purified allogeneic and xenogeneic thyroglobulin can induce the disease (Romball and Weigle, 1983). Among allogeneic thyroglobulins, the Tg from EAT poor responder mice has low antigenicity and the Tg from high responder has high antigenicity (Tomazic and Rose, 1976). Syngeneic thyroglobulin administered repeatedly in small doses is able to induce thyroid lesions even in the absence of adjuvant (Elrehewy et al., 1981). An optimal dose of Tg is required in order to induce significant titers of Tg autoantibodies and thyroid lesions in a reliable fashion, especially in poor responder strains (Vladutiu, 1983).

The successful use of thymectomy in conjunction with irradiation to provoke EAT has prompted the view that deletion of T suppressor cells with sublethal irradiation might underly the induction of the disease in this case. Neonatally thymectomized mice develop EAT which can be prevented by injection of either thymic cells from young donors or lymph node cells from old donors (Penhale et al., 1976; Kojima et al., 1976), supported a regulatory

role for T cells.

The involvement of T suppressor cells in EAT was also demonstrated with other immunization protocols. Braley-Mullen et al., (1978) observed that EAT induced by Tg plus CFA is suppressed if guinea-pigs are pre-treated with homologous Tg plus incomplete Freund's adjuvant. They suggested that a population of T suppressor cells are activated during the pre-immunization period which could then prevent the induction of disease. This suppression, however, could not be transferred into naive animals (Braley-Mullen et al., 1983). In contrast, Kong et al., (1982) and Parish et al. (1988a), using a similar protocol have shown that suppression of the EAT response to mouse Tg (MTg) + CFA, induced by pretreatment with soluble MTg, could be transferred to irradiated (200 rads) syngeneic recipients. This transferred suppression was further demonstrated to be MTg specific and it could be shown to involve both T helper and B cells since immunization with a modified antigen, namely MTg conjugated with DNP, did not abrogate the induced suppression (Parish et al., 1988a).

1.3.2 Spontaneous Autoimmune Thyroiditis (SAT)

SAT was first observed in chickens (Cole, 1966), particularly in the obese strain (OS), a selective inbreeding of Cornell strain chickens affected by hypothyroidism. OS chickens develop SAT within 1 to 5 weeks after hatching (Kite et al., 1969), characterized by high titers of Tg autoantibodies and thyroid infiltrates composed of plasma cells, B and T lymphocytes as well

as macrophages (Wick et al., 1985). Together with the SAT of Buffalo (BUF) rats, the disease of OS chickens is regarded as a suitable model for studying Hashimoto's thyroiditis. Because of the anatomical compartmentalization (bursa and thymus) of the immune system in birds(Fleischer, 1981), the avian model provides the unique opportunity for separately studying the B and T cell arms of the autoimmune response.

Chemical bursectomy studies have shown that an intact B cell system, though important for the generation of Tg autoantibodies, is not indispensable in the development of thyroid infiltration (Krömer et al., 1985). On the other hand, T helper/effector cells are able to initiate and maintain the disease on their own, the presence of B cells accelerating the process (Wick et al., 1985). In this model it has also been shown that the continuous presence of the autoantigen is necessary for the production of autoantibodies. Longitudinal studies in OS chickens have demonstrated that neonatal thyroidectomy prevents the development of Tg autoantibodies, an effect which was abrogated by administration of OS chicken Tg. The question whether alteration of the autoantigen plays a role in the induction of the disease was also addressed but no particular immunogenic determinants could be identified in the OS thyroglobulin (Pontes de Carvalho et al., 1982).

Neonatal thymectomy in both OS chicken (Welch, Rose and Kite, 1973) and BUF rat (Silverman and Rose, 1974) enhances the severity of SAT. This observation has lead to the suggestion that the removal of the thymus depletes the animal of T suppressor cells and that the development of SAT depends on the presence of a T cell

subset that has migrated to the periphery before thymectomy (Rose et al., 1981).

Different from the situation in the induced disease, antibody titers in SAT generally seem to correlate with the severity of thyroid pathology, strengthening the notion of a major role for autoreactive B cells or their antibodies in the pathogenesis of SAT. However, thyroid lesions may also occur in the absence of circulating autoantibodies (Bigazzi and Rose, 1975). Since autoantibodies are not detected in birds treated with thymectomy and anti-T cell serum (Pontes de Carvalho, Wick and Roitt, 1981) it seems most likely that humoral and cell-mediated mechanisms cooperate in the development of SAT.

As mentioned above, intrinsic defects of the thyroid function in the OS and also in the Cornell C strain (CS) have been implicated in the increased susceptibility to SAT in these genetically susceptible chickens. One of the abnormalities reported is an increased uptake of iodine, partially independent of TSH levels. Since administration of iodine increases autoantibodies to Tg, T_4 and T_3 and also the degree of lymphocytic infiltration of the thyroid in CS chicks this indicates that iodine might play a role in the development of the disease. Confirming this, inhibition of the iodine uptake by administration of potassium perchlorate (KClO4) reduced the severity of SAT in the highly susceptible OS chickens (Bagchi et al., 1985).

SAT has also been described in the BioBreeding/Worcester rats (BB/W) (Sternthal et al., 1981) and in hybrids generated by

intercrossing BB/W and BUF rats. In these hybrids severe forms of autoimmune thyroiditis are associated with the RT.1^{b} haplotype (Cole, Guttmann and Seemayer, 1985). As in the OS model, SAT in these rats is significantly increased by the administration of iodine. However, this effect is restricted to young animals (30 days old), adult animals being unaffected by iodine administration (Allen et al., 1986).

1.3.3 The non-obese diabetic mouse, a model for insulin dependent diabetes mellitus

The non-obese diabetic (NOD) mouse is currently used as a model of insulin dependent diabetes mellitus (IDDM). The strain was derived from Jcl-ICR cataract mice by brother sister matings (Makino et al., 1980). Both sexes develop insulitis starting at about six weeks of age and onset of diabetes in females follows at about 30 weeks of age. Development of overt diabetes is much less frequent in males and orchidectomy increases whereas ovariectomy reduces the diabetes incidence in NOD mice (Makino et al., 1981). Nearly 90% of the NOD mice population display mononuclear cell infiltration of the pancreatic islets leading to selective destruction of insulin producing β cells. The destruction of the β cells is thought to be an autoimmune response mediated by T cells (Harada and Makino, 1984; Bendelac et al., 1987) in which autoantibodies may also play a role.

The precise mechanism by which destruction of the β cells

occurs is still unknown. There are indications that 6 weeks is a critical age in the development of disease in the NOD and that some event at about that age triggers the onset of the disease. It has been postulated that before this event, critical antigenic determinants may not be present on the β cell (Wicker et al., 1986). There is also a considerable body of evidence to date implicating both $L3T4^+$ and $Ly2^+$ T cells in the destructive process (Bendelac et al., 1987; Miller et al., 1988). Furthermore, it has been shown that treatment of NOD mice with L3T4 antibody in vivo delays the onset of overt clinical symptoms (Shizuru et al., 1988). More recently it was observed that administration of silica particles, which interfere with macrophage function, could prevent β-cell destruction in NOD mice, suggesting that macrophages play an important role in the mediation of β-cell death (Charlton, Bacelj and Mandel, 1988).

The NOD is of the unique haplotype K^d $I\text{-}A^{nod}$ $I\text{-}E^0$ D^b. Non-expression of I-E is due to non-transcription of I-Eα. A role for the I-Eα chain in the development of diabetes has been tested in NOD by introducing the $E\alpha^d$ gene, leading to I-E expression. This could prevent the development of autoimmune insulitis in the transgenic NOD mice (Nishimoto et al., 1987). The specific I-A serotype is due to the unusual sequence of I-Aβ, I-Aα being of d-haplotype. The presence of serine in position 57 of the Aβ chain seems to be permissive for development of IDDM. This is analogous to the situation in the human, where the presence of aspartate in position 57 of the DQβ chain seems to reduce the susceptibility to the development of IDDM (Todd, Bell and McDevitt, 1987).

The NOD mice also develop lymphocytic infiltration in submandibular glands and antiduct epithelial antibodies (Miyagawa et al., 1986). Other manifestations of spontaneous autoimmunity have been observed e.g. autoantibodies to thyroglobulin and anti-nuclear antibodies, which indicate that NOD could also provide an animal model for thyroiditis and Sjögren's syndrome (Pontesilli et al., 1987, Anne Cooke - unpublished observations).

1.3.4 Insulin dependent diabetes mellitus in mice transgenic for MHC genes

A very recent approach to investigate the role of aberrant class II expression in autoimmunity is the development of mice transgenic for MHC genes. Since the insulin promotor upstream of the insulin gene has been cloned, three groups have chosen insulin dependent diabetes mellitus as their model. They transfected mice with constructs of the syngeneic I-A gene (Sarvetnik et al., 1988), the I-E^b gene (Lo et al., 1988) or an MHC class I gene (Allison et al., 1988) under the control of the insulin promotor. In all cases the transgene was selectively expressed in the β-cells of the islets of Langerhans, and in all cases the animals developed diabetes requiring insulin substitution; but surprisingly there was no or only minimal insulitis. In fact, the animals seemed to be tolerant to the transgene, since the development of diabetes in the animals carrying class I transgenes was independent of the degree of MHC mismatch (Allison et al., 1988), and tolerance to the I-E^b transgene product in mice not normally expressing I-E molecules could be

demonstrated by cellular assays (Lo et al., 1988). Interestingly it was noted that in contrast to mice constitutively expressing I-E (Kappler, Roehm and Marrack, 1987) the unresponsiveness to the aberrantly expressed I-E^b molecules was not due to deletion of T cells expressing Vβ17a in their receptor from the repertoire of the transgenic mice (Parham, 1988). This indicates that clonal deletion is not the only mechanism of T cell tolerance.

Since insulitis is virtually absent, it seems unlikely that an autoimmune mechanism can explain the development of diabetes in mice transgenic for MHC genes. It has been suggested that the internal metabolic pathways of the β-cells may be deranged by the overexpression of transgene products interfering with insulin secretion; in particular MHC molecules might "trap" the insulin in the presecretory compartment of the cells (Parham, 1988). Alternatively Pujol-Borrell and Bottazzo (1988) have suggested that induction of class II expression in highly specialized cells normally negative for class II might turn on an internal suicide pathway (Ucker, 1987) leading to the self-destruction of the islet cells.

1.3.5 Autoimmunogenic T cell lines in experimental autoimmune encephalomyelitis

Experimental autoimmune encephalomyelitis (EAE) is a rodent model for multiple sclerosis. In the rat the disease can be induced by immunization with myelin basic protein (MBP) or by

injection of encephalitogenic T cell lines (Ben-Nun, Wekerle and Cohen, 1981). These encephalitogenic T cell lines have been derived from spleen cells of immunized but also from naive animals by stimulation with MBP and expansion with IL-2 in vitro (Ben-Nun and Cohen, 1982), indicating that these autoreactive and potentially lethal T cells are not deleted from the normal T cell pool.

Encephalitogenic T cell lines, highly specific for an immunodominant epitope of MBP, are $CD4^+$ and MHC class II restricted (Ben-Nun and Cohen, 1982). In the presence of MBP they induce MHC class II expression on astrocytes in vitro (Fierz et al., 1985), respond to these in an MHC class II restricted manner with proliferation (Fontana, Fierz and Wekerle, 1984), and finally lyse the astrocytes (Sun and Wekerle, 1986).

Animals recovering from from an episode of EAE and those treated with non-pathogenic doses of MBP (Alvord et al., 1965) or encephalitogenic T cells (Ben-Nun, Wekerle and Cohen, 1981, and Driscoll, Kies and Alvord, 1982) become resistant to further induction of EAE. Sun and colleagues (1988) have been able to generate a $CD8^+$ T cell line from the spleen of an animal recovering from EAE induced by an encephalitogenic T cell line (S1), which specifically lyses the inducing (MBP specific) cell line in vitro in the absence of MBP and also neutralizes its encephalitogenic capacity in vivo, indicating that antigen- independent clone-specific interactions between T cells may play a role in suppression of autoimmunity. The observation that animals immunized with the protective T cell line are unable to develop resistance to the encephalitogenic T cell line S1 even after five

episodes of EAE induced by S1 (Sun and Wekerle, manuscript in preparation), further underlines the importance of these T cell interactions.

1.3.6 Tolerance induction by antibody treatment

Further information on the mechanisms of tolerance has come from treatment experiments with monoclonal antibodies. Antibodies against I-A molecules have been shown to prevent induction of experimental autoimmune encephalitis (EAE) (Steinman et al., 1981) and also to suppress the active disease process (Sriram and Steinman, 1983). In the BB rat treatment with anti-I-E homologue has been shown to confer protection from IDDM and SAT (Boytard et al., 1985). This points to a role for class II restricted cell interactions in the breakdown of tolerance in this model. This is confirmed by the reversal of EAE by treatment with anti-CD4 antibodies (Waldor et al., 1985) and also by the fact that all encephalitogenic T cell lines are of $CD4^+$ phenotype (Ben-Nun and Cohen, 1982).

Anti-CD4 treatment has also been effective in spontaneous models of autoimmune disease. In NZB/NZW F1 mice, a model for systemic lupus erythematosus, anti-CD4 antibodies delayed the onset of kidney disease and significantly prolonged the life of the animals (Wofsy and Seaman, 1985). In the NOD mouse long-term anti-CD4 treatment could reverse ongoing insulitis and prevent the onset of diabetes mellitus. Interestingly diabetes did not develop for long periods after cessation of the antibody treatment, even

though insulitis recurred in a relatively mild form (Shizuru et al., 1988). Treatment with anti-CD8 antibodies also prevents IDDM in NOD mice, probably interfering with an effector mechanism of β-cell damage, since $CD4^+$ cells are still present in the islets of treated animals (Charlton, Bacelj and Mandel, 1988).

Not always can the disease process be abrogated in this way. In the mouse model of collagen II induced arthritis, the induction of the disease can be prevented by anti-CD4 immune manipulation only if the antibody is administered before or shortly after immunization with collagen II. A delay by 14 days renders the treatment completely ineffective, even though at that point symptoms are not yet apparent (Ranges et al. 1985). It has been shown that anti-CD4 can prevent the induction of antibodies but does not interfere with mature humoral responses (Ranges et al., 1985). This might explain the differences observed between EAE and collagen arthritis with regard to antibody treatment: possibly autoantibodies play a more important role in collagen arthritis than in EAE.

Benjamin and Waldman (1986) have demonstrated that the prevention of an immune response by anti-CD4 leaves a "memory" in the immune system. After regaining immune competence the animals are specifically tolerant to the "immunizing" antigen, even though no effective immune response had taken place. The relevance of these observations for physiological acquisition and maintenance of tolerance remains an open question. In any case, there may be important clinical applications of similar techniques, for example in the prevention of graft rejection.

In most clinical situations of autoimmune disease, however, therapy will aim at termination of an ongoing disease process rather than its prevention. The animal models indicate that this will probably only be achieved with long term treatment to prevent the recruitment of more autoreactive cells and so gradually dampen down the disease process. The benefits of such a treatment would then have to be weighed against the problem of immunosuppression as a consequence of immune manipulation with anti-CD4 antibodies.

1.4 TOWARDS AN INTEGRATIVE VIEW OF AUTOIMMUNE DISEASE

1.4.1 Immune regulatory circuits: Idiotypic network

The network theory of the immune system as described initially by Jerne (1974) postulates functional interactions between immunocompetent cells in the absence of antigen. It thus predicts the internal activity of the immune system through idiotype-antiidiotype recognition (Coutinho 1980), as has recently been demonstrated in 'antigen-free' mice (Pereira et al., 1986).

The finding that autoantibodies of various origin carry cross-reactive idiotypes (Cooke and Lydyard, 1981; Zanetti, 1985) suggests that they may be under network influence which can be imagined to have either enhancing or suppressive effects on an ongoing autoimmune response. In EAT for example, D8 and P4 are predominant idiotypes on anti-Tg autoantibodies in the sera of

unrelated mice with EAT (Male et al., 1983). In humans Tg autoantibodies carrying not only cross-reactive but also private idiotypes have been demonstrated in Hashimoto's sera (Matsuyama, Fukumori and Tanaka, 1983; Delves and Roitt, 1984).

Many experiments have used idiotypes or antiidiotypes as surrogate antigens in immunization protocols, achieving autoimmunization or tolerization depending on the model system. Mice and rats immunized with anti-idiotype developed de novo autoantibodies to Tg, suggesting that such anti-idiotypes are per se capable of inducing autoimmunity and perpetuating ongoing autoimmune reaction (Zanetti, Rogers and Katz, 1984). On the other hand, heterologous anti-idiotypic antibodies, cross-reacting with BUF rat Tg autoantibody, when repeatedly injected into sublethally irradiated BUF rat with autoimmune thyroiditis caused a significant decrease in the levels of circulating Tg autoantibodies (Zanetti and Bigazzi, 1981). Also, immunization of mice with Tg specific T cells, attenuated by irradiation, vaccinates against the induction of EAT by both the intact cell line and mouse Tg + CFA (Maron et al., 1983). Furthermore, BALB/c mice first immunized with anti-idiotype antibodies and immediately challenged with Tg produce significantly lower amounts of autoantibodies than controls (Zanetti, Rogers and Katz, 1984).

Mimicry of hormones and hormone receptors by idiotypic or antiidiotypic antibodies has been hypothezised as a possible mechanism underlying autoimmune disease (Cooke, Lydyard and Roitt, 1984). With regard to thyroid autoimmunity anti-TSH receptor antibodies have been implicated in the pathogenesis of

Grave's disease. Such anti-receptor antibodies could be experimentally induced with anti-TSH antibodies (Gaulton and Greene, 1986).

In summary, the ability of idiotypic and antiidiotypic antibodies to manipulate the immune system supports the notion of a functional idiotype network. Often, however, the outcome of these experimental manipulations remains unpredictable, underlining the complexity of this regulatory system.

1.4.2 Hormone modulation of the immune response

Although the autoimmune process has mainly been investigated within the framework of an autonomous immune system, there is an increasing amount of information available stressing influences of other systems, particularly the endocrine system on the immune response. In fact, autoimmune diseases are more common in females and data from animal models of autoimmune thyroiditis indicate that progesterone enhances the disease whereas oestrogen partially suppresses it (Ansar Ahmed, Young and Penhale, 1983). The protective effect of male hormones is infered from the observation that orchidectomy potentiates the development of EAT in thymectomized and irradiated rats whilst testosterone administration to these animals reverts the effect of the orchidectomy (Ansar Ahmed and Penhale, 1982). Similar results were described by Okayasu, Kong and Rose (1981) in mice castrated and treated with 5-α-dihydrotestosterone. The effects of sex hormones are also reported in other experimental models of

autoimmunity like the NZB/NZWF_1 mice, an animal model for systemic lupus erythematosus (SLE) (Roubinian et al., 1978) and the NOD mice, a model of insulin dependent diabetes mellitus (IDDM) (Makino et al., 1981).

Other hormones also modulate the course of many autoimmune diseases. In rats immunized with Tg + CFA, continuous administration of thyroxine reduced the intensity of thyroiditis (Hassman et al., 1985). Similarly, in the OS chickens high dose thyroxine supplementation has been reported to cause a significant decrease in the severity of SAT (Sundick et al., 1977). Moreover, OS chickens afflicted with SAT display low concentrations of free, hormonally active serum glucocorticoid due to a marked increase in a corticosteroid binding globulin. Additionally, in vivo treatment of OS chickens with glucocorticoid hormones, leading to an increase of the level of active hormone normalizes the T cell hyperreactivity and significantly reduces the thyroid infiltration (Fässler et al., 1986). Since these chickens do not show the transient increase in serum glucocorticoid that normally follows administration of antigen (Schauenstein et al., 1987), this points to a primary defect in an immunoendocrine feedback loop which plays a role in their immune hyperreactivity (Krömer et al., 1988).

Pituitary hormones have also been implicated in the evolution of autoimmune processes. In Graves' disease the mitogenic response of peripheral blood lymphocytes (PBL) to PPD is suppressed, in a dose dependent manner, after incubation with βTSH (Wenzel et al., 1981). The finding of prolactin receptors on human

PBL (Russell et al., 1985) together with a dampening effect of the anti-prolactin agent bromocriptine on the immune response (Talal and Ansar Ahmed, 1987), suggests that prolactin may play a role in the regulation of cell-mediated immunity. In the clinical context of SLE, Lavalle et al., (1987) have shown high levels of prolactin in male patients and reported that treatment for SLE with chloroquine also reduces these prolactin levels.

Although information about the precise mode of action of these hormones on lymphocytes is still lacking, the available data suggest that hormone intervention may be therapeutically promising in the control of autoimmune diseases by directly modulating immune function.

1.5 AIMS OF THIS STUDY

The mechanisms leading to the destruction of the thyroid gland observed in human autoimmune thyroiditis are not yet understood. Since in man an experimental approach to the problem has limitations due to ethical considerations, animal models were used in an attempt to study the contribution of humoral and cellular immune reactions in the pathogenesis of autoimmune thyroiditis.

Genetic factors play a role in the development of murine thyroiditis. A further objective of the study was to examine the genetic basis of susceptibility and establish its linkage to the rat major histocompatibility complex (RT.1) (Chapter 3).

A solid phase radioimmunometric method was chosen to quantitate anti-thyroglobulin autoantibody titres and define epitope specificity of spontaneously arising as well as induced autoantibodies in the serum (Chapters 3 and 4).

The cellular infiltrates in the affected glands were phenotypically characterized using monoclonal antibodies in single and double immunofluorescence staining on serial cryostat sections (Chapters 5 and 6).

The expression of MHC class II molecules on thyroid epithelial cells, implicated to play a key role in the mechanisms leading to autoaggression, was analysed using a rat thyroid epithelial cell line (FRTL-5) in vitro as well as histology of thyroid glands from diseased animals (Chapter 7).

Finally, immunization protocols for induction of autoimmune thyroiditis in non-obese diabetic mice were standardized and a tolerization procedure established (Chapter 8)

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Animals

Inbred strain of rats

- Female inbred rats of the Cesarian Derived Fisher (CDF) ($RT.1^l$), August (AUG) ($RT.1^c$), Lewis (LEW) ($RT.1^l$) and Albino Oxford (AO) ($RT.1^u$) strains were purchased from OLAC, Bicester, U.K.
- Male and female strains of PVG-$RT.1^a$, PVG-$RT.1^c$ and PVG-$RT.1^u$ rats congenic for different haplotypes at the RT.1 locus were also obtained from OLAC.
- Male and female (PVG-$RT.1^c$xPVG-$RT.1^a$)F_1 and PVG-$RT.1^{a/c}$ recombinants at the RT.1 locus were kindly provided by Dr Geoff Butcher, Institute of Animal Physiology and Genetic Research, Cambridge, U.K.
- BB/E ($RT.1^u$) inbred rats were obtained from the Edinburgh colony of BB rats.

Apart from the BB/E, the animals were used for the induction of Experimental Autoimmune Thyroiditis (EAT), and were 4-6 weeks of age at the start of experiments since this is the procedure adopted by most other groups. They were also acclimatized to our animal holding facilities for at least one week before immunization.

Outbred rats

Sprague-Dawley rats for thyroglobulin preparation were from a breeding colony maintained at the Middlesex Hospital Medical School.

Mice

Male and female non-obese diabetic (NOD) mice were obtained from Dr E. Simps0n, CRC, Northwick Park, and used at 3-4 months of age. CBA/Ca and CBA/J mice were purchased from NIMR, Mill Hill, London. (NODxCBA)F1 were bred in our animal facility.

Rabbits

The female New Zealand White rabbit for raising the rabbit anti-rat whole immunoglobulin used in the immunoradiometric assay for anti-rat thyroglobulin autoantibodies was bred in our animal facility and was about 12 months old when the experiment was carried out. Apart from the BB rats some of which developed insulin dependent diabetes mellitus, the animals looked healthy and they were free from infections.

2.1.2 Sera and antisera

- Normal Mouse Serum (NMS), Normal Rabbit Serum (NRbS) and Normal Rat Serum (NRtS) were obtained from animals not submitted to any immunisation procedure. They were used to preblock Fc receptors on the cryostat sections examined by indirect immunofluorescence. NRtS and NMS were also used as normal control sera in all experiments designed to estimate the anti-Tg autoantibody activity.

- BB/E sera were obtained from the Edinburgh BB colony and were a kind donation of Dr A. Bone (Edinburgh, U.K.).

- Sera from BB Hybrids (BUF x BB and PVG.r8 x BB) as described by Colle, E. et al., 1985 were kindly provided by Dr E. Colle, Montreal, Canada.

- Mouse monoclonal antibody to human thyroglobulin was generously provided by Dr P. Shepherd, Guy's Hospital Medical School, London.

- Rabbit anti-rat gammaglobulin was prepared according to the technique described by L. Hudson and F. Hay as detailed in Methods.

- Fluorescein-conjugated rabbit anti-mouse immunoglobulin (Rb α M Ig^{*Fl}) was purchased from DAKO, High Wycombe, Bucks, UK.

- Fluorescein-labelled sheep anti-human immunoglobulin (Sh α H Ig^{*Fl}) was purchased from Wellcome Laboratories Ltd, Dartford, UK.

- Fluorescein-conjugated rabbit anti-rat immunoglobulin (Rb α Rt Ig^{*Fl}) was labelled as described in Methods.

- Apart from the ED.1 and the ED.2, the panel of mouse monoclonal anti-rat antibodies defined in Table 2.1 was a kind gift of Dr D. W. Mason (Sir William Dunn School of Pathology, Oxford, U.K.).

- ED.1 and ED.2 mouse monoclonal anti-rat antibodies whose specificities are shown in Table 2.1 were purchased from Serotec Ltd, Oxford, UK.

- Antiserum to the human microsomal antigen was obtained from a patient with Hashimoto's thyroiditis (positive at 1:160[2] dilution).

- Biotinylated horse anti-mouse immunoglobulin was purchased from Vector, Burlingame, California, USA.

- The mouse monoclonal antibody to dinitrophenol (αDNP) used as an

irrelevant monoclonal antibody in the indirect immunofluorescence procedures was kindly provided by A.-M. Varey.

CLONE	ISOTYPE	WORKING DILUTION	CD-NUMBER	SPECIFICITY
OX-6	IgG1	1:2		CLASS II (I-A HOMOLOGUE)
OX-8	IgG1	1:2	CD8	T CYTOTOXIC/SUPRESSOR, NK
OX-12	IgG2a	1:2		IG KAPPA LIGHT CHAIN
OX-17	IgG1	1:2		CLASS II (I-E HOMOLOGUE)
OX-18	IgG1	1:2		CLASS I
OX-19	IgG1	1:2	CD3	PAN T LYMPHOCYTE
OX-39	IgG1	1:8	CD25	IL-2 RECEPTOR
OX-42	IgG2a	1:30	CD21	C3b RECEPTOR
W3/25	IgG1	1:2	CD4	T HELPER, MACROPHAGES
ED.1	IgG1	1:500		MONOCYTE AND MACROPHAGES
ED.2	IgG2a	1:500		TISSUE MACROPHAGES
DNP	IgG1	1:2		DINITROPHENOL

TABLE 2.1: MOUSE MONOCLONAL ANTIBODIES.

2.1.3 Antigens

- Mouse, bovine, chicken, dog, guinea-pig, human, pig, rabbit, reindeer, wallaby and zebra thyroglobulins were a kind gift of Dr Brian R. Champion.
- Human thyroglobulins containing different molar ratios of iodine as defined by their thyroxine (T_4) content were generously provided by Peter Byfield.

- Rat thyroglobulin was extracted and purified from pooled thyroids of the Sprague-Dawley rat strain as described in Methods.
- Rat gamma globulin for the preparation of the rabbit anti-rat gamma globulin was purchased from the Sigma Chemical Company, Poole, Dorset, UK.

2.1.4 Adjuvants

- Complete Freund's Adjuvant (CFA) which was used in the immunization procedure for the induction of Experimental Autoimmune Thyroiditis (EAT) was supplied by Difco Laboratories, Detroit, Michigan, USA.
- Bordetella Pertussis (killed bacilli) included in the protocol for the induction of (EAT) was obtained from Wellcome Laboratories Ltd, Beckenham, UK.

2.1.5 Solutions

All solutions were routinely made up in singly distilled water and AnalR grade chemicals were used where possible.Those solutions likely to undergo bacterial degradation were stored at 4°C for short periods of time. The composition of the solutions used frequently in the protocols are detailed as follows:

- Phosphate Buffered Saline (PBS): Stock solution 0.15M pH 7.2.

  Sodium chloride (NaCl) - 80 g l^{-1}

  Potassium chloride (KCl) - 2.0 g l^{-1}

  Di-sodium hydrogen orthophosphate (Na_2HPO_4) - 11.5 g l^{-1}

Potassium dihydrogen orthophospate (KH_2PO_4) - 2.0 g l^{-1}

This volume was diluted up to 1:10 immediately before use to obtain the PBSx1 solution. Additionally it was supplemented with Sodium Azide (0.05%) plus Tween 20 (0.05%) to get PBS/T and then plus Bovine Serum Albumin (0.5%) to get PBS/BT. The PBSx1 solution was also supplemented with Formalin (Formaldehyde solution - 37.41% w/v) to obtain the 10% PBF solution used as fixative for tissue specimens.

- Borax Phosphate Buffer pH 7.0

  Solution A - Borax buffer 0.05 M

  Solution B - KH_2PO_4 0.1 M

Solution B was added to solution A to reach pH 7.0

- Toluidine-Blue Staining Solution

Toluidine-blue (1%) was solublised in borax phosphate buffer and filtered immediately before use for staining cryostat sections.

- Glycine Buffer pH 8.6

  Glycine - 14 g l^{-1}

  Sodium Chloride (NaCl) - 17 g l^{-1}

  Sodium Hydroxide (NaOH) - 0.7 g l^{-1}

  Sodium Azide (NaN_3) - 1.0 g l^{-1}

- Mounting Medium

  Diazabicyclo-octano (DABCO) - 2.5 g

  Glycine buffer pH 8.6 - 30 ml

  Glycerol - 70 ml

DABCO was dissolved in the glycine buffer and the pH readjusted to 8.6 with concentrated HCl. Then the solution was diluted dropwise in glycerol with magnetic stirring.

- Saturated Ammonium Sulphate (SAS)

Ammonium sulphate $(NH_4)_2SO_4$ - 1000 g

PBS x 1 - 1000 ml

Ammonium sulphate was dissolved at 50 °C with magnetic stirring and allowed to stand overnight at room temperature. Then the pH was readjusted to 7.2 with 1M ammonium hydroxide or 1M sulphuric acid.

2.1.6 Chemicals and biological reagents

- Iodogen for radioiodination of antibodies was supplied by Pierce Chemical Company, Rockford, USA.
- L-thyroxine for the inhibition assays was purchased from Sigma Chemical Company, Poole, Dorset, UK.
- Hypnorm (Fentanyl citrate 0.315 mg/ml + Fluanisone 10 mg ml^{-1}) used as anaesthetic by subcutaneous injection (0.25 ml Kg^{-1} body weight) for immunization procedures was obtained from Janssen Pharmaceutica - Kent, UK.
- Temgesic, which is Buprenorphine 0.3 mg ml^{-1} and was used as an analgesic, was given by subcutaneous injections (0.1 mg Kg^{-1} body weight) and was purchased from Reckitt & Colman, UK.
- Rat liver acetone powder for the adsorption of antisera was obtained from Sigma Chemical Company, Poole, Dorset, UK.
- Bovine serum albumin used for blocking nonspecific binding sites on immuno-assay coated plates was purchased from Sigma Chemical Company, Poole, Dorset, UK.
- Rhodamine 600 avidin D for immunofluorescence staining was

supplied by Vector Laboratories Inc., Burlingame, California, USA.

- Fluorescein isothiocyanate for the preparation of fluorochrome conjugated antisera was supplied by BDH Chemical Ltd., Poole, UK.

- Toluidine blue O used as histochemical stain in order to screen cryostat sections for immunofluorescence procedures was purchased from Sigma Chemical Company, Poole, UK.

- Calcium ionophore A23187 used in FRTL-5 experiments was purchased from Sigma, Poole, UK.

- Dibutyryladenosine cyclic monophosphate $(Bu)_2$cAMP used in FRTL-5 experiments was purchased from Sigma, Poole, UK.

- Long acting thyroid stimulator was a lyophilized standard preparation (LATS-B, coded 65/122) supplied by the National Institute for Biological Standards and Controls, London, UK.

- Recombinant interferon-gamma (rIFN-γ) was a generous gift of Dr P.H. Van der Meide, Primate Centre, TNO, Rijswijk, The Netherlands.

- Acridine Orange for counting cells was purchased from Raymond A. Lamb, Middlesex, UK.

- Ethidium Bromide for counting cells was purchased from Sigma Chemical Company, Poole, UK.

- Diazabicyclo-Octano (DABCO) used for avoiding fluorescence fading was obtained from Aldrich, Gillingham, Dorset, UK.

- D.P.X. mountant used for mounting H/E sections was supplied by BDH Chemical Ltd., Poole, UK.

2.1.7 FRTL-5 Reagents

- The original stock of cultured FRTL-5 cells were kindly supplied by Dr. S. Bidey, Manchester.
- Newborn fetal calf serum was supplied by Gibco, Paisley, Scotland.
- Fetal calf serum was supplied by Gibco, Paisley, Scotland.
- RPMI 1640 medium with L-glutamine was supplied by Gibco.
- Hank's balanced (Ca^{+2} / Mg^{+2} free) salt solution (HBSS) was supplied by Gibco.
- Coon's modified Ham's F-12 medium was supplied by Gibco.
- Minimal Essential Medium (MEM) non-essential aminoacids was supplied by Gibco.
- Insulin, Hydrocortisone, Glycyl-L-histidil-L-lysine (GHL) acetate and Somatostatin were supplied by Calbiochem, Cambridge Bioscience, Cambridge, UK.
- Bovine Thyroid Stimulating (TSH) hormone was obtained from Armour Pharmaceuticals, Eastbourne, UK.
- Penicillin / Streptomycin was supplied by Gibco.
- Ethylenediaminetetra-acetic acid (Na_2-EDTA) was obtained from BDH Chemicals Ltd, Poole, UK.

2.1.8 Other Materials

- Sodium [^{125}I]iodide for antibody labelling was supplied in radioactive doses of 10 mCi/0.1 ml by the Radiochemical Centre, Amersham, England.

- Ektachrome 200 film for fluorescence photomicrography was purchased from Kodak Ltd, UK.

- Plastic disposable products specified in brackets were obtained from the following manufacturers:

Sabre International Products Ltd, Reading, England (sterile syringes).

Sarstedt, West Germany (microfuges and LP3 tubes).

Sterilin Ltd, Feltham, England (titertex-96 well polyvinylchloride round bottomed microtiter plates, universals, bijous and pipettes).

Nunc, Denmark (microtitre 24 well flat bottomed plates and polystyrene10 cm diameter petri dishes)

Gelman Sciences, UK (acrodisc filters 0.2 μM)

- Multispot microscope slides were purchased from Hendley, Essex, England. Prior to use they were routinely soaked in 10% quadralene "2000" (Fison, Scientific Equipment Division, Loughborough, UK) and then rinsed thoroughly in tap-water and distilled water. The same procedure was adopted with all other glassware using common detergents.

2.2 METHODS

2.2.1 Preparation of rat thyroglobulin

Pooled thyroids from Sprague-Dawley rats were homogeneised with PBS x1 using an ultra-turrax homogeneiser. The homogenate was centrifuged at 4°C for 10 minutes at 5500 g and the supernatant filtered through a cotton wool mesh. The thyroid extract was then fractionated by sequentially salting-out at 37% and 45% saturated ammonium sulphate (SAS) pH 7.2.

To give 37% saturation a volume of SAS (= 0.587 x Volume of extract) was added dropwise, on stirring, at room temperature (RT) and the final solution was allowed to stand at RT for about 30 minutes. The precipitate was then spun off (5500 g for 10 minutes at 4°C), the supernatant removed and brought to 45% SAS (= 0.1455 x Volume of supernatant) under the same conditions as above.

The thyroglobulin recovered on the second fractionation was washed in 45% SAS and finally solublised in PBS. This solution was then dialysed against 3 changes of PBS (for 4, 16 and 24 hours each) at 4°C and the protein content was estimated by optical absorbance at 280 nm using a UV spectrophotometer.

The final preparation was analysed by SDS-PAGE (Laemmli, 1970) using a 4.5% acrylamide stacking gel and a 7.5% acrylamide separation gel. Electrophoresis was performed at 10 mAmp for exactly 3.5 hours. Gels were stained with 0.025% Coomassie Brilliant Blue-R (Sigma, Poole, UK) in 50% (v/v) methanol and 5% (v/v) acetic acid in singly distilled water and destained using 10%

acetic acid. The loading dose was 5µg of protein. The predominant component was 330,000 MW and on destaining appeared to be a doublet. This represents most likely the reduced thyroglobulin glycoprotein subunits which may vary in their carbohydrate moieties (Ronin et al., 1986). Some of the thyroglobulin was not reduced and ran at 660,000 MW. No proteins of lower molecular weight which might correspond to immunoglobulin molecules or immunoglobulin light or heavy chains were seen (Fig. 2.1). Furthermore, contamination with IgG was undetectable when the product was tested in the ELISA with $F(ab)'_2$ anti-rat IgG.

To obtain the concentration of thyroglobulin [Tg] in mg ml^{-1} we used the formula:

$$[Tg] = E_{280} \times F \times L$$

where:

E is the absorbance at 280 nm (optical density)

F is the factor for thyroglobulin (F = 1.0)

L is the length of cell in centimeter

The amount of thyroglobulin recovered per rat thyroid was approximately 1.3 mg and was stored as frozen 1 ml aliquots at 10 mg ml^{-1}.

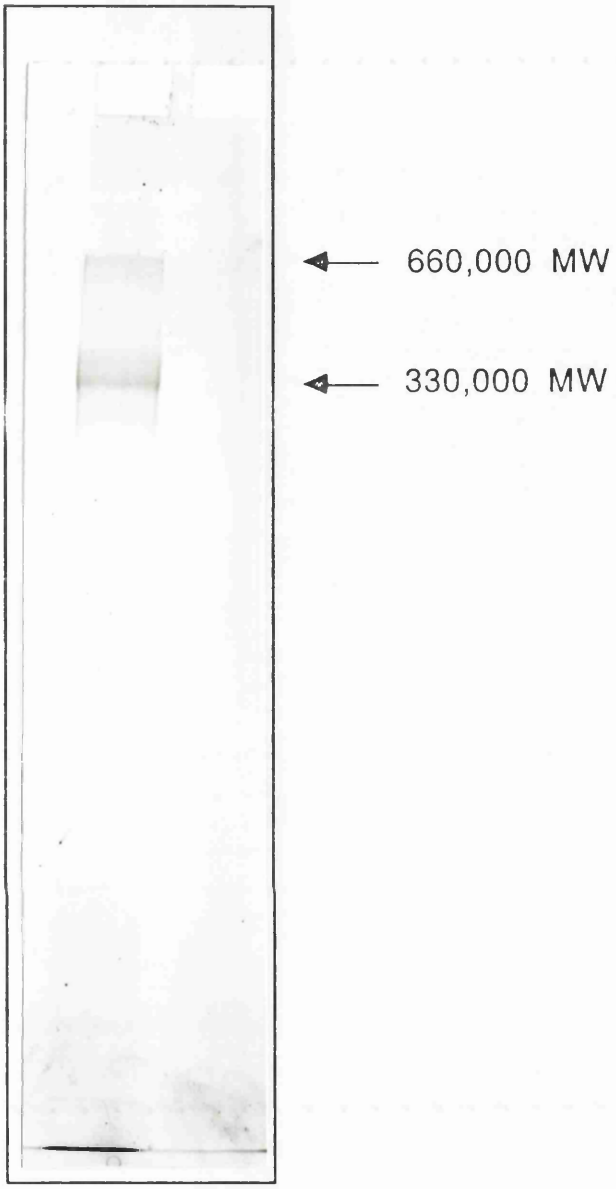

FIGURE 2.1: SDS-PAGE ANALYSIS OF THE THYROGLOBULIN PREPARATION. Loading dose 5µg protein, 7.5% acrylamide separation gel. The main component at 300,000 MW corresponds to the thyroglobulin subunits, the band at 660,000 MW is unreduced thyroglobulin.

2.2.2 Immunization protocols

Preparation of whole rabbit anti-rat immunoglobulin

A female New Zealand White (NZW) rabbit of about 12 months old was injected with approximately 2 ml of an emulsion of rat gammaglobulin (500 µg in 1 ml PBS) in Freund's Complete Adjuvant (CFA) volume : volume.

The protein was added to the adjuvant dropwise and emulsified by repeated aspiration through the needle of a syringe until a white cream emulsion was obtained. Approximately 1 ml of this emulsion was given twice with two weeks interval by intramuscular injection into each hindquarter of the rabbit.

Two weeks later a 5 ml sample test bleed from the peripheral ear vein was obtained. The blood was allowed to clot at 37°C for 1 hour and then allowed to retract overnight at 4°C. The serum was then tested for its antibody activity by interfacial ring test and by double diffusion in two dimensions (Ouchterlony's method).

Induction of rat-EAT

Experimental Autoimmune Thyroiditis (EAT) in rats was induced by a modification of the method of Lillehoj, Beisel & Rose (1981). Rat thyroglobulin in PBS was emulsified with an equal volume of CFA and a 200 µl volume of emulsion containing 2 mg of rat thyroglobulin was injected as divided doses in the four footpads on each of days 0 and 7. On day 0 only, 8×10^8 killed pertussis bacilli were also injected but in the dorsal side of the footpad. All injections were routinely preceded by subcutaneous administration

of 75 µl Hypnorm given as anaesthetic and followed immediately by 100 µl of the analgesic Temgesic given by the same route.

Animals were generally sacrificed at day 21 under carbon dioxide atmosphere. In some experiments as noted, the day of sacrifice and the thyroglobulin dose were also varied.

Induction of mouse-EAT

CBA/Ca, (CBAxNOD)F1 and NOD mice were immunized with 50µg syngeneic thyroglobulin using various immunization protocols: 1) 50µg MTg i.v. and at the same time 20 µg LPS i.p. repeated once after seven days; 2) 50µg MTg in CFA i.p. once; 3) 25µg MTg in CFA into each hind foodpad once. In control groups MTg was replaced by PBS. For tolerization 200µg MTg in PBS was administered twice with 7 day interval into the dorsal tail vein.

Test bleeds were taken from the retroorbital sinus on day 14 of the experiment and four weeks later the mice were sacrificed under carbon dioxide atmosphere and the thyroid gland removed.

2.2.3 Determination of antibody activity and specificity

Interfacial ring test

This test was carried out to confirm that a rabbit anti-rat gammaglobulin activity was present in serum of the primed animal. A 100 µl volume of the tested serum was deposited into a small glass tube and an equal volume of 10 mg ml^{-1} of the original rat gammaglobulin solublised in PBS was carefully layered over the serum.

The positive reaction was ascertained by the detection of a precipitate between the two phases in solution.

Double immunodiffusion (Ouchterlony method)

The species and class specificities of the antiserum obtained from the rabbit primed with rat gammaglobulin was determined by immunodiffusion in agar according to the Ouchterlony technique. A volume of 2% agar melted in barbitone buffer pH 8.2 was layered onto a pre-coated slide standing on a levelled surface and allowed to set. Then the agar was punched in order to give 5 wells which were filled with the test antiserum and four known antigen activities until the meniscus just disappeared. The slides were placed in a humid chamber and incubated overnight at 37°C.

Whenever lines of precipitation were seen the gel was washed for 24 hours in PBS to remove free protein from the agar. After that the gel was transfered to a gel bond film and covered with Whatman 1 paper to dry out overnight. Finally, the gel bound film was stained with Comassie brilliant blue to develop the precipitate for the analysis of its specificity.

2.2.4 Affinity purification of antibodies

The rabbit anti-rat gammaglobulin was purified by affinity chromatography using Sepharose 4 B as matrix for immobilization of the antigen. To couple the antigen to the beads these were first activated with cyanogen bromide. Cyanogen bromide (5 ml at 50 mg ml^{-1}) was mixed with Sepharose 4 B (100 mg in 15 ml distilled water) and left for 10 min at room temperature with magnetic

stirring. The pH was maintained at10-10.5 by dropwise addition of 2M NaOH.

Activated beads were rinsed on a sintered glass funnel (with PBS) and washed into a glass beaker. Rat-gammaglobulin (5 mg ml^{-1} sepharose) was added and left rolling overnight at 4°C to allow attachment to the matrix. Unbound protein was removed by washing the beads on a sintered glass funnel and the protein concentration of the eluate was measured using a UV spectrophotometer to calculate the amount of protein bound. Finally the immunoadsorbent was packed into a 5 ml syringe barrel and equilibrated with PBS-azide.

Then 10 ml of 40% SAS precipitated rabbit anti-rat gammaglobulin (7 mg ml^{-1}) was passed through the column. A first fraction consisting of unbound protein was washed away (with PBS) until the absorbance (Uvicord at 280 nm) was below 0.1%. The bound antibody was eluted with 0.05 mM diethylamine pH 11.5. This fraction was collected into 1M Phosphate buffer pH 7.2 to achieve fast neutralization and prevent denaturation of the antibody. When the elution was completed the column was washed with PBS until the UV register returned to the baseline. The fraction containing the purified antibody was dialysed against PBS and the protein concentration estimated at 280 nm.

2.2.5 Radioiodination of proteins

The affinity purified anti-rat gammaglobulin was labelled with sodium [^{125}I]iodide by the iodogen technique. 200 µl volume of

100 μg ml^{-1} iodogen was solublised in dichloromethane in a glass tube and evaporated under nitrogen atmosphere in order to leave a thin film of iodogen in the bottom of the iodination tube. Then, 200 μg of anti-rat gammaglobulin in 200 μl PBS was deposited into the tube followed by 10 μl $Na^{125}I$. After 10 minutes incubation at room temperature with occasional gentle shaking labelled protein was separated from free radioiodide by gel filtration. This was carried out on a 10 ml sephadex G-25 column equilibrated and eluted with PBS/BT. Fractions of 250 μl were collected and those containing the initial protein peak were pooled. Radioiodide incorporation varied from 70-90% which corresponded to iodinated protein of specific activity between 4 and 6 μCi μg^{-1}.

The integrity of the ^{125}I-anti-rat gammaglobulin was tested by running a radioimmunometric assay for anti-thyroglobulin antibody using an standard experimental autoimmune serum of known activity as a positive control.

2.2.6 Surgical and tissue-processing procedures

Thyroidectomy

Animals were sacrificed under carbon dioxide atmosphere. The thyroid was approached through a ventral midline incision extending from the top of the sternum to the anterior border of the submaxillary gland. The salivary glands were separated and the pretracheal muscles divided by blunt dissection. The thyroid gland was removed in block with the larynx and the trachea. The thyroid lobes were then separated at the isthmus through a section dividing

the trachea into two halves.

This procedure allowed dissection without crushing the thyroid structure and therefore provided specimens with the integrity required for cryostat and paraffin sectioning.

Tissue-processing

The right thyroid lobes were reserved for immuno-fluorescence studies and snap-frozen in isopentane at -70°C using acetone with dry ice. Consecutive cryostat sections of 4 microns were taken along the whole lobe structure and collected onto multispot microscope slides. In 50 micron intervals, one representative section was collected separately onto eight spots slides. This was used to screen for mononuclear infiltration by staining with toluidine-blue. This procedure was always done before the immunofluorescence analysis.

The left lobes were fixed in 10% PBF, processed for routine histology and stained with haematoxylin and eosin. Sections were scored blindly for mononuclear infiltration at six different levels and graded as follows: 0 = no lesions; 1^+ = 1 -2 foci < 500 μm; 2^+ = any lesion > 500 μm or more than two discrete lesions; 3^+ = 30 - 70% of the section area infiltrated and 4^+ = > 70% of the area infiltrated.

2.2.7 Histochemical staining techniques

Haematoxylin and eosin

Paraffin embedded sections were dewaxed with xylene followed by absolute ethanol and descending grades of alcohol to water. After 20 minutes incubation on Ehrlich's acid haematoxylin, sections were washed in running tap water until blue (about 2 minutes) and differentiated in 1% HCl-70% Ethanol (about 2-3 dips). They were then washed in running tap water until blue (about 1 minute), counterstained in 1% Eosin (for 2 minutes) and differentiated in running tap water. Differentiation was examined under microscope until the desired colour was obtained. Then, sections were dehydrated through ascending grades of ethanol from 95% to 100% to ensure complete removal of water and treated with two changes of xylene to remove any trace of alcohol. Finally, sections were mounted using DPX.

Toluidine-blue

To identify the areas suitable for investigation by immunofluorescence, representative sections were stained with toluidine-blue.

Freshly cut sections were fixed in methanol for 1 minute at room temperature, dried with blotting paper and incubated with 1% toluidine-blue for about 5 minutes at room temperature. After that the sections were throughly washed out in running tap water until no more dye could be eluted and allowed to dry with cold ventilation. The unmounted sections were then immediately analysed for mononuclear cell infiltration by light microscopy.

2.2.8 Assays for anti-thyroglobulin autoantibodies

Solid phase immunoradiometric assay (IRA)

Titertex 96 well microtitre plates were coated with 10 μg ml^{-1} of thyroglobulin in PBS for 24 hours at 4°C and unbound sites blocked with 5 mg ml^{-1} bovine serum albumin, immediately before incubation with test sera. Samples were then applied to plates and serially diluted. Following a 2 hour incubation plates were washed 3 times with PBS/T and Tg-autoantibody binding was detected by adding ^{125}I-labelled affinity purified rabbit anti-rat $F(ab')_2$ at 0.4 μg ml^{-1}. Two hours later plates were washed again (3 times with PBS/T), cut up and individual wells counted on a gamma counter.

Anti-thyroglobulin autoantibody levels were expressed as the titre ($-\log_2$) giving 50% of the maximal activity of a positive standard EAT serum after correction for binding of label to control wells tested with normal rat serum, which was usually much less than values obtained at a 1:256 dilution of an average positive serum. Using this procedure a clear comparative picture of the binding ability of the antisera to thyroglobulin could be obtained. Such values always lie on the linear portion of the binding curve and hence can be evaluated by using the linear equation of the straight line ($\mathbf{y = mx + c}$).

Enzyme linked immunosorbent assay (ELISA)

Flow 96 well plates were coated overnight at 4°C with 10 μg ml^{-1} mouse thyroglobulin in 0.05 M carbonate-bicarbonate buffer pH 9.6 and were blocked before use with PBS/BT. Sera were assayed using doubling dilutions for 5 wells and a positive control was

included on each plate. Rabbit anti-mouse polyvalent immunoglobulin conjugated to alkaline-phosphatase was used at 1:500 as developing antibody. The optical density at 405 nm of each well was read on a titertek ELISA plate reader.

2.2.9 Competitive binding inhibition assay

To characterize the fine binding specificity of induced (I-TgAb) and spontaneous (S-TgAb) autoantibodies a competition assay was used to examine the ability of Thyroxine (T_4) or thyroglobulin (Tg) to inhibit antibody binding to Tg on the solid phase.

For that, rat sera containing high thyroglobulin autoantibody titres were appropriately diluted and incubated overnight at 4°C with different concentrations of T_4 (1 mg ml^{-1} to 0.1 µg ml^{-1}) or with rat Tg (0.5 mg ml^{-1} to 0.1 µg ml^{-1}). The preincubated sera were then tested by immunoradiometric assay as described above for their ability to bind at room temperature to plates coated with human TgA (T_4 = 4.0 residues per mole) or human TgC (T_4 = 0.08 residues per mole).

Normal rat serum at the same dilution served as a control in these experiments.The percentage inhibition was calculated as follows:

$$\% \text{ INHIBITION} = 1 - \left[\frac{\text{cpm with competitor - cpm control}}{\text{cpm with no competitor - cpm control}} \right] \text{X } 100$$

2.2.10 Preparation of conjugated antibodies

Fluorescein isothiocyanate (FITC) conjugation

The rabbit anti-rat antiserum was precipitated with 40% saturated ammonium sulphate. The gammaglobulin fraction was dialysed against 3 changes of 0.25M, pH 9.0 carbonate-bicarbonate buffer for 6 hours at 4°C. The protein concentration was estimated by optical absorbance at 280 nm (F for gammaglobulin = 0.69) and adjusted to 20 mg ml^{-1}. Then, fluorescein isothiocyanate (50 µg mg^{-1} protein) was added and the mixture left rolling for 16 hours at 4°C. The FITC-conjugated rabbit anti-rat IgG was separated from the free fluorochrome by passage through a G-25 sephadex column equilibrated with PBS.

2.2.11 Prevention of non-specific binding

To avoid nonspecific immunofluorescence staining, Fc receptors were blocked and the antibodies were adsorbed and titrated.

Blocking of Fc receptor binding

Nonspecific binding of the antibodies to Fc receptors on cryostat sections was blocked with an excess of normal rabbit immunoglobulin. Sections were pre-incubated with normal rabbit serum (NRbS) and all monoclonal antibodies were also diluted in 10% NRbS.

Adsorption of antibodies

To avoid nonspecific binding of the polyclonal mouse sera to cross-reactive epitopes on the rat section, conjugated-antisera were adsorbed with rat liver acetone powder (20 mg ml^{-1} antibody). The mixture was left rolling overnight at 4°C wrapped in aluminium foil and then centrifuged at 400 g for 10 minutes at 20°C. The supernatant was aliquoted and stored at -20°C until use.

The same procedure was applied to the biotinylated horse anti-mouse immunoglobulin and to the human antimicrosomal antiserum.

Antibody titration

To define the working dilution of the monoclonal antibodies titration curves on cryostat spleen sections were carried out for each specificity using the indirect immunofluorescence technique. Optimal dilutions for rat monoclonal antibodies are specified on Table 2.1.

To titrate the rabbit and horse adsorbed antisera, Ox-6 was used as primary antibody. This was incubated on spleen cryostat sections and the binding visualized with various dilutions of each conjugate diluted in PBS. In the case of the biotin-avidin system, horse anti-mouse biotinylated antibody and the avidin rhodamine conjugate were used at equal dilutions and titrated out together. Appropriate dilutions for the rabbit FITC-conjugate and the horse biotinylated antibody were 1:20 and 1:100 respectively.

A similar protocol was adopted to define the working dilution of human anti-microsomal antiserum and the FITC-conjugate used to reveal its binding to rat thyroid cryostat sections. These were 1:10 (anti-microsomal) and 1:60 (sheep FITC-conjugate).

2.2.12 Indirect immunofluorescence (IIF) on cryostat sections

To characterize the inflammatory infiltrate in EAT, consecutive 4 μm cryostat sections were stained with a panel of mouse monoclonal antibodies the specificities of which are shown in Table 2.1. For that two protocols were adopted:

Single-specificity staining

Air-dried unfixed sections were incubated with single monoclonal antibodies and the cellular distribution of binding was visualized after incubation with appropriated fluoresceinated rabbit IgG antiserum. The optimal dilutions of both primary and secondary antibodies were determined by standard titration curves.

Monoclonal antibodies were diluted in 10% normal rabbit serum and the FITC-conjugate, previously adsorbed with rat liver acetone powder, diluted in PBS. All incubations were performed at room temperature for 25 minutes with a 20 minutes washing period in PBS after each incubation.

Sections were mounted in 70% glycine buffered glycerol containing 2.5 g% of DABCO in order to retard fading during microscopy and photomicrography (Johnson et al., 1982). The fluorescence was examined and photographed using a Zeiss Photomicroscope II equipped with epifluorescence illumination.

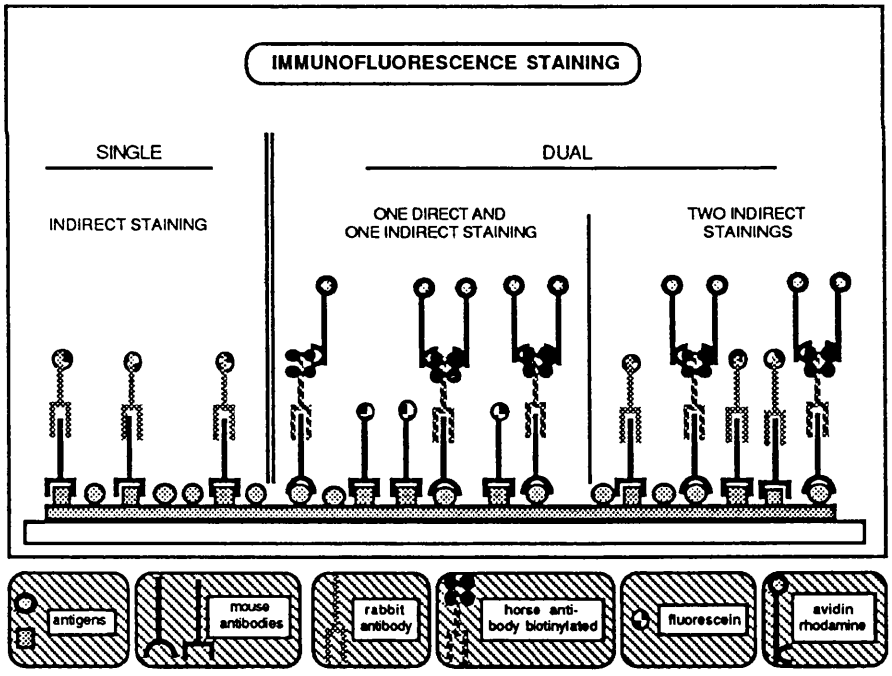

FIGURE 2.2: DIAGRAM OF SINGLE AND DUAL IIF STAINS.

Dual-specificity staining

To quantify the expression of more than one leukocyte antigen simultaneously, and also to relate mononuclear infiltration to class II, MHC antigen expression on the thyroid epithelial cells double-labelling methods were used. This method relied on staining with a first monoclonal antibody which was then detected and blocked by addition of biotinylated horse anti-mouse IgG and developed with rhodaminated avidin D. Antibody with a second specificity was then added and visualized with FITC rabbit anti-mouse IgG and FITC anti-human IgG (for human anti-microsomal antibody).

PROTOCOL	FIRST SPECIFICITY (RHODAMINE)	SECOND SPECIFICITY (FLUORESCEIN)
1	OX-8 OX-12 OX-19 OX-42 W3/25 OX-39 ED.1 ED.2	OX-6
2	OX-8	OX-19
3	OX-6	ANTI-MICROSOMAL

TABLE 2.2: DUAL-SPECIFICITY STAINING.

Three double-fluorescence antibody combinations were employed as shown in Table 2.2. The first allowed enumeration of leukocyte subsets as a proportion of Ox-6 positive leukocytes. This approach was valid since essentially the entire leukocyte infiltrate in EAT was consistently Ox-6^+. The second allowed quantitation of the Ox-8^+ (CD8) subset as a proportion of total OX-19^+ (CD3) cells and also NK cells (Ox-8^+, Ox-19^-). The third combination permitted simultaneous evaluation of epithelial class II and microsomal antigen expression using a human serum positive for anti-microsomal antibodies, which crossreacted with rat tissues.

For lymphocyte subpopulation analysis (protocol 1 and 2), rhodamine-labelled cells were expressed as the percentage of fluorescein-labelled (Ox-6 and Ox-19) cells. Counts were made in representative infiltrated areas over ten high power fields (630x), corresponding to a section area of 0.5 mm^2. To facilitate accurate cell countings 270° of the eyepiece field was occluded and the fluorescein was counted in separate single quadrants of each field.

The specificity of this double staining procedure was checked out in consecutive sections either in spleen and thyroid by incubating the secondary antibodies in the absence of the primary antibodies or by using the unrelated monoclonal antibody DNP. In addition, absence of cross reactivity between two layers was verified by omiting one of the primary antibodies in the presence of the second antibody (conjugate). Further negative controls included application of the anti-mouse FITC conjugate on sections stained with the human anti-microsomal antibody and also incubation of the

anti-human FITC as secondary antibody on sections with mouse monoclonals as primary antibodies.

2.2.13 FRTL-5 cell cultures

The cultured FRTL-5 cells are a non-transformed continuous line of epithelial cells derived from the Fisher rat thyroid. The morphology, TSH-dependent growth characteristics and functional responses of this cell line has been described previously (Ambesi-Impiobato, Parks and Coon, l980; Bidey et al., 1984; Vitti et al., 1983).

FRTL-5 cells, obtained as frozen cell suspensions, were plated and cultured to confluency over a 14 day incubation period in Coon's modified Ham's F-12 medium containing 5% newborn calf serum, 100 U ml^{-1} penicillin and 100 µg ml^{-1} streptomycin, and a hormone growth factor supplement of thyroid-stimulating hormone, TSH (10 mU ml^{-1}), Insulin (10 µg ml^{-1}), Hydrocortisone (10^{-8} mol l^{-1}), Transferrin (5 µg ml^{-1}), Glycyl-L-hystidyl-L-lysine acetate (10 ng ml^{-1}) and somatostatin (10 µg ml^{-1}).

Cells were cultured in 10 cm diameter petri dishes in a humidified atmosphere containing 5% CO2 in air in a water-saturated cell incubator at 37°C. Upon attainment of confluency, monolayers were detached from 24-well plates by exposure to PBS containing 1mg ml^{-1} BSA and 0.2 mg ml-1 disodium EDTA for 15 minutes at 37°C. For studies of MHC antigen induction, washed cell suspensions in calcium- and magnesium-free HBSS containing 1% BSA were replated on glass cover slips at a density

of 2 x 10^5 ml^{-1} in 24-well tissue culture plates (0.5 ml cell suspension per well) and exposed to test agents for 60 to 84 hours.

2.2.14 Immunofluorescence of FRTL-5 cells on coverslips

To investigate modulation of class II MHC antigen expression, FRTL-5 monolayers were grown on sterile 13-mm glass cover slips in 24 well plates in the absence of TSH and incubated for 3 days with 90 U ml^{-1} rat rIFN-γ. Aditionally, monolayers were cultured in parallel with no IFN-γ and with IFN-γ + DB-1, a mouse monoclonal antibody with specific neutralizing capacity for rat and mouse IFN-γ (Van der Meide et al., 1986). Cover slips were removed from culture wells, rinsed in BSS-1% BSA, then in RPMI (without additives) and blotted off on absorbent paper. After that, cover slips were put on support and cell monolayers were incubated with 50 µl of the Ox-6 antibody, followed by FITC rabbit anti-mouse IgG. Finally, the FRTL-5 cells were fixed with 5% acetic acid in absolute ethanol at -20°C for 10 minutes and washed in PBS . The incubations were performed at room temperature for 30 minutes with two rinses in BSS-1% BSA and RPMI after each incubation. The Ox-6 antibody and the FITC-conjugate were diluted as described before using the same diluents. Cover slips were flipped over and mounted onto glass slides with a drop of 70% glycine buffered glycerol containing 2.5% of DABCO and the edges sealed with nail varnish. Fluorescence was viewed under a Zeiss Photomicroscope II equipped with epifluorescence illumination.

2.2.15 Immunocytostaining and flow cytometry

Cell monolayers were detached from test plates by exposure to divalent cation-free buffer. The cell suspensions were washed, and exposed sequentially to a) the rat monoclonal antibodies OX-6, OX-17, OX-8 and b) to fluoresceinated rabbit anti-mouse immunoglobulin, prior to flow cytometric analysis.

Cells were analysed at 250 mW laser power in a Coulter EPICS C system and the fluorescence distribution was assessed in terms of log green fluorescence (LGFL), peak channel, and forward angle light scatter signal. In order to express antigen density in simple quantitative terms, treated cells were arbitrarily gated on the LGFL of unstained control cells (or OX-8 stained negative control cells).

2.2.16 Photomicrography

Fluorescence was photographed using a Zeiss Photomicroscope II equipped with epifluorescence illumination and Ektachrome 200 film exposed with index of 400 to 800 ASA followed by normal development. In double fluorochrome experiments double exposure photographs were taken using normal green and red filters successively.

2.2.17 Statistics

All data were expressed as the mean (X) and standard error (SE) of the results from at least 3 experiments.

Since the parameters analysed (e.g. Tg autoantibody titers, index of thyroiditis and cell counts) do not show normal destribution, the significance of differences between groups was determined using the nonparametric MannWhitney U test (Zar, 1974). Data were analysed using a Hewlett Packard (HP 41CV) calculator and the Stat Pac HP-41C.

To compare Tg autoantibody levels in different assays they were expressed as the titre ($-\log_2$ dilution) giving 50% of the maximal activity of a positive standard EAT serum. A program to calculate these titres was developed in the HP 41 CV calculator, using the linear equation of the straight line.

CHAPTER 3

INDUCED THYROGLOBULIN AUTOANTIBODIES

3.1 INTRODUCTION

Induced autoantibodies to thyroglobulin (Tg) represent one of the hallmarks of experimental autoimmune thyroiditis (EAT) although their precise role in the pathogenesis of the disease remains speculative. Evidence both in favour and against a humoral etiology for EAT is fairly abundant among different animal models (Lewis and Rose, 1985). In the rat, attempts to transfer disease with serum from immunized animals to healthy recipients has failed (Rose, Molotchnikoff and Twarog, 1973). On the other hand, Ansar Ahmed and Penhale (1981) observed a close correlation between Tg-autoantibody titres and the severity of thyroiditis following thymectomy and irradiation. However, Kotani et al., (1981), using a similar protocol, found a clear-cut dissociation between Tg-autoantibody titres and the severity of thyroid damage.

Whether or not actively implicated in the development of EAT, Tg- autoantibodies are induced in virtually all strains of rat by either immunization with thyroglobulin emulsified in Freund's complete adjuvant (CFA) (Jones and Roitt, 1961) or following thymectomy and irradiation (Penhale et al., 1973). Most of those autoantibodies are produced in the bone marrow but the spleen and the cervical lymph nodes are also involved (Weetman et al., 1982). The autoantibodies are polyclonal, of IgG class, the isotype IgG_{2a} being the most frequent (Cohen and Weetman, 1987). Observations in

the mouse model, where IgG1 subclass is predominant, suggest that the pattern of distribution between IgG subclasses does not contribute to the susceptibility to EAT (Pontes de Carvalho and Roitt, 1982) since it was similar both in high and low responder strains. Because there is no association between Tg-autoantibody titre or IgG subclass and the severity of the disease the question still remains whether differences in epitope recognition could distinguish pathogenic from non-pathogenic autoantibodies.

The autoantibody response to rat-Tg and CFA depends upon several factors. Twarog and Rose (1970), observed that the inclusion of a coadjuvant like pertussis vaccine in the Tg+CFA protocol sustained the antibody levels indefinitely. More recently, Hassman et al. (1985) reported a significant increase in the anti-Tg levels in rats following treatment with lithium chloride immediately after immunization with Tg plus CFA. Increasing age has been noted to reduce the response to the same effective immunizing dose (Weetman and McGregor, 1984). Despite all these observations the role for Tg autoantibodies in the pathogenesis of EAT has not yet been clearly defined.

Genetic factors involved clearly play a role, since inbred strains vary in their susceptibility to thyroiditis and induction of Tg-autoantibodies (Penhale et al., 1975). However, attempts to relate the autoantibody response to MHC-RT.1 alleles has not shown any linkage (Lillehoj, Beisel and Rose, 1981). Such linkage cannot be definitely ruled out, since thyroiditis induction may be affected by genes outside the MHC which obscure linkage studies. Evidence for an influence of sex hormones on disease susceptibility has been provided by the observation that female rats from both

high and low responder strains developed, higher levels of autoantibodies than the corresponding males (Lillehoj, Beisel and Rose, 1981), and administration of progesterone augments whereas oestrogen depresses the levels of anti-Tg autoantibodies (Ansar Ahmed, Young and Penhale, 1983). One approach to investigate the role of MHC linked genes would be the use of intra-RT.1 recombinant strains of rats which have only recently become available.

In this study we initially measured the autoantibody response to Tg using different doses of thyroglobulin and distinct time-points in order to establish standard conditions. These observations were correlated with severity of thyroiditis as determined by histological analysis. The experiments were undertaken in CDF inbred strain of rats and extended to three other inbred strains differing in their RT.1 haplotypes. Then we looked at the fine specificity of the autoantibodies by investigating the binding pattern of induced rat-Tg autoantibodies to a panel of thyroglobulins extracted from different species. Finally, we immunized a congenic and intra-RT.1 recombinant strain of PVG rats attempting to define the genetic location of the immune response gene to thyroglobulin in terms of both autoantibody response and thyroiditis.

3.2 RESULTS

Time course for the induction of rat-EAT

CDF inbred rats were immunized twice with 4 mg of rat-Tg in CFA given 7 days apart. Pertussis vaccine was also administered as coadjuvant at the time of the first injection. Controls included animals given CFA and CFA plus pertussis vaccine only. Groups of animals from each category were sacrificed on days 14, 21 and 28 after the first injection.

Tg autoantibodies were not detected in groups receiving adjuvants alone or in combination. On the other hand Tg autoantibodies developed at the three time-points in animals given rat-Tg + CFA. As shown in Table 3.1, anti-Tg autoantibodies were significantly higher at day 28 ($p<0.05$) but the differences in titres between the two first time-points were of no statistical significance ($p>0.05$).

TIME-POINT (DAY)	ANTI-TG TITRES	THYROIDITIS INDEX	PROPORTION WITH INFILTRATES
14	10.4 ± 0.2^{b}	0.8 ± 0.4^{c}	3/5
21	11.7 ± 0.2^{b}	3.8 ± 0.2^{a}	5/5
28	12.5 ± 0.1^{a}	3.8 ± 0.2^{a}	5/5

TABLE 3.1: THYROGLOBULIN TIME DEPENDENCE FOR THE INDUCTION OF EAT IN CDF INBRED RATS. Anti-Tg titres and thyroiditis index are expressed as X ± SE. $P<0.05$ compared to a) 14 days b) 28 days c) 21 and 28 days.

Although some animals developed small infiltrations by day 14, thyroiditis was not commonly seen before day 21 revealing that Tg-autoantibodies and thyroiditis production can be dissociated during the early stages of the EAT induction (Table 3.1). A severe thyroiditis involving mononuclear infiltration and also thyroid damage was detected on days 21 and 28, but the differences between these points were not statistically significant ($p>0.05$). In all subsequent experiments the serological and histological changes were evaluated 21 days after immunization.

Thyroglobulin dose dependence for the induction of EAT

The dose of rat thyroglobulin required for induction of EAT was established initially by immunizing CDF rats with a range of doses of rat-Tg (0.5, 1, 2 and 4 mg) given as two single injections one week apart. Animals were sacrificed 21 days after the first immunization, according to our previous observations.

RAT-TG DOSE MG	ANTI-TG TITRE	THYROIDITIS INDEX	PROPORTION WITH INFILTRATES
0.5	11.6 ± 0.2	0.3 ± 0.3 [a]	1/4
1.0	11.7 ± 0.2	1.2 ± 0.4 [b]	4/5
2.0	11.7 ± 0.1	3.0 ± 0.5 [c]	5/5
4.0	12.1 ± 0.3	4.0 ± 0.0 [d]	4/4

TABLE 3.2: THYROGLOBULIN DOSE DEPENDENCE FOR THE INDUCTION OF EAT IN CDF INBRED RATS. Anti-Tg titres and thyroiditis indices are expressed as X ± SE. $P< 0.05$ compared to [a] 2 mg and 4 mg [b] 4 mg [c] 0.5 mg [d] 0.5 mg and 1.0 mg.

High titres of Tg-autoantibodies were shown to develop in all groups. Any differences in titres between the groups were of no statistical significance ($p>0.05$). However, a positive correlation was observed between the dose of thyroglobulin administered and the index of thyroiditis as defined by the numbers of mononuclear cells present in the thyroid infiltrate. As shown in Table 3.2, a reliable thyroiditis was only seen following the injection of a total dose of 4 to 8 mg thyroglobulin ($p<0.05$). Differences in the severity of thyroiditis between these doses were not statistically significant ($p>0.05$). We therefore used a total final dose of 4 mg in all subsequent experiments for the induction of rat-EAT.

Strain differences in the response to induced rat-EAT

In order to examine strain differences in the rat response to immunization with rat thyroglobulin plus adjuvants, we used inbred strains of rats differing in MHC haplotypes. Autoantibody titres and thyroiditis indices induced by rat thyroglobulin in Albino Oxford (AO) ($RT.1^u$), Cesarian Derived Fisher (CDF) ($RT.1^l$), August (AUG) ($RT.1^c$) and Lewis (LEW) ($RT.1^l$) are shown in Table 3.3. Significantly higher titres of thyroglobulin autoantibodies were induced in AUG and CDF than in LEW rats ($p<0.05$). LEW developed significantly higher titres of autoantibodies than AO.

All AUG, CDF and LEW rats developed severe thyroiditis whereas in the AO strain the few animals which developed thyroiditis showed minimal infiltration. No significant differences in the indices of thyroiditis were seen between CDF, LEW and AUG rats. Again there was no direct relationship between the titres of

autoantibodies and the severity of the thyroiditis developed by the animals.

STRAINS-HAPLOTYPE	ANTI-TG TITRE	THYROIDITIS INDEX	PROPORTION WITH INFILTRATES
CDF - $RT.1^{l}$	11.4 ± 0.2^{b}	4.0 ± 0.0^{a}	5/5
AUG - $RT.1^{c}$	11.2 ± 0.3^{b}	4.0 ± 0.0^{a}	5/5
LEW - $RT.1^{l}$	9.9 ± 0.4^{a}	3.4 ± 0.2^{a}	5/5
AO - $RT.1^{u}$	7.1 ± 0.7^{c}	0.8 ± 0.5^{c}	2/5

TABLE 3.3: STRAIN DIFFERENCE IN THE RESPONSE TO INDUCED RAT EAT. Anti-Tg titres and thyroiditis indices are expressed as $X \pm SE$. $P < 0.05$ compared to a) AO b) AO and LEW c) LEW, CDF and AUG.

Genetic control of rat-EAT

To further analyse the MHC associated genetic factors influencing the induction of rat-EAT we used congenic PVG rats with differing MHC haplotypes ($RT.1^{c}$, $RT.1^{a}$ and $RT.1^{u}$). We also included AUG and AO rats in this study as controls. As shown in Table 3.4, animals sharing the $RT.1^{c}$ haplotype (PVG-$RT.1^{c}$, AUG) developed the most severe thyroiditis and the highest titres of thyroglobulin autoantibodies ($p < 0.05$). On the other hand, strains with the $RT.1^{u}$ haplotype (PVG-$RT.1^{u}$, AO) developed poor thyroiditis despite the presence of high titres of thyroglobulin autoantibodies in the PVG-$RT.1^{u}$ strain (Figure 3.1).

These data indicate that genes located in the RT.1 complex must influence the response to thyroglobulin, animals with the $RT.1^{c}$ haplotype being high responders whereas animals of $RT.1^{u}$ and $RT.1^{a}$ haplotypes are poor responders with respect to thyroiditis induction.

STRAINS-HAPLOTYPES	ANTI-TG TITRE	THYROIDITIS INDEX	PROPORTION WITH INFILTRATES
$PVG\text{-}RT.1^{u}$	13.2 ± 0.2^{a}	1.4 ± 0.4^{d}	4/5
$PVG\text{-}RT.1^{c}$	13.3 ± 0.2^{a}	3.4 ± 0.2^{e}	5/5
$PVG\text{-}RT.1^{a}$	11.3 ± 0.2^{b}	0.6 ± 0.4^{d}	2/5
$AUG\text{-}RT.1^{c}$	11.9 ± 0.5^{b}	4.0 ± 0.0^{e}	4/4
$AO\text{-}RT.1^{u}$	6.8 ± 1.8^{c}	0.2 ± 0.2^{d}	1/5

TABLE 3.4: SUSCEPTIBILITY TO INDUCED EAT IN A PVG-RT.1 CONGENIC STRAIN OF RATS. Anti-Tg titres and thyroiditis indices are represented as X ± SE. $P<0.05$ compared to a) AO and $PVG\text{-}RT.1^{a}$ b) AO c) $PVG\text{-}RT.1^{u}$, $PVG\text{-}RT.1^{c}$, $PVG\text{-}RT.1^{a}$ and AUG d) $PVG\text{-}RT.1^{c}$ and AUG e) AO, $PVG\text{-}RT.1^{a}$ and $PVG\text{-}RT.1^{u}$.

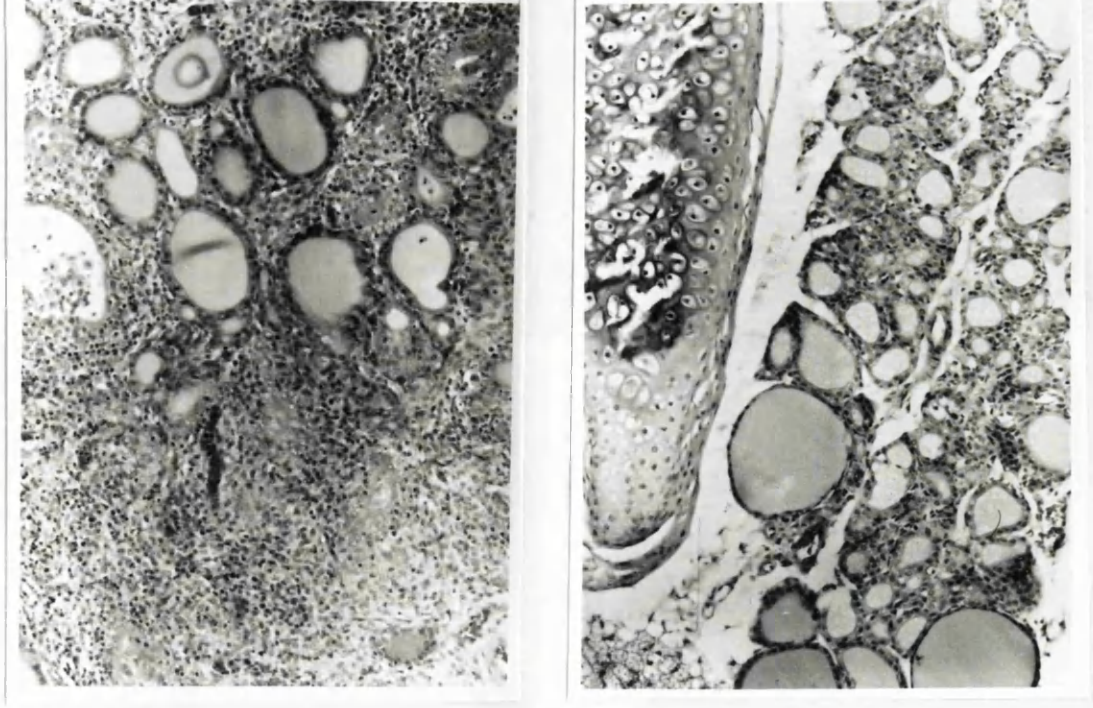

FIGURE 3.1: PHOTOMICROGRAPHS OF THYROIDS FROM RATS IMMUNIZED WITH 2 MG TG+CFA TWICE AND PERTUSSIS VACCINE. Thyroditis in the high responder PVG-RT.1c strain, illustrating diffuse lymphocytic infiltration with reduction in the number of thyroid follicles (left). Normal thyroid in the poor responder AO strain (right). H & E staining. Magnification x100.

MHC-RT.1 location of the immune response gene to Tg (Ir-Tg)

The MHC influence on the responsiveness to rat thyroglobulin was further investigated using intra-RT.1 recombinant strain of PVG rats. (PVG-RT.1^c x PVG-RT.1^a)F_1 and also PVG.R1 animals which have the $A^aB^cD^cC^c$ haplotype at the RT.1 subregions were immunized with 2 mg of Tg plus CFA twice and pertussis vaccine and evaluated 21 days after the first injection. PVG-RT.1^c AND PVG-RT.1^a male and female rats were included in this study as control for the proposed high and low responder haplotype respectively.

High titres of Tg-autoantibodies were detected in all groups investigated (Table 3.5). Female PVG.R1 developed significantly

lower titres than their sex-matched control PVG-RT.1^{a} and PVG-RT.1^{c} ($p < 0.05$). However, the differences between male animals (PVG-RT.1^{c} x PVG-RT.1^{a})F_1 and PVG-RT.1^{c} and PVG-RT.1^{a} were not statistically significant ($p > 0.05$).

STRAIN - RT.1	SEX	ANTI-TG TITRES	THYROIDITIS INDEX	PROPORTION WITH INFILTRATES
(PVG-RT.1^{c} x PVG-RT.1^{a}) F_1	M	12.0 ± 0.4	3.5 ± 0.5 ^{b}	4/4
PVG. R1	F	11.9 ± 0.4 ^{c}	3.8 ± 0.2 ^{a}	6/6
PVG-RT.1^{c}	F	13.5 ± 0.2	3.7 ± 0.3	3/3
PVG-RT.1^{c}	M	12.6 ± 0.1	4.0 ± 0.0	4/4
PVG-RT.1^{a}	F	13.3 ± 0.2	1.8 ± 0.3	4/4
PVG-RT.1^{a}	M	12.7 ± 0.2	1.5 ± 0.3	4/4

TABLE 3.5: MHC-RT.1 LOCATION OF THE IR-TG. Thyroiditis indices and anti-Tg titres are expressed as X ± SE. $P < 0.05$ compared to a) F-PVG-RT.1^{a} b) M-PVG-RT.1^{a} c) F-PVG-RT.1^{a} and F-PVG-RT.1^{c}.

Female PVG-RT.1.R1, male (PVG-RT.1^{c} x PVG-RT.1^{a})F_1 and male and female PVG-RT.1^{c} developed severe thyroiditis (Table 3.5). Significantly higher thyroiditis indices were found in (PVG-RT.1^{c} x PVG-RT.1^{a})F_1 and also PVG-RT.1.R1 as compared to their sex-matched control PVG-RT.1^{a} ($p < 0.05$). On the other hand, no significant difference in these indices were observed between

(PVG-RT.1^{c} x PVG-RT.1^{a}) F_1, PVG-RT.1.R1 and PVG-RT.1^{c} rats ($P > 0.05$).

These results confirm our previous observations that the RT.1^{c} haplotype is high responder whereas the RT.1^{a} is a poor responder in terms of EAT induction. They also indicate that the so-called immune response gene to thyroglobulin (Ir-Tg) maps to the MHC-RT.1 region.

Species binding specificity of induced Tg-autoantibodies

Tg-autoantibodies induced in AUG, CDF, LEW and AO rats by inoculation of Tg plus CFA only and Tg plus CFA and pertussis vaccine were tested for their ability to recognize a panel of thyroglobulin extracted from different species. In both protocols 2 mg of Tg were administered twice with pertussis vaccine being given with the first injection only. All test sera in the relevant groupings were shown to contain high titres of Tg- autoantibodies.

Sera obtained from either high or low responder strain of rats immunized with Tg plus CFA and pertussis vaccine revealed similar patterns of binding. They all recognized thyroglobulin from most species with a tendency to relatively lower binding to human Tg than to the other Tg species (Figure 3.2), but only in the case of rat versus human was statistical significance achieved ($p<0.05$ for all 4 species). The level of binding between strains varied according to Tg-autoantibody titres present in the sera, showing the same hierarchy of binding (AUG>CDF>LEW>AO) for all Tg species tested.

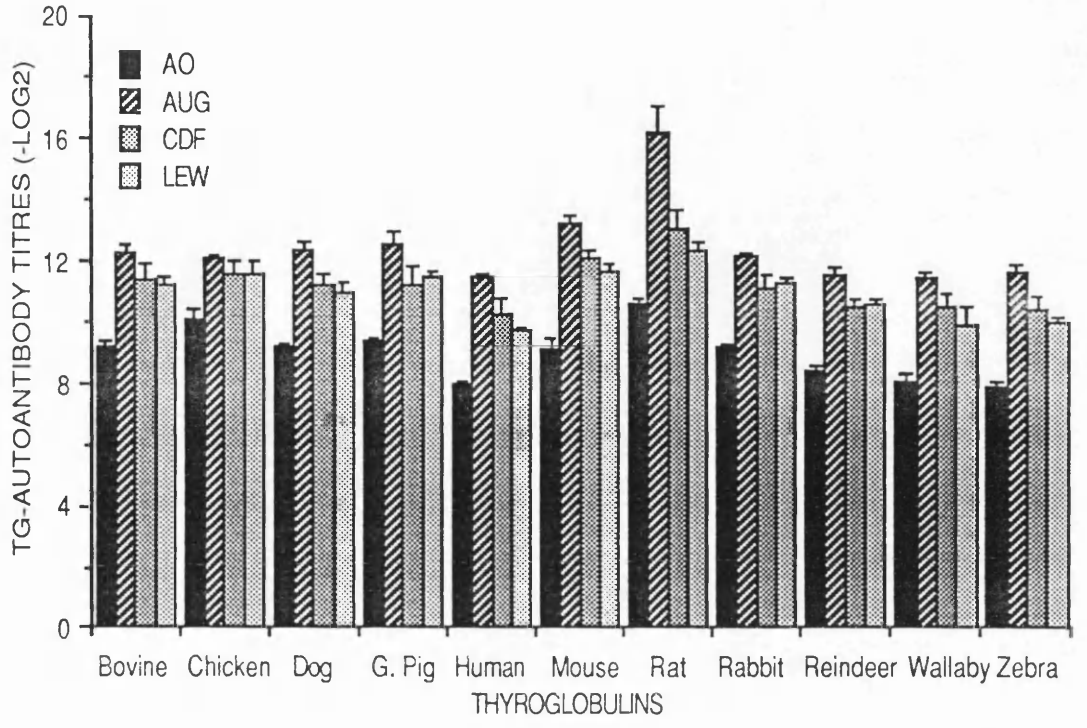

FIGURE 3.2: BINDING CAPACITY OF INDUCED TG-AUTOANTIBODIES TO A PANEL OF TG FROM DIFFERENT SPECIES. Anti-Tg titres of high and low responder sera (n=4) are expressed as titres ($-\log_2$) giving 50% of the maximum binding of a positive control EAT serum.

To see whether such binding pattern of Tg-autoantibodies was attributable to the use of pertussis vaccine in the immunization protocol, we tested sera from CDF rats immunized with Tg plus CFA alone and Tg plus CFA and pertussis vaccine. As shown in Figure 3.3, the inclusion of pertussis vaccine in the immunization protocol enhanced Tg-autoantibody titres produced but did not alter the basic pattern of recognition of the Tg panel.

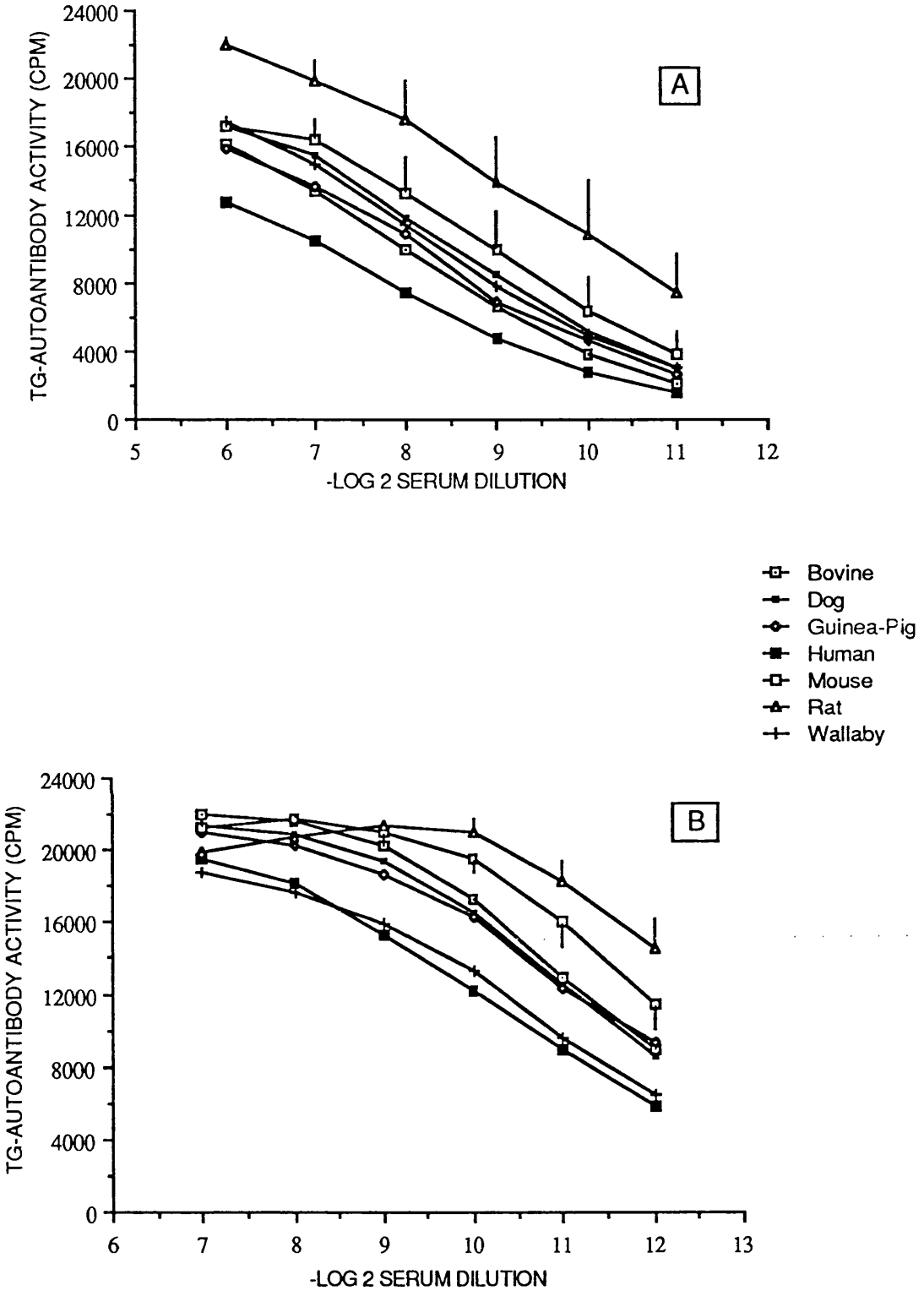

FIGURE 3.3: BINDING PATTERN OF INDUCED TG-AUTOANTIBODIES TO A PANEL OF TG. **A** and **B** show the binding profile of sera from rat-Tg/ CFA and rat-Tg/CFA+pertussis immunized rats, respectively.

3.3 DISCUSSION

Anti-Tg autoantibodies and thyroid infiltration are used to assess the induction of anti-Tg response in all animal models of experimental autoimmune thyroiditis (EAT). In rats immunized with Tg+CFA it has been shown that anti-Tg levels vary with the dose of Tg administered (Weetman and McGregor, 1984) as well as with the adjuvant (Twarog and Rose, 1969) and the strains used (Rose, 1975).

CDF inbred rats injected with 0.5, 1, 2 or 4 mg of Tg all developed high anti-Tg titres, but no statistical difference could be detected among the groups. This is in contrast with Weetman and McGregor (1984), who used Wistar rats and observed a dose dependence when injecting Tg doses ranging from 0.01 to 1 mg. Administration of pertussis vaccine simultaneously with thyroglobulin in emulsion with CFA into rats of the Lewis strain has been demonstrated to enhance the severity of EAT in terms of both thyroid damage and anti-Tg titres when compared with the injection of Tg + CFA only (Twarog and Rose, 1969). This effect may have obscured a dose response in our study. The mode of action of pertussis vaccine as adjuvant remains unknown, but it has been reported to potentiate IgG and IgE antibody response (Munoz, 1985) and also lymphocyte recirculation, resulting in lymphocytosis (Weiss and Hewlett, 1986). Also the purity of thyroglobulin used by Weetman and McGregor (1984) is not konwn, so that the doses may not be quite comparable.

Relatively severe thyroiditis has been reported as early as 10 days after immunization with rat-Tg + CFA (Jones and Roitt, 1961). Our time dependence studies for the induction of EAT in CDF

rats showed that although anti-Tg antibody titres and some thyroid damage could be detected following 14 days of immunization, thyroiditis was most reliably observed by 21 days. In our experiment we also included two additional control groups of animals given CFA or CFA+pertussis vaccine only. Neither Tg autoantibodies nor thyroid lesions were detected in either of these groups ruling out any direct effect of the adjuvants in the pathogenesis of EAT.

The amount of thyroglobulin injected markedly affected the severity of thyroid lesions. Although some thyroiditis could be induced with 1 mg of Tg, lesions were only reliably obtained following the administration of 2 or 4 mg of Tg. These differences in the dose response underline the dissociaton of Tg-autoantibody titres and thyroid lesions.

T cell and B cell responses do rely on different mechanisms of antigen presentation. It has been shown that B cells can present antigen at much lower concentration than conventional antigen presenting cells (APC's), since they take it up via their specific surface receptors (Hutchings et al., 1987). If autoreactive B cells were able to present antigen in this way to their T helper cells whilst T cytotoxic/ suppressor lymphocytes relied on conventional APC's, this might explain the difference in sensitivity observed between humoral and cellular response to Tg. This remains speculative at this moment, since it cannot be excluded that B cells can present antigen to all T cell subpopulations.

It is known that thyroglobulin is present in low amounts in the circulation (Roitt and Torrigiani, 1967), but in contrast with conditions normally used for immunization, it does not normally

induce thyroiditis. The physiological Tg concentration may be too low to trigger autoreactive B and T cells, particularly in the absence of adjuvants. Also, in the case of immunization the antigen is taken up by the peripheral immune system, whereas in the physiological situation it is secreted as a soluble protein directly into the systemic circulation, avoiding the lymph nodes.

It is known that some strains of rat are more susceptible to EAT than others (Rose, 1975). Among the inbred strains of rats that we immunized with Tg + CFA and pertussis vaccine, the incidence and severity of disease were consistently higher in the August and CDF strains with respect to both anti-Tg titres and thyroid damage. Albino Oxford rats, albeit defective in the cell mediated response, developed high titres of anti-Tg autoantibodies suggesting that in rat-EAT as well as in the mouse model (Tomazic and Rose, 1977), Tg autoantibody levels are not a reliable indicator of EAT susceptibility.

Several groups have investigated the genetic basis for high or low responsiveness to Tg. In the mouse, some experiments indicate that genes associated with the major histocompatibility complex (MHC) govern the response to Tg (Vladutiu and Rose, 1971). Tomazic, Rose and Shreffler (1974) suggested that the immune response gene to Tg (Ir-Tg) is located in the I-A subregion of the H-2 complex. In the rat, variations in Tg responsiveness among different strains have been related to the MHC haplotype of the strain (Penhale et al., 1975). However, attempts to map the Ir-Tg to the rat MHC (RT.1) have failed. Lillehoj, Beisel and Rose (1981) using an F_1 and backcross progeny of a poor and a high responder strain of rats, were unable to establish any linkage of the so-called

Ir-Tg to the RT.1 complex either with regard to Tg-autoantibody levels or thyroid damage. Furthermore, animals with the same MHC haplotype (PVG-RT.1^c and AUG) showed considerable differences in their responsiveness to Tg, on the bases of both anti-Tg autoantibody titres and the thyroiditis intensity (Lillehoj and Rose, 1982).

In this study the differential induction of thyroiditis among inbred and congenic strains of rats with different MHC-RT.1 haplotype were compared. In two separate experiments it was observed that animals with the RT.1^c haplotype (AUG and PVG-RT.1^c) developed most severe disease which correlated strongly with the anti-Tg autoantibody titres. On the other hand, rats with the haplotype RT.1^u (AO and PVG-RT.1^u) showed the lowest thyroiditis indices in spite of high circulatory anti-Tg autoantibody levels. This was particularly impressive in rats on the PVG-RT.1^u background. Our results with congenic strains, clearly show that the RT.1^c and RT.1^u haplotype MHC genes on identical genetic background, are directly associated with high and low responses to EAT induction respectively. This is in contrast with Lillehoj and Rose (1982), who could not demonstrate such a clear relationship using non-congenic strains. This implies that genes outside the MHC also influence the response to Tg.

In the mouse it has been shown that high responder status is dominant with regard to susceptibility to EAT (Rose et al., 1973). Therefore a comparative study was carried out in the rat. (PVG-RT.1^c x PVG-RT.1^a)F_1 hybrids and PVG-RT.1^c congenic rats developed similarly high indices of thyroiditis in contrast to the lowest indices observed with the PVG-RT.1^a. This suggests that

high response to Tg (RT.1^{c} linked) is dominant over low response (RT.1^{a} linked) in the inheritance of susceptibility to thyroiditis. To more precisely map the Ir-Tg genes within the MHC complex intra-RT.1 recombinant strains of congenic PVG rats recombinant for high (RT.1^{c}) and the low (RT.1^{a}) responder MHC haplotypes were used. The PVG.R1 strain which has haplotypes "c" at the B, D and C subregions and "a" at the A subregion of the RT.1-MHC complex is a high responder strain in terms of both Tg autoantibody titres and thyroditis index. This suggests that class II genes (B and D) and/or the C locus of class I MHC play a major role in the genetics of susceptibility to EAT whilst the A subregion of class I makes no important contribution. Other intra-RT.1 recombinant strains are needed in order to dissect the contribution of the B, D and C subregions respectively and also to identify the influence of other haplotypes.

Although the amount of Tg-autoantibody generated was strain dependent it did not show any relationship with MHC haplotype. Since Tg-autoantibody titres were not correlated with the responsiveness to Tg, the following questions arose: i) What is the role of autoantibody in the development of thyroiditis? ii) Does differential epitope recognition allow to distinguish pathogenic from non-pathogenic Tg-autoantibodies? To answer these questions the fine specificity of Tg-autoantibodies induced in various strains of rats differing in the RT.1-MHC haplotypes were examined by testing their binding capacity to a panel of Tg species. Differences in binding affinity between the strains did not reveal any differential epitope recognition by high and low responder sera, but simply reflected Tg-autoantibody titres.

In summary, an antibody mediated mechanism is unlikely to be the primary trigger of thyroiditis, since passive transfer studies have invariably been negative and poorly iodinated thyroglobulin induces a good antibody response but no thyroiditis (Champion et al., 1988). However antibody might enhance the severity of the cell-mediated immunity seen in later stages of the EAT. The fact that thyroiditis was never seen in the absence of high levels of Tg-autoantibodies and evidence of immune-complex deposition in the areas of thyroid damage (Clagett, Wilson and Weigle, 1974) support this notion.

CHAPTER 4

SPONTANEOUSLY ARISING RAT THYROGLOBULIN AUTOANTIBODIES

4.1 INTRODUCTION

Spontaneously arising thyroglobulin (Tg) autoantibodies have been detected in both BUF (Noble et al., 1976) and BB (Like, Appel and Rossini, 1982) rat strains as well as in hybrids between them (Colle, Guttmann and Seemayer, 1985). Autoantibodies directed against rat Tg can be demonstrated in the sera of these animals from 9 weeks of age onwards (Voorbij, Kabel and Drexhage, 1986). Evidence from the obese strain chicken indicates the need for Tg in the development of such autoantibodies (Pontes de Carvalho, et al., 1982). This suggests that recognition of Tg autoantigenic determinants by immunocompetent cells is necessary for the antibody response. The molecular nature of such determinants and their position in the overall Tg structure are not yet well characterized. Three approaches have been employed attempting to answer these questions.

Heat treatment of Tg as well as enzymic digestion have been used to determine the topology of autoantigenic determinants on Tg. Chan et al., (1987) have shown that heat treated human-Tg is less efficient in inhibiting the binding of human Tg-autoantibodies than native Tg. This suggests that Tg autoantibodies do not recognize particular linear amino-acid sequences in the Tg molecule but probably conformational determinants which are altered when the

molecule is physically denatured as has been described with other proteins (Refetoff et al., 1984). Enzymatic fragmentation of Tg also reduces the Tg antibody binding to their epitopes on native Tg (Male et al., 1985).

The second approach has been the use of monoclonal antibodies to human or mouse thyroglobulin to investigate the number of independent epitopes expressed on the molecule. Using this procedure Ruf et al. (1983) defined six antigenic regions on the human Tg. In the mouse, Kotani et al. (1985) using a set of 14 monoclonal antibodies identified five distinct patterns of cross-reactivity compatible with the existence of five antigenic regions. Furthermore, many of these antibodies not only bound to mouse Tg but also to a panel of other Tg species. Similar patterns of binding have been observed with polyclonal human Tg autoantibodies in that they recognized non species specific epitopes on thyroglobulin (Kohno, Nakajima and Tarutani, 1985). These data suggest the existence of common determinants shared between species and point towards the conclusion that autoantigenic determinants involve conserved regions of the Tg molecule.

Autoantigenic epitopes of Tg have also been mapped by cross-inhibition analysis. In these studies the binding of various populations of monoclonal and polyclonal autoreactive T and B cells specific for mouse Tg have been tested using a panel of 25 Tg species variants (Champion et al., 1987). This protocol revealed the existence of at least six different epitopes, three recognized by T cells (in association with I-A^k on antigen presenting cells) and three by B cells (monoclonal autoantibodies). Further information on the number of autoreactive epitopes on the mouse thyroglobulin

obtained with cross-blocking studies has indicated a minimum of eight autoantigenic epitopes on mouse Tg (Rose et al., 1982). Studies on spontaneous human Tg autoantibodies suggest recognition of few epitopes in human Tg (Roitt, Campbell and Doniach, 1958; Nye, Pontes de Carvalho and Roitt, 1980). This may reflect the potential human repertoire of autoreactive T or B cells or alternatively, this observation might indicate differences between spontaneously generated and induced Tg autoantibodies, since the monoclonal and polyclonal Tg autoantibodies used in the mouse studies have been raised with immunization in contrast with the situation in man.

Dietary iodine has been shown to influence the spontaneous incidence of thyroiditis in humans (Boukis et al., 1983), chickens (Bagchi et al., 1985) and rats (Allen, Appel and Braverman, 1986; Sundick et al., 1986) suggesting that iodinated tyrosines or thyroid hormones residues, Thyroxine (T_4) or Tri-iodothyronine (T_3) within the thyroglobulin molecule may be important recognition elements for autoreactive T or B cells. Autoantibodies that react with T_3 and T_4 have been demonstrated in both human thyroid disease (Byfield et al., 1982) and in thyroiditis of O.S. chickens (Brown, Bagchi and Sundick, 1985). Thus it is likely that most Tg autoantibodies recognize the highly conserved 'hormonogenic' region of Tg molecule and that the iodothyronine-reactive autoantibodies occurring in the human are generated against Tg (Pearce et al., 1981). Using displacement studies the authors have shown two classes of Tg autoantibodies. One, comprising 70% of the anti-Tg antibodies reacted with the T_4 containing region, and the other

containing the remaining 30% was directed against other parts of the Tg molecule. On the other hand, it was demonstrated by enzymatic cleavage that fragments of human Tg, which were recognized by both auto- and heterologous Tg autoantibodies were not related to the "hormonogenic" determinants on the molecule (Nye, Pontes de Carvalho and Roitt, 1980). Using cross-inhibition analysis the authors came to the conclusion that neither tyrosine nor the iodinated residues contribute to the composition of the autoantigenic determinants in human thyroglobulin.

In this chapter the cross-reactivity of spontaneously arising Tg autoantibodies and the epitope-specificities of polyclonal Tg autoantibodies was tested using a large panel of thyroglobulins from different species. The importance of the thyroxine residue as an important autoantigenic site for the recognition by autoreactive B cells was also studied. Furthermore the patterns of binding of antisera obtained from BB/E and different lines of BB hybrids was investigated in an attempt to correlate the MHC haplotype with the epitope-specificity of Tg autoantibodies.

4.2 RESULTS

Species binding specificity of spontaneously arising thyroglobulin autoantibodies (S-TgAb)

Seventy-five sera from BB hybrids and 90 sera from BB/E rats were screened for the presence of autoantibodies to rat Tg. Of these, 28 BB hybrids and 30 BB/E sera were positive for S-TgAb, giving at least 2 times the background binding of normal rat serum. Sera giving the highest binding to rat Tg were then selected for further analysis. The RT.1 genotype and the derivation of these lines of spontaneously autoimmune rats has been described elsewhere (Colle, Guttmann and Seemayer, 1985; Varey et al., 1987).

The ability of these sera to bind to a panel of thyroglobulins obtained from 12 different species was investigated. Three discrete patterns of binding emerged. The first group manifest the ability to bind to thyroglobulin of many species (Figure 4.1).

This pattern resembled that one seen with induced rat TgAb, as described in the Chapter 3. The second group which included all of the BB/E (Figure 4.2) and some of the BB hybrid sera (Figure 4.3) had a preference for binding to rat and mouse Tg with little reactivity with other species Tg. A similar binding pattern has been described in induced mouse TgAb (Champion et al., 1988).

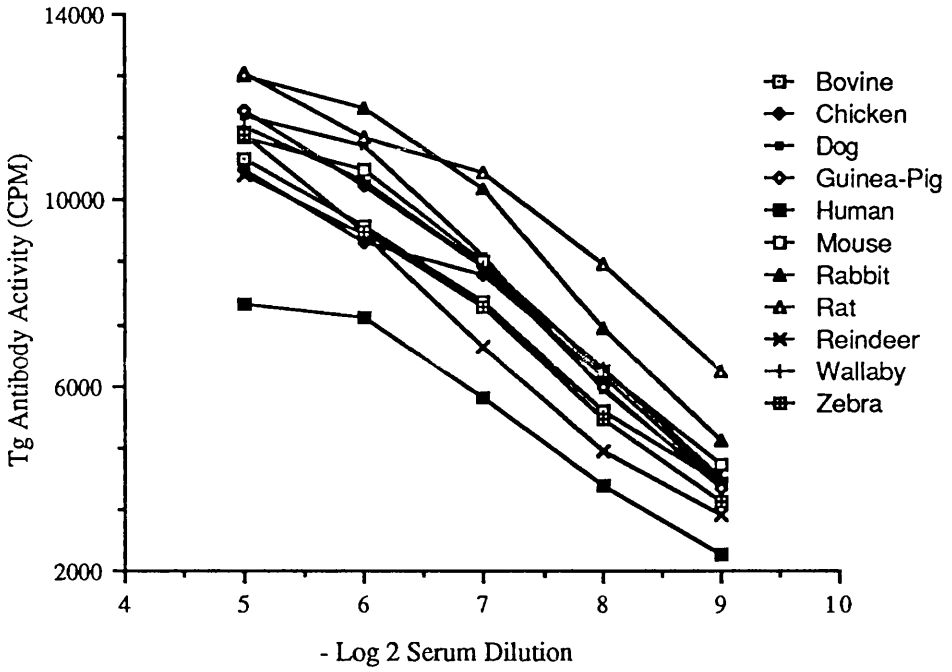

FIGURE 4.1: REPRESENTATIVE PATTERN OF BINDING OF A BB HYBRID (RT.1$^{u/u}$) SERUM FROM GROUP 1. Doubling dilution of serum were tested in a solid-phase radioimmunoassay for their ability to bind to a panel of Tg from 12 different species.Antibody binding was detected with an ^{125} I-labelled affinity purified anti-Ig rabbit anti-rat F(ab')$_2$.

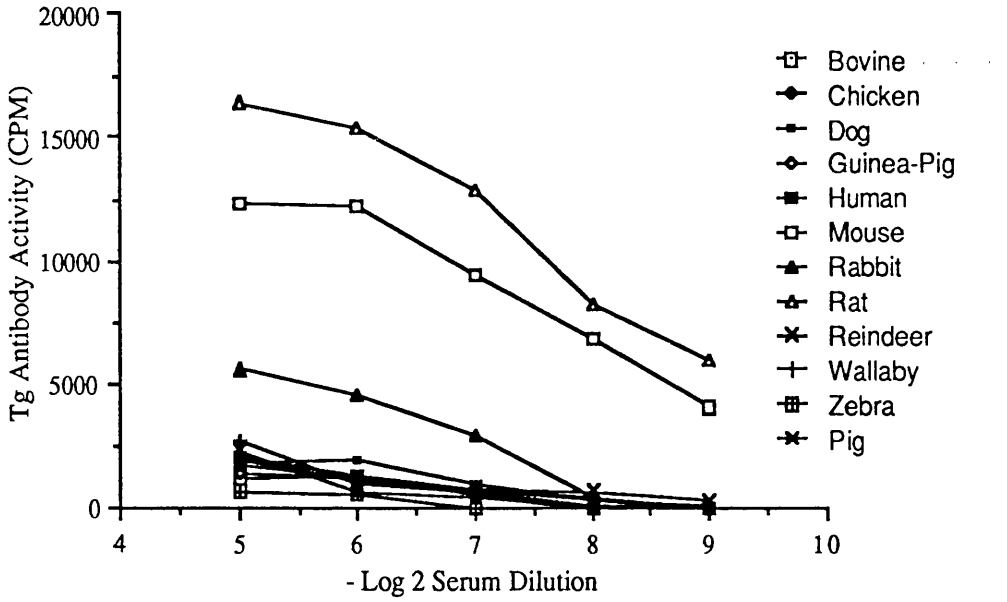

FIGURE 4.2: REPRESENTATIVE PATTERN OF BINDING OF A BB/E (RT.1$^{u/u}$) SERUM FROM GROUP 2. Serum tested in the same conditions as mentioned above.

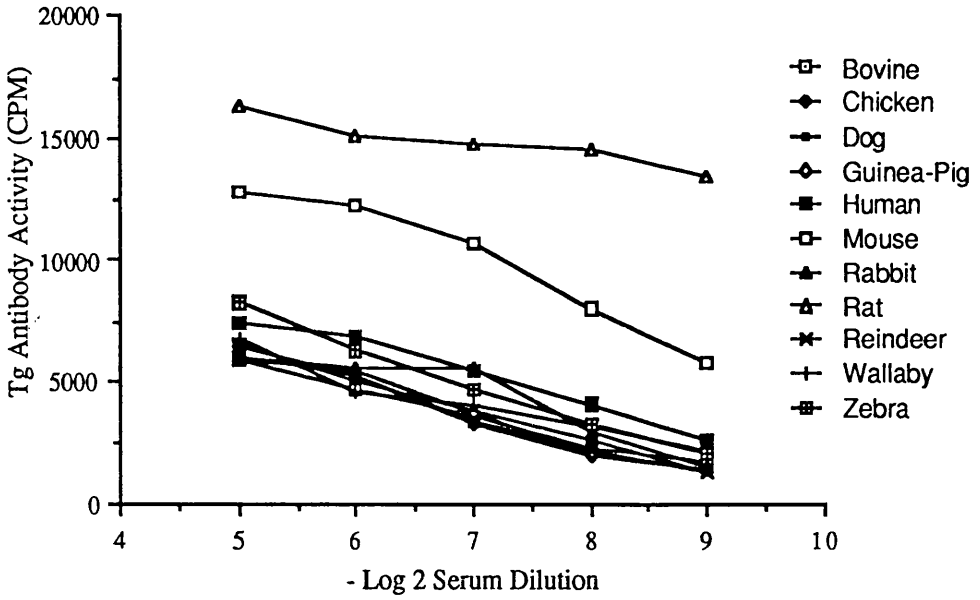

FIGURE 4.3: REPRESENTATIVE PATTERN OF BINDING OF A BB HYBRID (RT.1u/u) SERUM FROM GROUP 2. Serum tested under experimental conditions described above.

The last group contained the remaining BB hybrid sera and showed a higher degree of binding to dog than to rat Tg and Tg of other species (Figure 4.4). These results are summarized on Table 4.1.

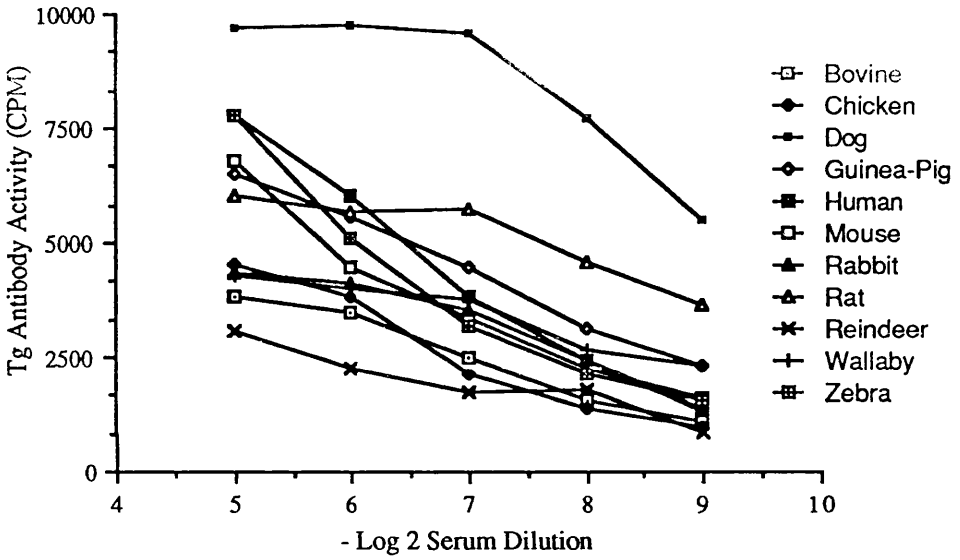

FIGURE 4.4: REPRESENTATIVE PATTERN OF BINDING OF A BB HYBRID (RT.$1^{b/b}$) SERUM FROM GROUP 3. Serum tested as above.

THYROGLOBULINS	GROUP 1	GROUP 2		GROUP 3
	BB HYBRIDS n = 5	BB HYBRIDS n = 3	BB/E n = 8	BB HYBRIDS n = 2
BOVINE	+++	+	±	+
CHICKEN	+++	+	±	+
DOG	+++	+	±	+++
GUINEA-PIG	+++	+	±	+
HUMAN	++	+	±	+
MOUSE	+++	+++	+++	++
PIG	+++	+	±	+
RABBIT	+++	+	±	+
RAT	+++	+++	+++	++/+++
REINDEER	+++	+	±	+
WALLABY	+++	+	±	+
ZEBRA	+++	+	±	+

TABLE 4.1: BINDING OF SPONTANEOUSLY ARISING TG AUTOANTIBODIES TO VARIOUS THYROGLOBULIN SPECIES. Sera from BB hybrids resulted from an original cross between either a BB male and a female BUF or PVG.r8 as described by Colle, Guttmann and Seemayer,1985. All BB hybrids in groups 1 and 2 including BB/E are RT.1^u at the class II MHC loci; animals in group 3 are RT.1^b at the class II loci. ± : 50% over the background (bg = 2500 cpm @ 1:64 dilution) ; + : 2 - 3 times bg; ++ : 3 - 5 times bg; +++ : > 5 times bg.

Relationship between species cross reactivity and thyroxine related epitopes

Although group 1 sera were able to bind to thyroglobulin from most species, they were relatively poor at recognizing human thyroglobulin. Since human Tg was routinely prepared from thyroids of thyrotoxic patients, which have abnormally low iodine content, it seemed possible that some of the antibodies in this group 1 sera

were recognizing iodination related epitopes on the Tg molecule. To investigate this possibility we studied the binding to a small panel of human Tg with different iodine contents. In this study we included an induced TgAb serum, which as described in Chapter 3 bound to the panel as Group 1 S-TgAb. Some representative results are shown in Figures 4.5 and 4.6 from which it can be seen that induced and S-TgAb from this group bind best to human Tg with the higher iodine content, Tg-A (T_4 = 4.0 residues per mole). Thyroglobulin containing the lowest molar ratio of iodine, Tg-C (T_4 = 0.08 residues per mole) being least well recognized. BB/E sera (group 2) were retested for their ability to bind human Tg but still failed to recognize human Tg irrespective of the degree of iodination (Figure 4.7).

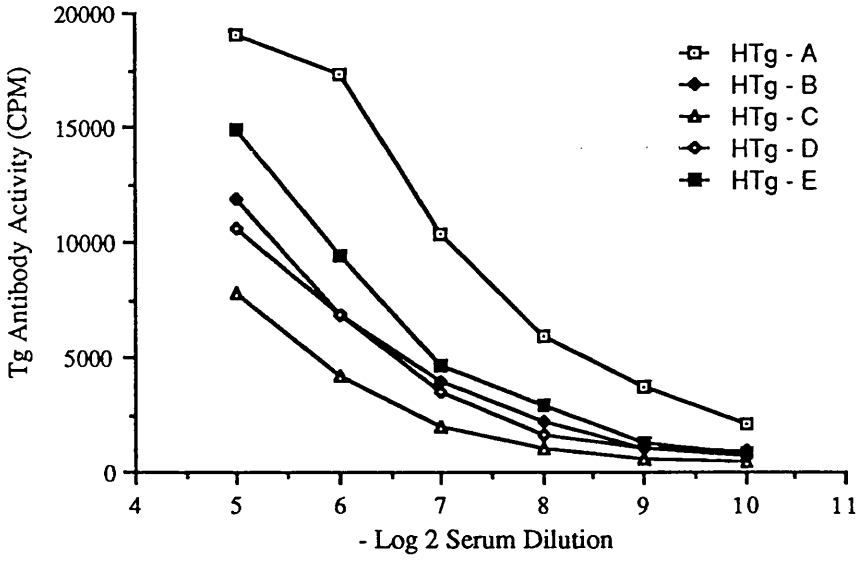

FIGURE 4.5: BINDING PROFILES OF A BB HYBRID S-TGAB SERUM WITH PREPARATIONS OF HUMAN TG CONTAINING DIFFERENT MOLAR RATIOS OF IODINE: HTg-A (T_4=4.0), HTg-B (T_4=0.9), HTg-C (T_4=0.08), HTg-D (T_4=1.02) and HTg-E (T_4=1.0).

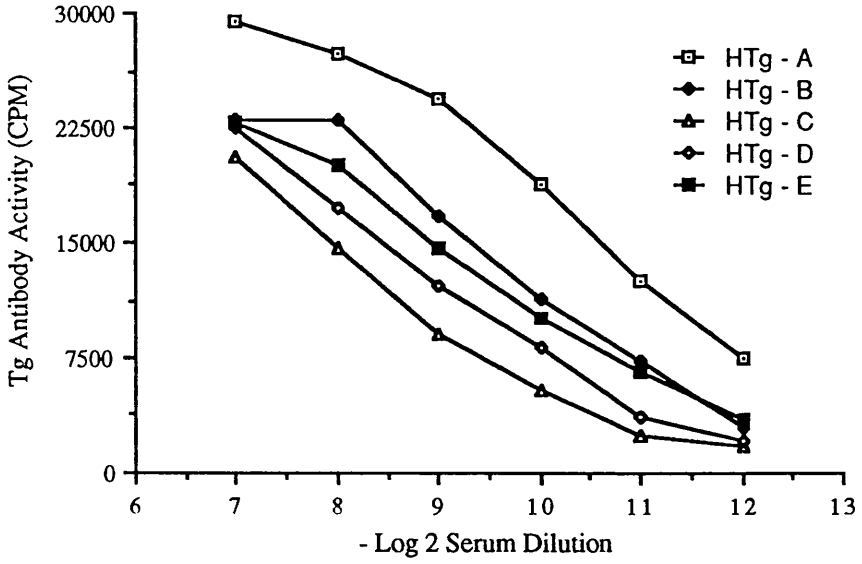

FIGURE 4.6: BINDING PROFILES OF AN INDUCED TGAB WITH PREPARATIONS OF TG CONTAINING DIFFERENT MOLAR RATIOS OF IODINE. HTg-A (T_4=4.0), HTg-B (T_4=0.9), HTg-C (T_4=0.08), HTg-D (T_4=1.02) and HTg-E (T_4=1.0).

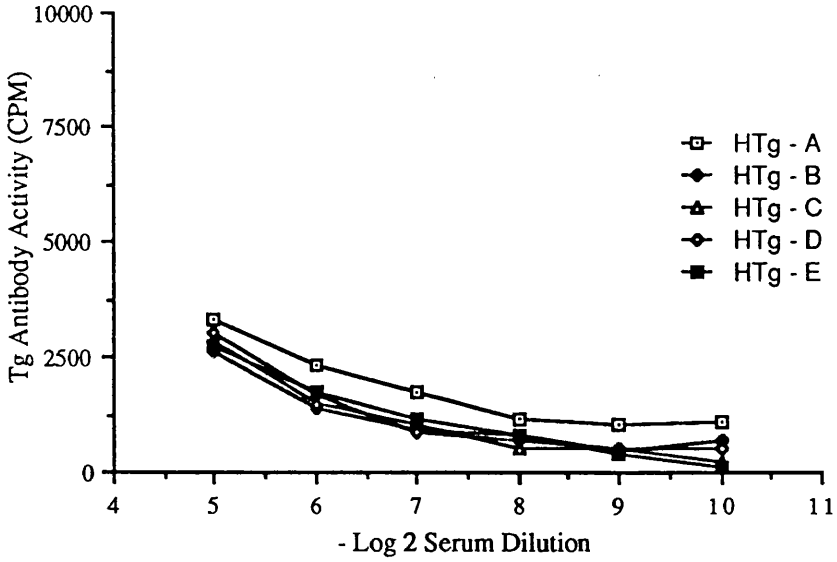

FIGURE 4.7: BINDING PROFILES OF A BB/E S-TGAB SERUM WITH PREPARATIONS OF TG CONTAINING DIFFERENT MOLAR RATIOS OF IODINE. HTg-A (T_4=4.0), HTg-B (T_4=0.9), HTg-C (T_4=0.08), HTg-D (T_4=1.02) and HTg-E

(T_4=1.0).

To ensure that these findings were not attributable to differences in the amount of the different human thyroglobulins bound to the plates, the ability of a mouse monoclonal to human Tg to bind to these different thyroglobulin coated plates was determined. As shown in Figure 4.8 this monoclonal antibody to human Tg binds equally to all the human Tg preparations. This indicates that a major proportion of antibodies in group 1 sera recognize a site on human Tg which is dependent upon the degree of iodination.

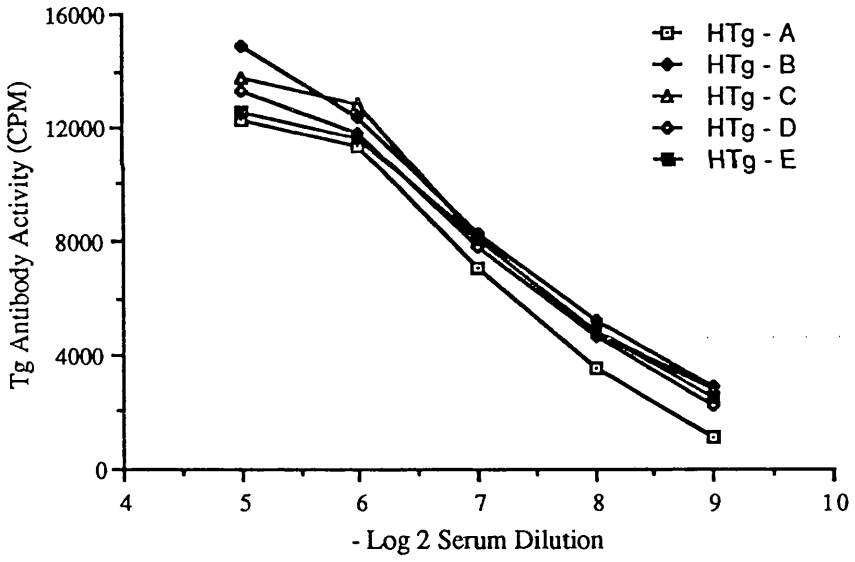

FIGURE 4.8: BINDING OF A MOUSE ANTI-HUMAN THYROGLOBULIN MONOCLONAL ANTIBODY TO HUMAN THYROGLOBULINS WITH DIFFERENT IODINE CONTENT.

The ability of group 1 sera to recognize Tg from many different species and the effect of iodine content on the binding to human Tg suggested that they might recognize thyroxine (T_4)

residues within the Tg molecule. Therefore we examined the ability of T_4 to inhibit the binding of group 1 sera to human Tg with either a low (Tg-C) or a high (Tg-A) iodine content. Thyroxine clearly inhibited the binding of S-TgAb and induced TgAb to bind to Tg-A but had little effect on their binding to Tg-C (Figure 4.9). As might be expected, the binding of induced TgAb to both Tg-A and Tg-C was completely inhibited by rat Tg. However, rat Tg inhibited the binding of S-TgAb to Tg-A but not the binding to Tg-C. This observation suggests that some of the spontaneous antibody activity against thyroglobulin is specific for human Tg and does not cross react with rat Tg.

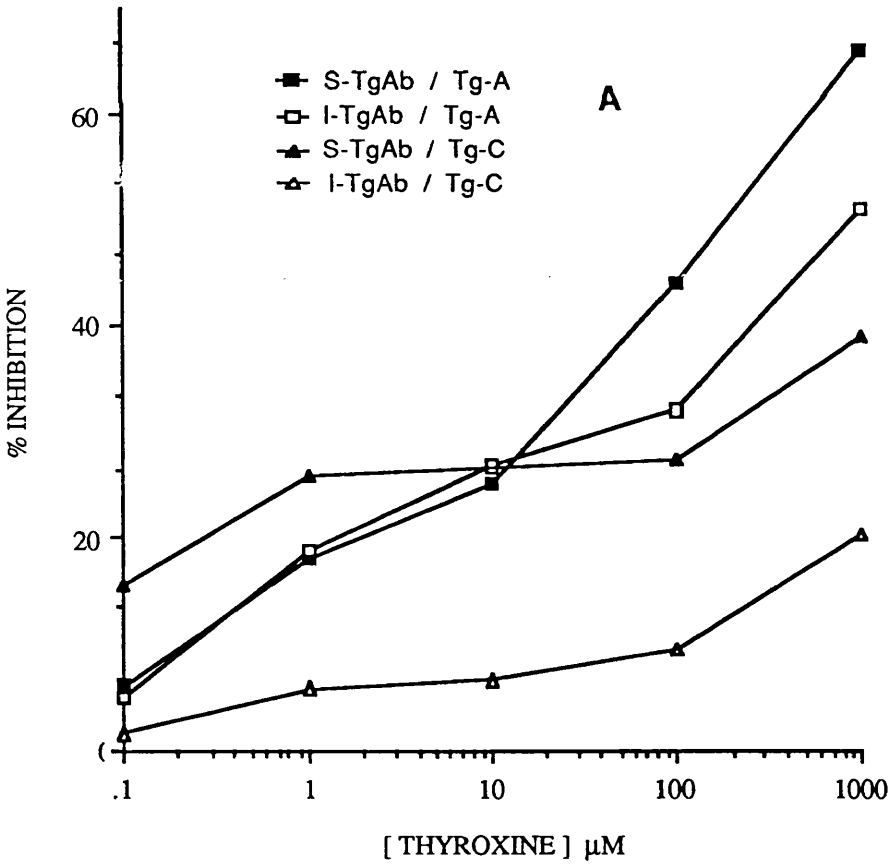

FIGURE 4.9-A: Legend see p. 110.

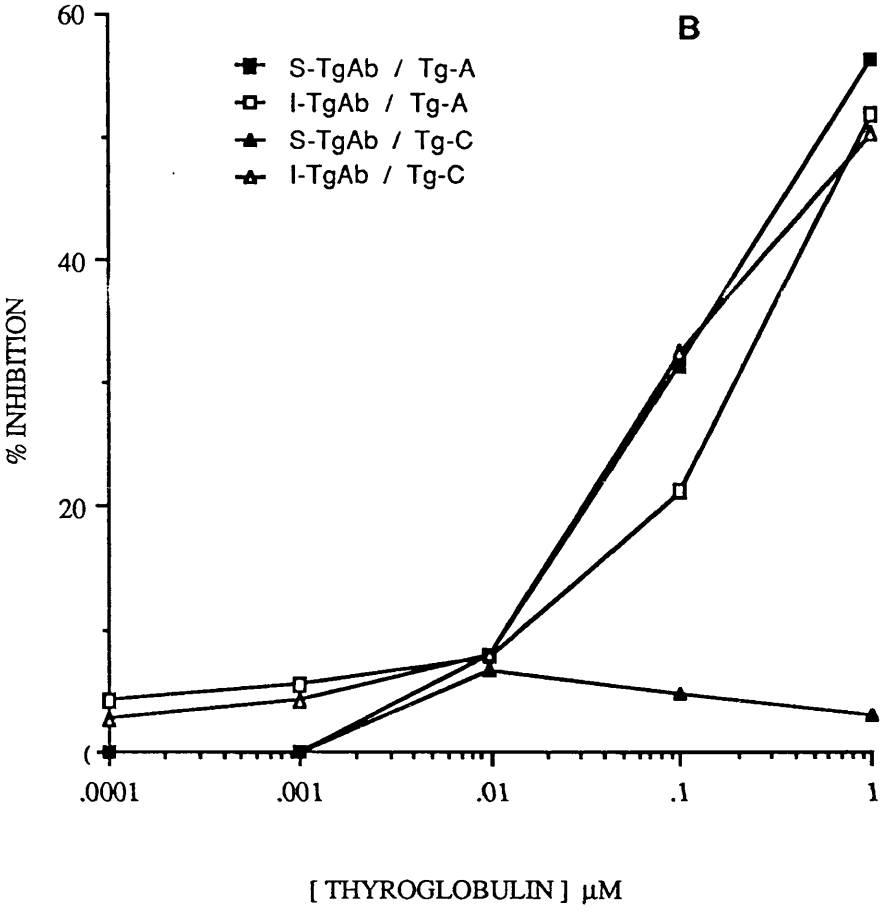

FIGURE 4.9-B.

FIGURE 4.9: INHIBITION OF BINDING OF SPONTANEOUS AND INDUCED TG AUTOANTIBODIES TO HUMAN THYROGLOBULINS WITH HIGH (Tg-A) AND LOW (Tg-C) IODINE CONTENT BY A) THYROXINE AND B) RAT THYROGLOBULIN.

4.3 DISCUSSION

Thyroglobulin is a 660 kD glycoprotein, the complete amino-acid sequence of which has only recently become available for the bovine (Mercken et al., 1985) and the human (Malthiéry and Lissitzky, 1987) molecules. The protein is known as the main antigen involved in experimental thyroid autoreactivity although precise characterizaton of the immunogenic determinants of the molecule is still lacking. This question was approached by examining the binding patterns of spontaneously arising Tg autoantibody using: i) thyroglobulins isolated from different animal species, and ii) human thyroglobulins manifesting different levels of iodination. The data shown here provide evidence for restricted epitope recognition by Tg autoantibodies in the rat.

Initial examination of sera from spontaneously autoimmune BB/E or BB hybrid revealed that approximately 30% contained Tg autoantibodies. Since the BB hybrid lines are of $RT.1^{u}$, $RT.1^{b}$ or mixed haplotypes (Colle, Guttmann and Seemayer, 1985) it was possible to establish that the presence of these autoantibodies was also not related to MHC haplotype. Sera with high titers were further examined and could be divided into three distinct groups according to their reactivity with the panel of Tg species variants. The BB/E and some BB hybrid sera (group 2) bound strongly to rat and mouse Tg, but only weakly to the other Tg species tested. Two BB hybrid sera (group 3) also reacted strongly to rat and mouse Tg, but additionally bound to dog Tg even more strongly. The remainder of the BB sera (group 1) were found to be highly cross-reactive, binding all twelve Tg species tested including the evolutionary

distinct chicken Tg.

Although the presence of Tg autoantibodies in the spontaneously autoimmune rats did not show any relationship with MHC haplotype, the cross-reactivity profiles of the ten BB hybrid sera examined showed a good correlation with MHC haplotype. Animals giving group 1 profiles all had the $RT.1^{u}$ haplotype at the class II MHC loci. Some with recombinant haplotype (PVG.r8) carried the $RT.1^{a}$ allele at class I A locus. Those with group 2 profiles were also $RT.1^{u/u}$ whereas those in group 3 were homozygous for the $RT.1^{b}$ haplotype of the Buffalo rat. The numbers are quite small but the correlation was supported by the BB/E sera which are $RT.1^{u/u}$ and also give a group 2 pattern of binding. Since B cell recognition of antigen does not involve MHC molecules, it seems likely that the basis of the observed association of epitope specificity with MHC haplotype is related to recognition of Tg by T cells. Breeding studies in the Buffalo rat have shown that the presence of at least one $RT.1^{b}$ haplotype favours the occurrence of a more severe thyroiditis than that seen in animals without an $RT.1^{b}$ haplotype (Colle, Guttmann and Seemayer, 1985).

As described in chapter 3, TgAb could be induced in several rat strains of different MHC haplotypes. Although the amounts of antibody elicited were strain dependent, all patterns of binding specificity were identical with the group 1 sera from spontaneously autoimmune rats. As expected this binding of induced antibodies to Tg from different species was completely inhibited by rat Tg. Because of the apparent dominance of highly cross reactive antibodies, the nature of the epitope(s) recognized by group 1 sera was further analysed. In these studies human Tgs of different iodine

content, which reflected the average number of thyroxine residues per mole of Tg, were used. It was observed that high titre activity of both I-

TgAb and S-TgAb in group 1 sera depended on Tg iodination. Inhibition experiments confirmed that thyroxine was the recognized determinant since T_4 could block the binding of these sera to Tg with a high iodine content but could not block binding to human Tg with a low iodine content. Thus, group 1 sera contain a dominant set of antibodies which recognize thyroxine. These may account for the high cross reactivity of group 1 sera. Interestingly, rat Tg could not inhibit the binding of some group 1 S-TgAb sera to poorly iodinated human Tg. This could mean that these sera see human-specific epitopes different from thyroxine or that there are some determinants, normally hidden by thyroxine, which are only seen on poorly iodinated Tg, rat or human. The latter possibility could be tested by competition assays utilizing poorly iodinated thyroglobulin obtained from rats treated with aminotriazole.

It is interesting to note that induced thyroglobulin autoantibodies in the rat show a different patterns of binding specificity from the induced autoantibodies in the mouse (Champion et al., 1988). Although it is possible that this may reflect a species difference in tolerance induction in the B cell pool during ontogeny it seems to be unlikely since mice can be induced to make antibody against thyroxine when given chicken Tg (Chan et al., 1986). The observed differences between mice and rats may therefore rather reflect the immunization protocol or the way in which thyroglobulin can be presented to the T cell in the context of different MHC haplotypes.

The very restricted pattern of binding specificity of group 2 and 3 rat sera suggests a limited epitope recognition. In all cases group 2 sera bound rat and mouse Tg more effectively than the other Tg species. Group 3 sera were particularly interesting in that they bound dog Tg better than mouse and rat Tg.

The fact that both spontaneous and induced Tg autoantibodies are directed against conserved sites suggests that B cell tolerance is not always directed toward conserved regions of the autoantigen as originally deduced from studies on the autoantigenic determinants of haemoglobin and cytochrome C (Reichlein, 1972; Jemmerson, Morrow & Klinman, 1982).

Taken together these data show that autoantibodies arising spontaneously in rats prone to develop autoimmune thyroid lesions recognize very few epitopes on Tg. Additionally, they provide evidence that conserved regions of the Tg molecule are likely to be involved in the rat Tg autoantigenicity. Further analysis of the sera may provide some clues to their mode of induction.

CHAPTER 5

CELL POPULATIONS IN THE INFILTRATES OF RAT SPONTANEOUS AUTOIMMUNE THYROIDITIS

5.1 INTRODUCTION

Spontaneous autoimmune thyroiditis (SAT) in chickens (Wick, Sundick and Albini, 1974) and in BUF (Silverman and Rose, 1975) and BB rats (Wright et al., 1983) is used as a model of Hashimoto's thyroiditis. Apart from the presence of Tg-autoantibodies in the serum, both diseases develop manifestations of cell-mediated immunity. One characteristic feature of the thyroid cellular infiltrate is the predominance of plasma cells and B lymphocytes which appear to be forming germinal centres (Bech et al., 1984). Although this suggests that a humoral mechanism is involved in the pathogenesis of this process, numerous observations point to a role of T lymphocytes and macrophages in the initiation and progression of the disease (Wick et al., 1985). Different lines of investigation have been followed in order to elucidate the role of those cells in the damage to the thyroid follicle.

In the obese strain chicken, in vivo depletion of B cells by cyclophosphamide bursectomy significantly delays the development of SAT but does not prevent T cell infiltration, indicating that antibody-mediated mechanisms enhance rather than trigger the

thyroid lesions (Krömer et al., 1985a). Successful depletion of B cells was confirmed by phenotypical analysis which revealed that 77% of the infiltrate consisted of T cells and that less than 1% were B cells. Moreover, no Tg-autoantibodies were detected in the sera of such animals, again supporting the view that, although autoantibodies could increase the severity of the disease, they are not indispensable in the initiation of thyroid lesions (Polley, Bacon and Rose, 1981). However, when the birds were neonatally thymectomized and then treated with anti-thymocyte serum thyroiditis did not develop (Pontes de Carvalho, Wick and Roitt, 1981). Together with the finding that lymphocytes infiltrating the thyroid spontaneously release IL-2 in vitro (Sundick et al., 1984) these data suggest a primary role for T cells in the pathogenesis of SAT. Neonatal thymectomy alone, however, enhances the severity of the disease. Rose et al., (1981) postulated that the faster maturation and emigration of T helper cells leads to an imbalance between T helper and T suppressor cell function in the periphery of the newly hatched animal. Thymectomy at that stage would prevent suppressor mechanisms from becoming fully operative, so that thyroiditis can develop. The use of anti-thymocyte serum in conjunction with thymectomy would prevent the disease by killing mature T-helper cells already present at the time of hatching.

In contrast to the OS chicken very little is known about the phenotypes of cells involved in the cell mediated immunity in SAT of rats. Voorbij, Kabel and Drexhage (1986) examining the thyroid and its cervical draining lymph nodes in BB/W rats from 2 weeks of age onwards demonstrated that the lymphocytic accumulation in the

thyroid gland was preceded by an earlier lymph node swelling. The typical thyroid infiltrate composed of T helper, T cytotoxic/suppressor and B cells was virtually absent until the animals were 20 weeks old. However, an increasing number of MHC class II positive APC's was present in the thyroid throughout the whole process. Long before B cells arrived in the thyroid, Tg-autoantibodies could be detected in the serum, implying that at least in the early stages of the disease autoantibodies are produced outside the gland (Voorbij and Drexhage, 1985).

In order to get some information on the pathogenesis of this spontaneous disease we characterized the cellular infiltrate in the thyroid of BB/E rats sacrificed in the course of other studies, using surface markers for monocyte subsets and MHC class II determinants with both single and double indirect immunofluorescence techniques.

5.2 RESULTS

Twelve BB/E rats of about 90 days of age, comprising 6 diabetic- prone and 6 animals from the non-diabetic BB subline (Walker et al., 1986) were initially screened for circulating islet cell surface antibodies (ICSA) and then sacrificed in the course of a study being carried out by A.-M. Varey on insulin dependent diabetes mellitus (IDDM). Thyroids obtained from these animals were snap-frozen and sequential sections were examined for the presence of mononuclear cell infiltration using toluidine-blue staining. Foci of thyroiditis were found in 3 rats, 2 of them from the diabetic-prone group and being ICSA positive. The infiltrated glands were indistinguishable in size from the normal BB/E thyroids and no metabolic or hormonal signs of hypothyroidism were noticed in these animals.

The infiltrations examined by toluidine-blue were multifocal, restricted to the neighbourhood of thyroid epithelium, and occasionally caused disruption of the normal follicle structure. No germinal centres could be identified in any of the infiltrated glands.

The cell phenotypes composing these infiltrates were initially characterized by single immunofluorescence staining, using a panel of rat monoclonal antibodies as described in Table 2.1. Controls using an irrelevant monoclonal antibody did not show any fluorescence. This study revealed that the vast majority of the infiltrating cells were macrophages although B lymphocytes and T lymphocytes, mainly of the $CD4^+$ subset, were also present. No

difference could be observed in these cell populations between diabetic-prone and non-diabetic BB/E rats. The small number of animals investigated, however, precludes a definite interpretation of these findings.

To define the proportion of cell subsets in the infiltrate we performed double immunofluorescence stains using the monoclonal antibody OX-6, FITC-conjugated, as the second layer. As shown in Table 5.1, macrophages were the predominant subset accounting for nearly 40% of the cell infiltrate, the majority of them bearing the C3b receptor (Figure 5.1). B lymphocytes were commly present in the infiltrate, and the use of the anti-kappa monoclonal antibody also revealed immunoglobulin deposition in both the interstitium and inside the damaged follicles (Figure 5.2). The T lymphocyte population comprised both CD4 and CD8 positive cells, although the former as judged by the number of $W3/25^{+}$ cells were more frequent (Figure 5.3). This is also suggested by the number of cells stained with the monoclonal marker OX-19. Figures of peripolesis were not observed, even though some follicles were invaded by the subsets found in the infiltrate in a typical manner.

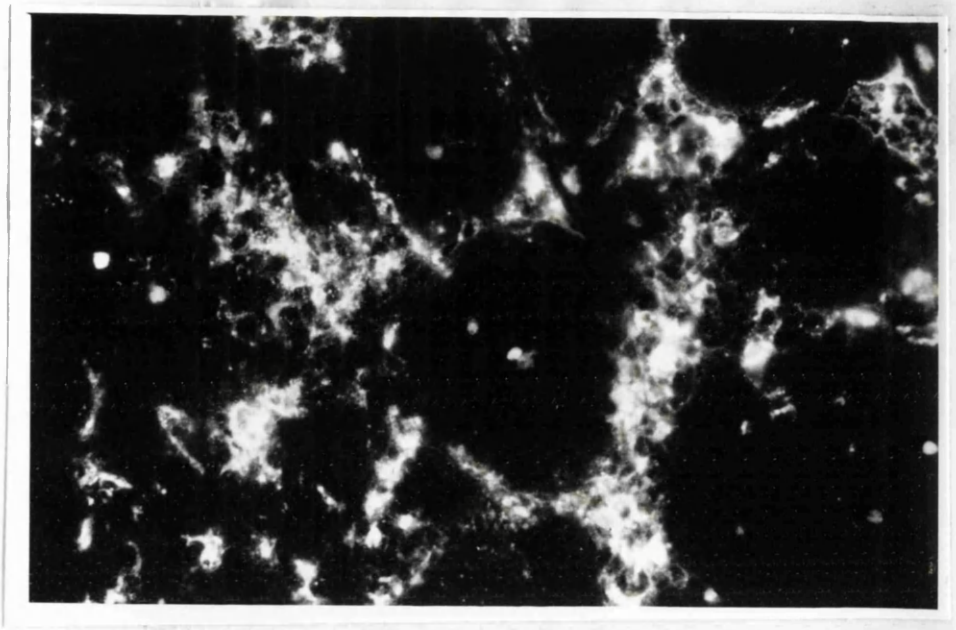

FIGURE 5.1: FOCUS OF THYROIDITIS IN BB/E RAT 5013 STAINED WITH OX-42. The picture shows a massive infiltration of macrophages from the C3b-receptor positive
subpopulation. Magnification x540.

PHENOTYPES	CELL PERCENTAGES		
	BB/E 5013	BB/E 4989	BB/E 5236
OX-8	9.4 ± 3.5	10.5 ± 1.3	8.6 ± 2.1
OX-12	28.5 ± 2.3	29.6 ± 2.1	22.0 ± 2.0
OX-19	29.9 ± 3.1	29.2 ± 2.8	23.5 ± 0.2
OX-42	38.2 ± 5.3	36.8 ± 7.5	42.7 ± 2.3
W3/25	70.4 ± 5.6	73.6 ± 4.3	68.6 ± 4.7
ED.1	43.8 ± 4.6	34.1 ± 7.5	48.3 ± 0.4
ED.2	15.8 ± 1.0	13.9 ± 1.6	11.6 ± 4.3

TABLE 5.1: SAT PHENOTYPES ON THE BB/E RAT. Percentages were calculated over all foci of infiltration and represented as the % of OX-6^+ cells. Data obtained from 2 diabetic-prone (5013 and 4989) and 1 non-diabetic (5236) BB/E rats are shown as X±SE.

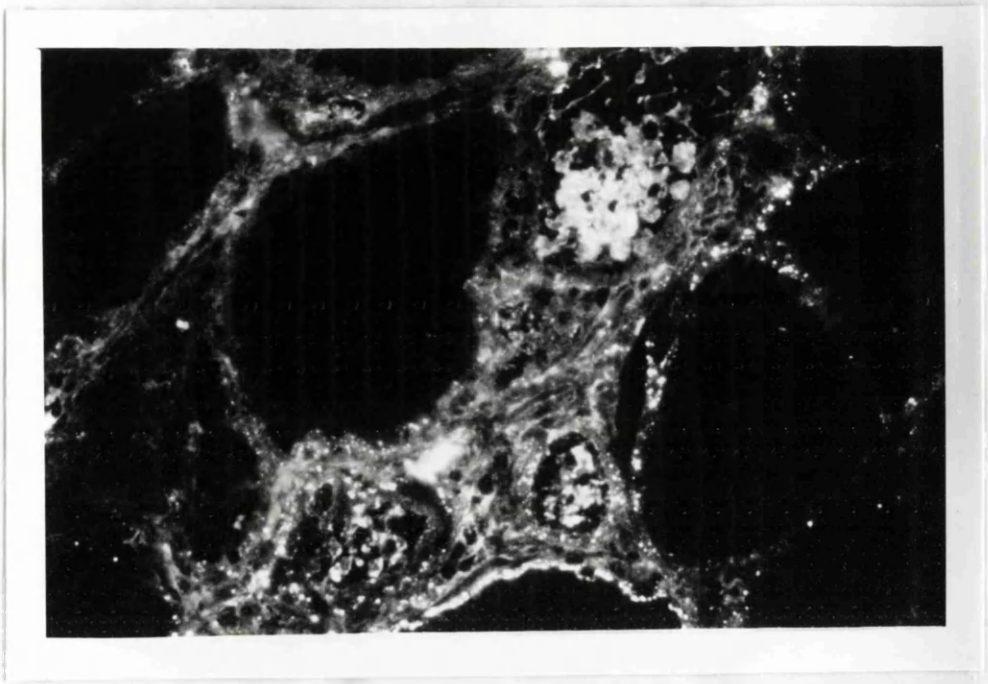

FIGURE 5.2: INDIRECT FLUORESCENCE STAINING OF SAT INFILTRATE IN BB/E RAT 5013 WITH OX-12. The photomicrograph shows a diffuse deposition of immunoglobulin and B lymphocytes. Magnification x540.

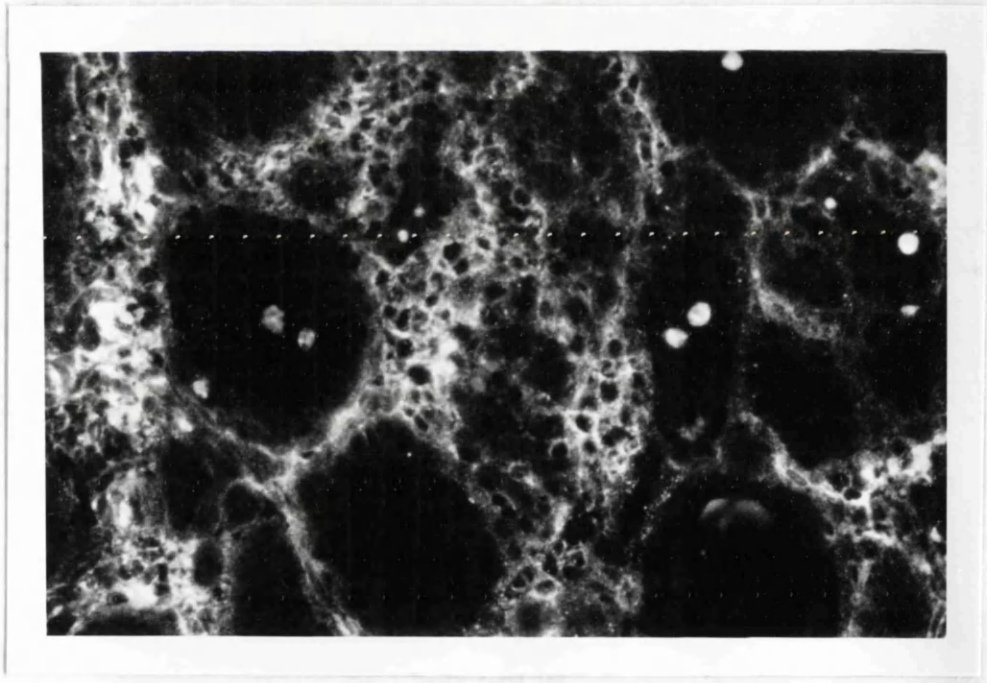

FIGURE 5.3: SAT INFILTRATE OF BB/E RAT 5013 WITH W3/25. Magnification

x540 Even though a large portion of the infiltrating leukocytes were positive for the Ia antigen determinant, detected by the OX-6 monoclonal antibody (Figure 5.4), the staining was less bright than in the rat-EAT infiltrate (Chapter 6). Their failure to bind the marker for the IL-2 receptor (OX-39), suggests again that these leukocytes are poorly activated. No differences were observed in composition of the thyroid infiltrates of the diabetic-prone and the non-diabetic BB/E rats.

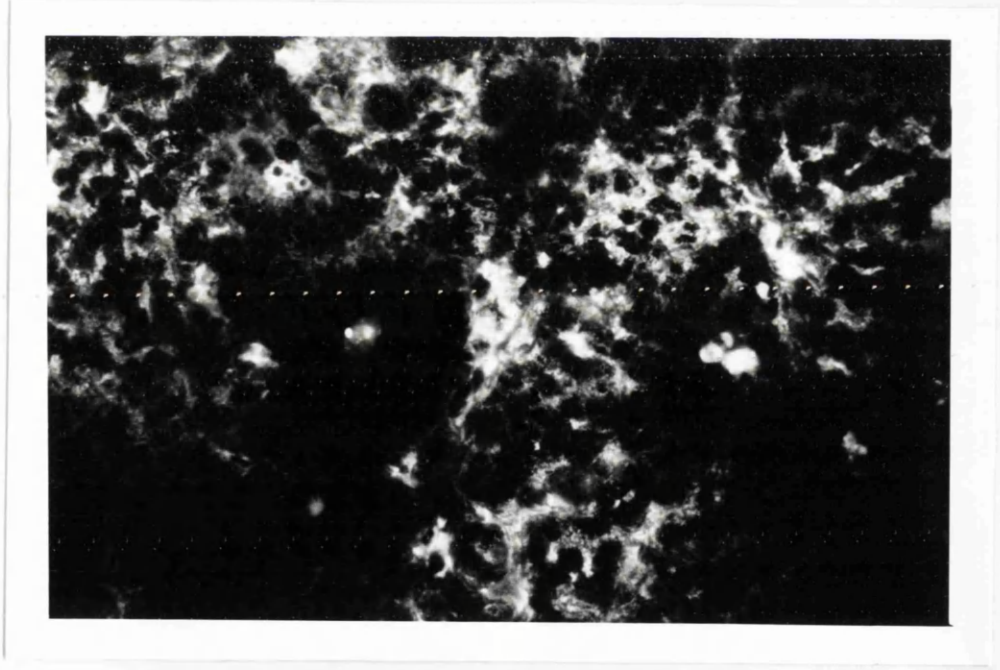

FIGURE 5.4: FOCUS OF LEUKOCYTE INFILTRATION IN BB/E RAT 5013 EXPRESSING Ia ANTIGEN (OX-6 POSITIVE). Magnification x540.

5.3 DISCUSSION

The Bio Breeding/Edinburgh (BB/E) rats, a line derived from the BB/Worcester (BB/W), spontaneously develop insulin-dependent diabetes mellitus with a mean age of onset of 96 days of age following lymphocytic infiltration and subsequent destruction of the pancreatic beta-cells (Dean, et al., 1985). Spontaneous occurrence of lymphocytic infiltration in the thyroid has also been demonstrated in the parental line (Boitard, et al., 1985; Yanagisawa et al., 1986). This evidence together with the well-known coexistence of Hashimoto's thyroiditis and diabetes mellitus in man (Neufeld et al., 1980) indicates a common autoimmune basis for these processes and points to the BB rat strain as a model of Hashimoto's thyroiditis. We approached this question in two sublines of the BB/E rats using histological and immunofluorescence analysis of thyroid sections obtained from 12 animals in order to characterize the contribution of cell-mediated immunity to the pathogenesis of SAT in the rat.

SAT was found in 3 BB/E rats, 2 animals being diabetic-prone and 1 iabetic-resistant. The number of animals investigated is too small to analyse but previous observations in the BB/W showing that spontaneous thyroiditis tends to be more frequent in diabetic-prone than in diabetic-resistant animals (Sternthal et al., 1981) would make it worthwhile to study more rats. Even though double staining of spleen sections showed that in the rat T lymphocytes (OX-19$^+$) express Ia (OX-6) antigens (fig. 6.5) OX-6 cannot be regarded as a leukocute marker in the strict sense.

Future studies should therefore include pan-leukocyte markers (OX-1, OX-3) in the panel. Within these limitations it can be stated that the infiltration was composed of approximately 40% macrophages, 30% B and 30% T lymphocytes. A great proportion consisted of leukocytes positive for the W3/25 marker. Similar ratios have been described in lymph nodes and pancreatic islets of BB/W rats (Like et al., 1983) strengthening the notion of a common mechanism underlying these two diseases, in which macrophages and $CD4^+$ cells might play an important role. Although $CD8^+$ ($OX\text{-}8^+$) cells were also present, their contribution to the infiltrate never reached more than 30% of the T cell population.

On the other hand, the substantial number of B lymphocytes in the interstitium and immunoglobulin deposition, not only in the interstitium but also inside the damaged follicle, is consistent with the notion of the participation of antibody mediated autoimmunity in the pathogenesis. In this respect, antibody mediated cytotoxicity involving macrophages with Fc- and C3b-receptors could be an efficient effector mechanism either directly through antibody-binding to the Fc-receptors or indirectly via the complement cascade.

Although B lymphocytes were noticed in the interstitium, they were never observed in peripolesis (= migration through an epithelial cell layer), a feature which is characteristic of the SAT of chickens (Wick et al., 1982). Also, germinal centres were never seen in these infiltrated glands. Since in the BB/W B cells were the last subset to arrive in the gland, immigrating after $CD4^+$ and $CD8^+$ T cells (Voorbij, Kabel and Drexhage, 1986), it is possible that

germinal centre would have developed later. To confirm this hypothesis an experiment should be designed to follow the course of the disease for a period beyond the time animals were investigated in this study.

In contrast to the human, the disease in this rat line is a focal rather than a diffuse process. The study of Sternthal et al. (1981) suggested that BB/W rat may not be a good model of Hashimoto's thyroiditis since metabolic and hormonal manifestations of hypothyroidism as assessed by levels of TSH and thyroid hormones could not be convincingly demonstrated. However, the more recent work of Yanagisawa et al., (1986) suggests that metabolic effects may be age related since in their study 20 week old rats manifest depressed plasma thyroid hormone levels, 11 and 5 week old animal being normal.

The RT.1-MHC haplotype in the BB/E line may somehow influence the severity of the thyroiditis, since in BUF/BB hybrids the incidence and the severity of this disease was influenced by the RT.1 haplotype. $RT.1^{b}$ confers susceptibility to severe thyroiditis, whereas the $RT.1^{u}$ haplotype leads to marked islet destruction but a mild degree of thyroiditis (Colle et al., 1985). Future studies will aim at a more systematic long-term follow up of the development of spontaneous autoimmune thyroiditis in the BB rat, assessing the disease status of the animals not only histopathologically but also on endocrine, metabolic and serological levels.

CHAPTER 6

CELL PHENOTYPES IN THE INFILTRATES OF RAT EXPERIMENTAL AUTOIMMUNE THYROIDITIS

6.1 INTRODUCTION

Experimental autoimmune thyroiditis (EAT) in rats induced by inoculation of rat thyroglobulin (Jones and Roitt, 1961) constitutes a powerful experimental model for the analysis of thyroid autoimmunity. Although the model has been extensively studied, the pathogenic effector mechanisms at the cellular level are not yet well characterized. The precise role of T helper, T suppressor, T cytotoxic cells, and macrophages still remains speculative. Partly, this reflects the limitations of the approaches used to characterize the involvement of cell mediated immunity in induction of the disease.

Lillehoj and Rose (1981) using the T cell mitogens PHA and Con A, to assess T cell subsets in low and high EAT responder rat strains, defined two T cell subpopulations in lymph nodes of immunized animals. They suggested that the Con A responsive T cell subpopulation ($T_{Con\ A}$), fully active in high and low responder strains, would act as inducer cells whereas the PHA responsive T cell subpopulation (T_{PHA}), found less effective in the high responder strain, would modulate the response. The lack of specific phenotypic markers to quantitate the various subpopulations of T cells rendered it impossible to correlate these mitogen responses

with the presence or absence of inducer , suppressor or cytotoxic T cells.

In mouse EAT, a cytotoxic assay was employed to ascertain whether or not cytotoxic T cells are implicated in the effector mechanisms leading to the thyroid damage. Creemers, Rose and Kong (1983) observed that a cytotoxic effect of viable lymph node cells (LNC) from mouse Tg (MTg) + CFA immunized animals to thyroid monolayers is MTg specific and H-2 restricted. This cytotoxicity was abolished by pretreating LNC with monoclonal antibodies to Ly 2.1.The authors thus concluded that cytotoxic cells are generated in immunized mice and may well be an important phenotype in the EAT induced in mice. The role of suppressor cells in the course of rat EAT induced by Tg + CFA was inferred from two observations: i) splenectomy carried out within 4 days of immunization markedly reduced the thyroid damage. ii) infusions of splenocytes, taken from immunized donors during the later phase of the disease, decreased the incidence of the disease although this effect was small and statistically not significant (Weetman et al., 1984). Further indirect evidence for suppressor cell function in EAT is provided by the observation that administration of splenic cells to rats with thyroiditis, induced by thymectomy and irradiation, completely abolished the autoimmune response (Penhale et al., 1976). Additionally, a relative T suppressor cell deficit in the splenic lymphocyte population has been demonstrated in animals with thyroiditis (McGregor et al., 1983).

The availability of monoclonal antibodies which specifically recognize determinants associated with rat leukocytes from various lineages (Mason et al., 1983; Dijkstra et al., 1985) has

provided new insights in the composition of EAT infiltrates. Using a panel of monoclonal antibodies to rat leukocyte determinants Cohen, Dijkstra and Weetman (1988) have shown that macrophages and T lymphocytes comprising equal numbers of CD4 and CD8 cells were the mononuclear cells most frequent in the EAT infiltrate of neonatally thymectomized BUF rats. The proportion of these populations did not vary between early and late stages of the disease. The authors also detected a reduction of the $CD8^+$ subpopulation in the peripheral blood which showed a good correlation with the development of EAT.

Similar observations have also been reported by Smith et al. (1987) in rats developing EAT following thymectomy and irradiation. On the other hand, AUG rats immunized with Tg + CFA developed EAT infiltrates predominantly composed of B lymphocytes, although T cells were also present in smaller numbers (Hassman et al., 1986).

In this chapter the cell phenotypes in the thyroid infiltrates from different strains of rats, varying in the RT.1-MHC, immunized with Tg + CFA and Pertussis vaccine are described. These infiltrates were also characterized in CDF rats immunized with various doses of thyroglobulin and sacrificed at different time points. Phenotyping was carried out by indirect immunofluorescence using the panel of mouse monoclonal antibodies specific for leukocyte surface markers shown in Table 2.1 (Materials and Methods) with single and dual staining on consecutive cryostat sections.

6.2 RESULTS

Time course of cell phenotypes in the infiltrates

In CDF rats immunized twice with 2 mg of rat-Tg in CFA (total dose 4 mg Tg/animal), as described in Chapter 3, cell phenotypes in the EAT infiltrate were characterized by single immunofluorescence staining. Controls (irrelevant mouse monoclonal antibody) did not show any staining. The EAT infiltrate showed qualitative differences in the relative contribution of cell subsets during the evolution of the disease process. Whilst generally the numbers of all cell phenotypes increased with time, indirect single-stain immunofluorescence, as presented in Figure 6.1, revealed that (OX-19$^+$ and W3/25$^+$) T cells constituted much of the infiltrate at early time points and expressed class II determinants (I-A and I-E homologues, OX-6$^+$ and OX-17$^+$ respectively).

OX-8$^+$ cells had also reached substantial numbers in the infiltrate only at day 28. Since the increase in the OX-8$^+$ cells was paralleled by the increase in the OX-19$^+$ population, this indicates that the OX-8$^+$ cells are largely T cytotoxic/suppressor cells. No OX-12$^+$ cells (B lymphocytes with κ light chains) were found in the inflammatory infiltrates at the three time points investigated but the possibility cannot be excluded that B cells expressing λ light chains were present.

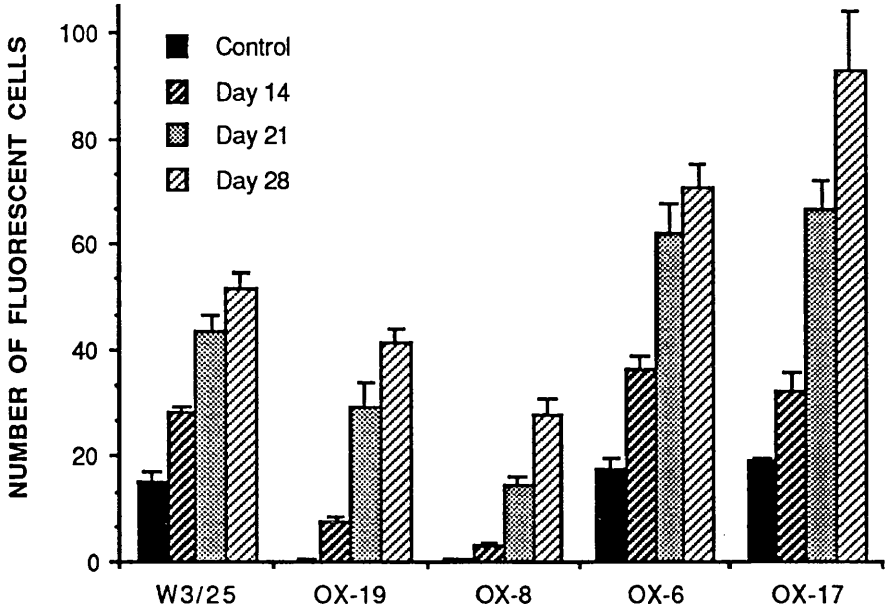

FIGURE 6.1: CELLULAR PHENOTYPES IN THE TIME COURSE OF EAT INDUCED IN CDF RATS. Animals were immunized with rat-Tg + CFA and Pertussis vaccine. Data are expressed as X±SE of the number of fluorescent cells per high power field (x630).

Thyroglobulin dose-dependence

In CDF rats immunized with various Tg-doses, the number of mononuclear cells infiltrating the thyroid gland increased with the amount of antigen injected. This was consistent with the finding of the dose-dependence of the histologic thyroiditis index (Chapter 3).

Although in these experiments double staining was not performed, cell counts from consecutive sections lead to the conclusion that the infiltrating cells are mainly T-lymphocytes (OX-19$^+$, W3/25$^+$) and macrophages (OX-42$^+$, W3/25$^+$) as is seen in Figure 6.2. Both these populations showed a dose-dependent increase in parallel with the total OX-6+ cell count. In contrast, the

number of cells bearing the ED-2 determinant, expressed on resident tissue macrophage (Dijkstra et al., 1985), did not differ between the four experimental groups. The data imply that the vast majority of leukocytes present in the thyroid infiltrate express class II determinants recognized by the OX-6 monoclonal antibody, suggesting a state of activation. Unlike the situation in spontaneous thyroiditis, no κ light chain$^+$ B lymphocytes (OX-12$^+$) were detected in the infiltrates irrespective of the immunization protocol. It would be of interest to study the phenotypes of the infiltrating cells by flow cytofluorometry after releasing them from the tissue using collagenase.

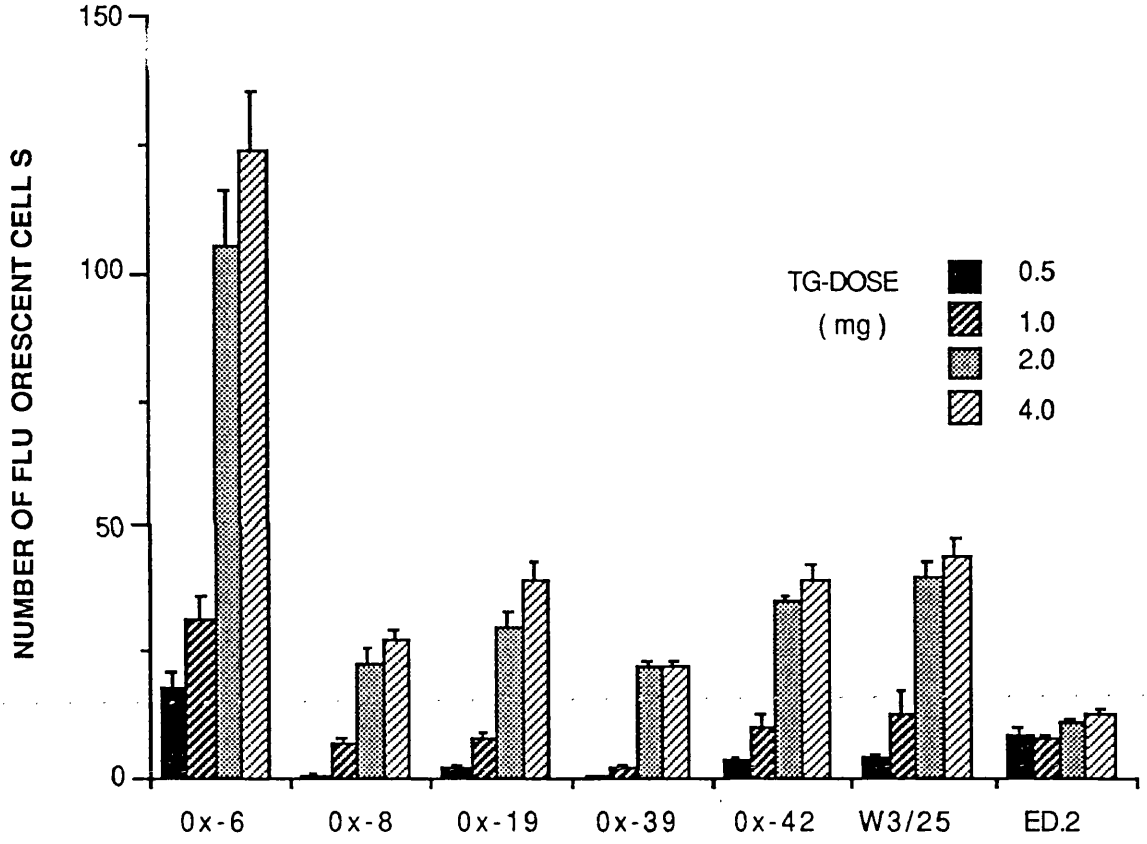

FIGURE 6.2 CELL PHENOTYPES IDENTIFIED IN THE INFLAMMATORY INFILTRATE OF CDF RATS IMMUNIZED WITH VARIOUS DOSES OF TG. Data obtained from 4 rats are expressed as X±SE of the number of fluorescent cells per high power field (x630).

Strain differences in the rat EAT cell phenotypes

In all the strains examined most of the cells which composed the infiltrate were found scattered throughout the interstitium, but in areas of severe infiltration they were frequently seen lining the thyroid epithelium. In densely inflamed areas, mainly in the AUG and CDF strains, where the infiltration was most striking there was peripolesis and extension into the thyroid follicles (Figure 6.3). No particular cell phenotype was regularly associated with this observation.

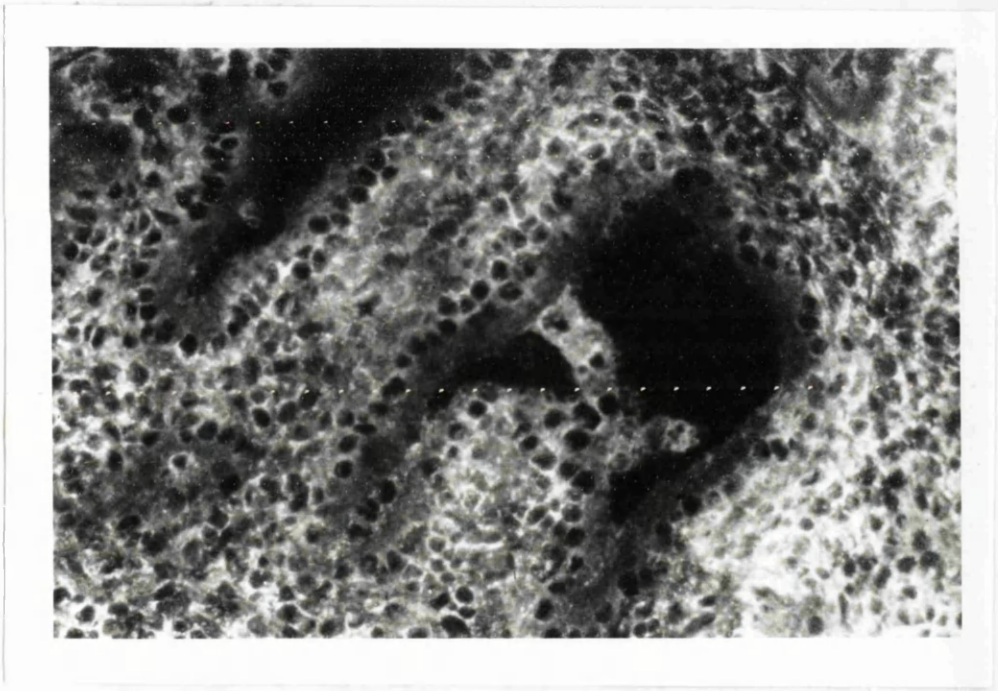

FIGURE 6.3: IIF STAINING OF THE THYROID INFILTRATE IN AUG RAT WITH OX-6 MONOCLONAL ANTIBODY (MoAb). The photomicrography illustrates Ia^+ lymphocytes in peripolesis. Magnification x540.

B lymphocytes, identified by the OX-12 monoclonal antibody were notably absent from the inflammatory infiltrate irrespective of the rat strain examined, although they were easily detected in large numbers using positive control sections of spleen. Moreover, the OX-12 antibody, specific for anti-kappa light chains, revealed extensive interstitial immunoglobulin deposits, particularly within damaged follicles present in heavily infiltrated areas (Figure 6.4). Again, no difference was found in this regard in all four inbred strains of rat studied.

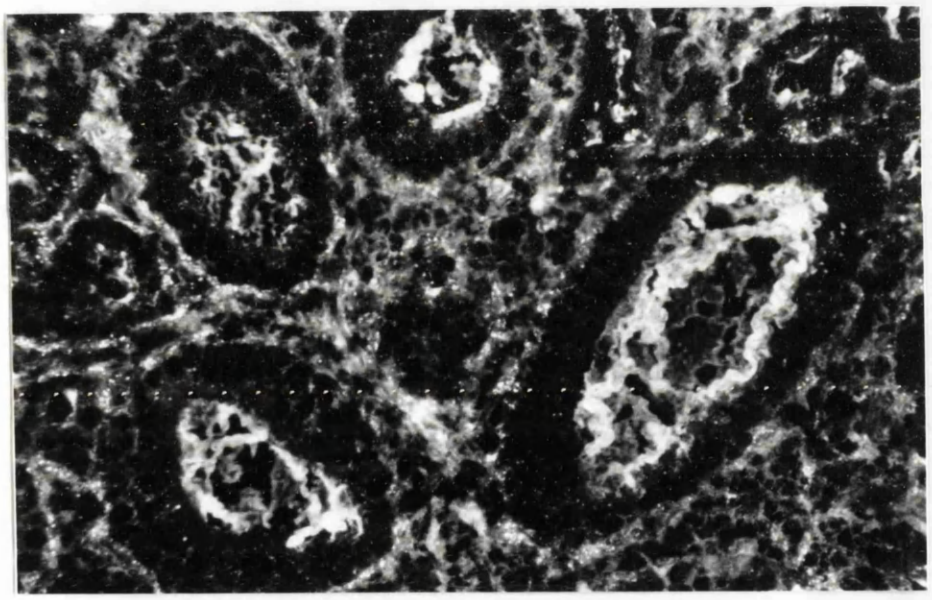

FIGURE 6.4: EAT INFILTRATE IN CDF RAT STAINED WITH OX-12 MoAb. The picture shows a diffuse deposition of immunoglobulin in the interstitium and within the damaged follicles. Magnification x540.

Since OX-6$^+$ cells are present in the inflammatory infiltrate the relative contribution of macrophages and T lymphocytes to the Ia$^+$ infiltrate was assessed. Using the double-labelling immunofluorescence technique we could analyse the OX-6$^+$ infiltrate and also identify the different subpopulations of T lymphocytes.

These phenotypes were characterized in thyroid cryostat sections from CDF rats immunized twice with 2 mg of rat-Tg, which was above the threshold dose for reliably inducing EAT as defined by either histological (H/E) or immunofluorescence (IIF) criteria. Sections were sequentially incubated with two monoclonal antibodies of different specificities. The first layer was visualized with rhodamine and the second layer with fluorescein-conjugated antibodies according to the protocol detailed in Table 2.2 (Materials and Methods). This system was standardized in spleen sections as exemplified in Figures 6.5 and 6.6. Substitution of the first monoclonal antibody with normal mouse serum or an irrelevant antibody completely abolished the binding of rhodamine to the tissue sections, and no fluorescein staining was seen in the absence of the second specific monoclonal antibody.

FIGURE 6.5-A:
OX-6

FIGURE 6.5-B:
OX-19

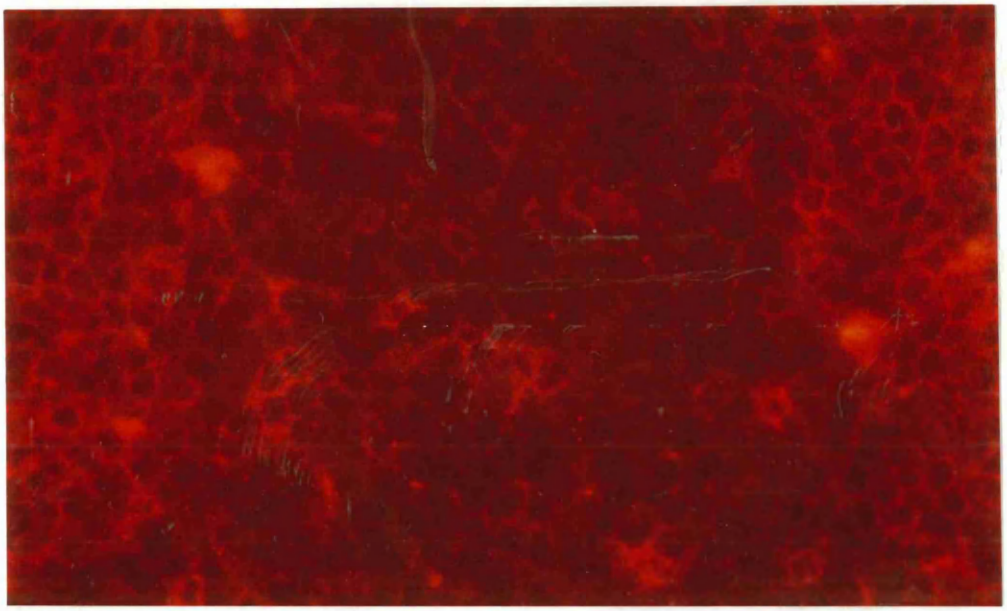

FIGURE 6.5-C:
OX-6/OX-19

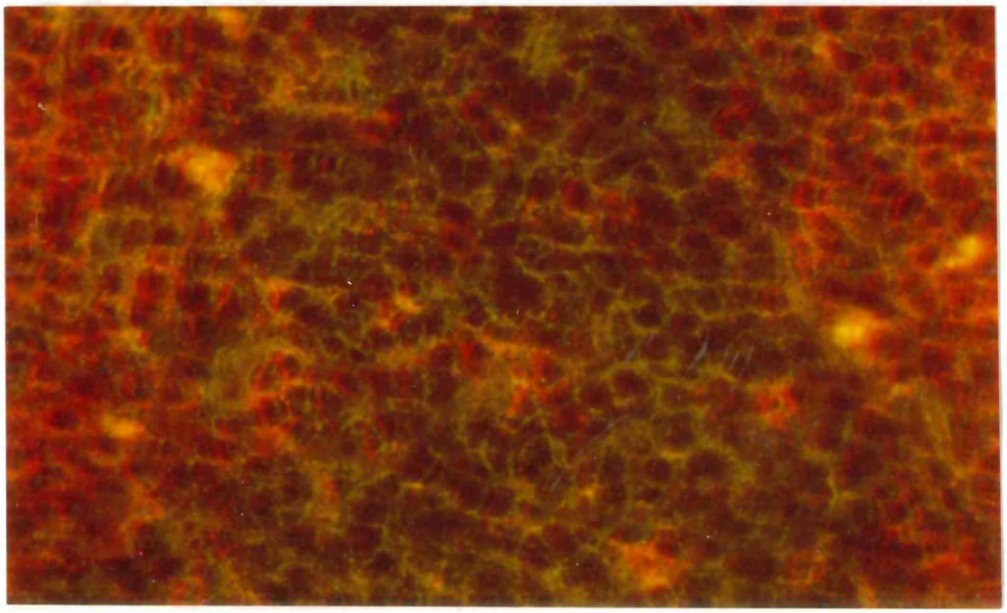

FIGURE 6.5 DOUBLE IIF STAINING ON SPLEEN USING OX-6 (GREEN) AND OX-19 (RED). A and B show single, C double exposure. Magnification x540

FIGURE 6.6-A:
OX-6

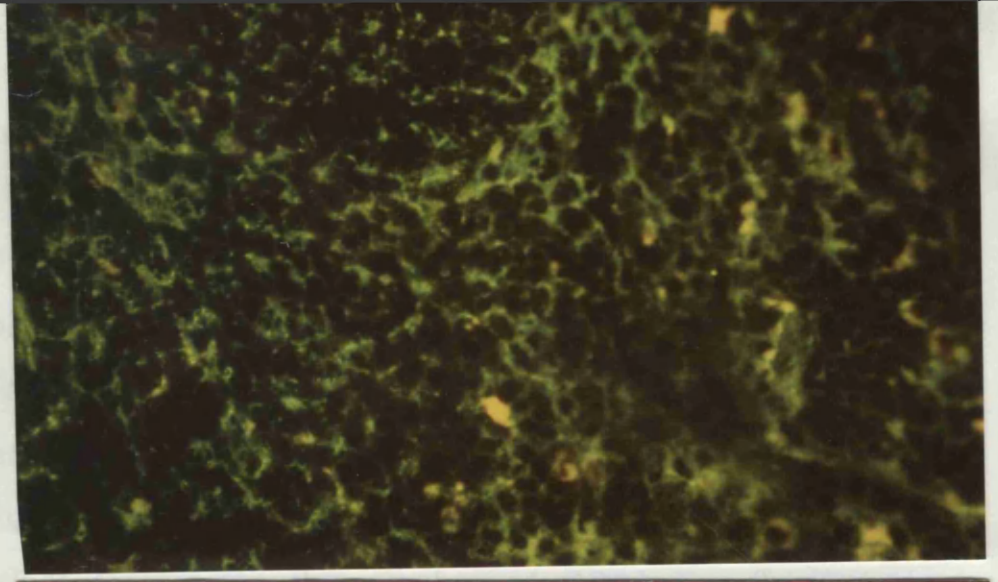

FIGURE 6.6-B:
ED.1

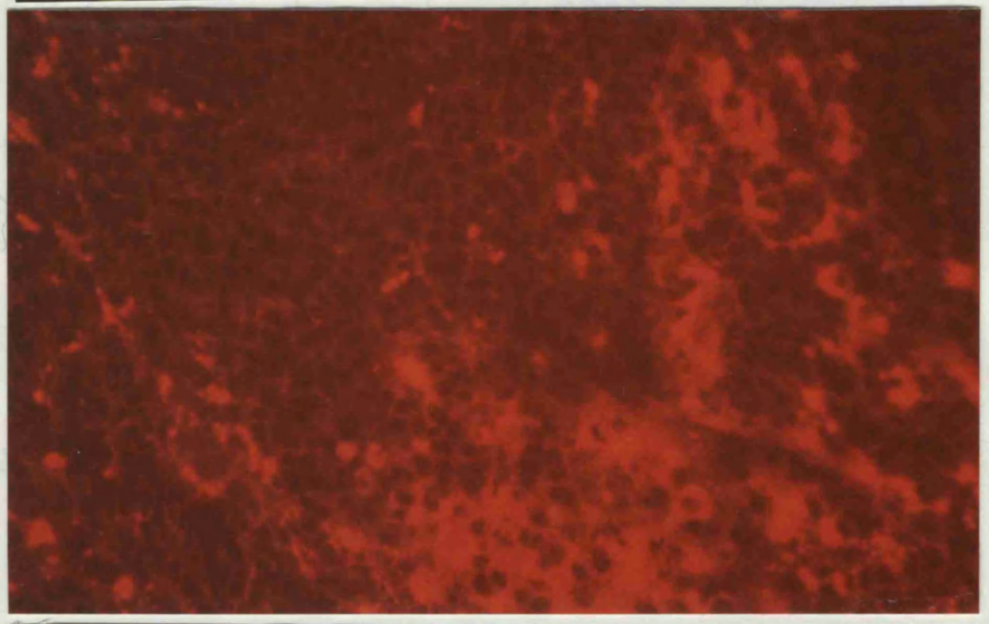

FIGURE 6.6-C:
OX-6/ED.1

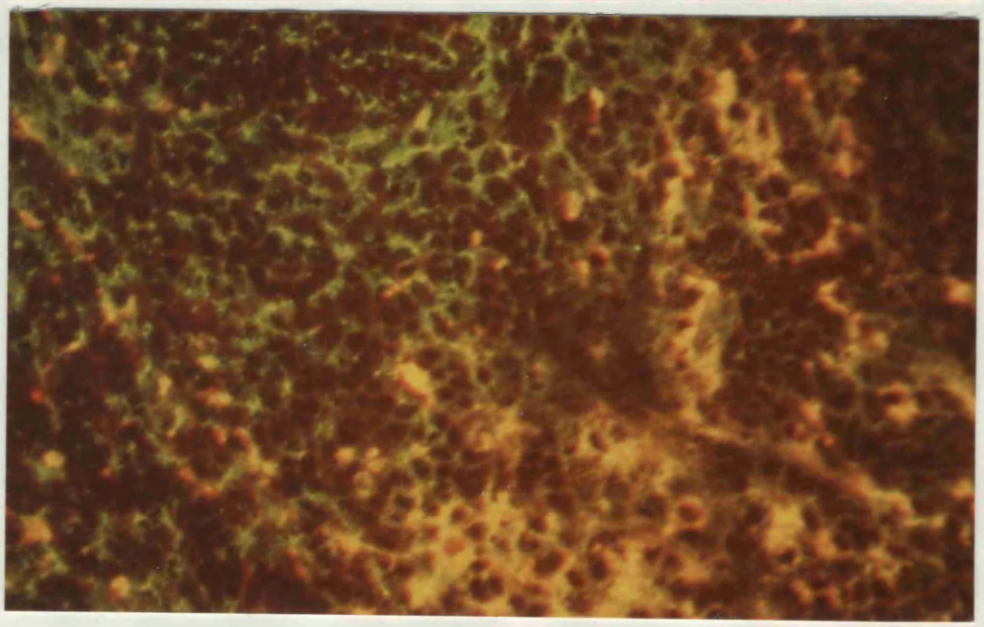

FIGURE 6.6 DOUBLE IIF STAINING ON SPLEEN USING OX-6 (GREEN) AND ED.1 (RED). A and B show single, C double exposure. Magnification x540

Comparing the percentages of OX-6$^+$ subpopulations identified by this procedure it was clear that macrophages (OX-42$^+$ and ED.1$^+$) (Figure 6.7-A) and T lymphocytes (OX-19$^+$) (Figure 6.7-B) were the dominant cell types, comprising approximately 45-55% and 36-38% of the total Ia$^+$ infiltrate respectively. The proportion of cells expressing IL-2 receptor (OX-39$^+$) (Figure 6.7-C) was essentially identical with that of total T cells (OX-19$^+$) confirming T cell activation. Comparing the number of CD8$^+$ T cells (OX-8$^+$) (Figure 6.7-D) with the total T cell counts (OX-19$^+$), the CD8$^+$ T cell subset represents approximately 60% of the OX-19$^+$ population, the CD4$^+$ T cell subpopulation presumably constituting most of the remainder. This was confirmed using the OX-8/OX-19 dual-specificity staining which demonstrated that almost 60% of the OX-19$^+$ population belonged to the T cytotoxic/suppressor phenotype (OX-8$^+$, OX-19$^+$) (Figure 6.8). This also showed that NK cells (OX-8$^+$,OX-19$^-$) (Woda et al, 1984) were a rare phenotype in the EAT infiltrate of CDF rats, accounting for only 7% of the total OX-8$^+$ population (Table 6.1).

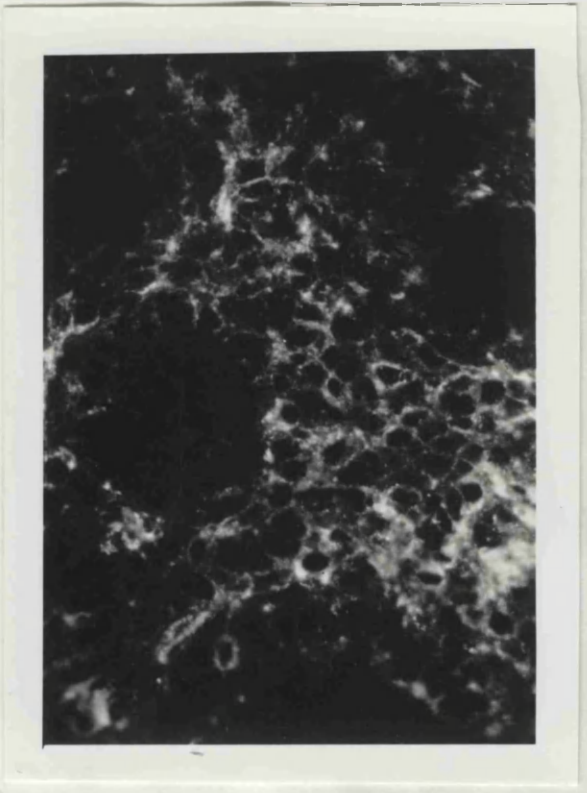

FIGURE 6.7-A , (x 400)

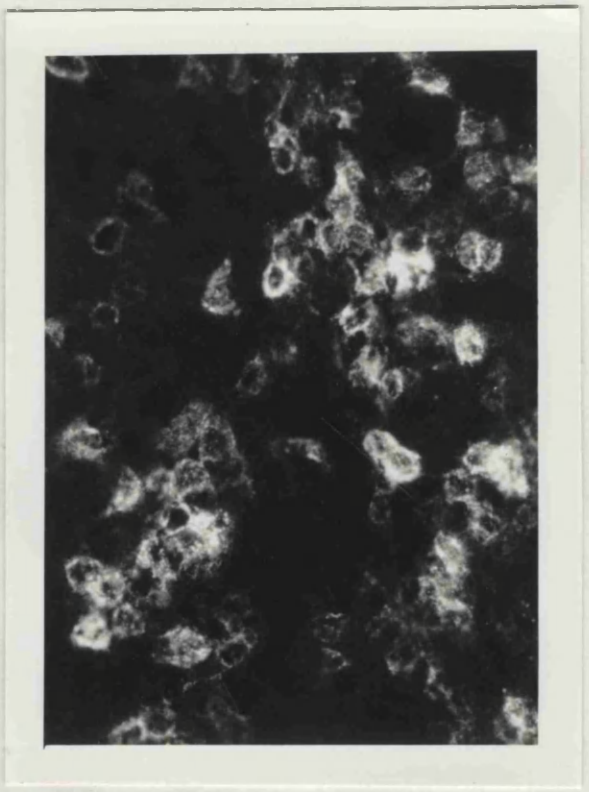

FIGURE 6.7-B, (x 400)

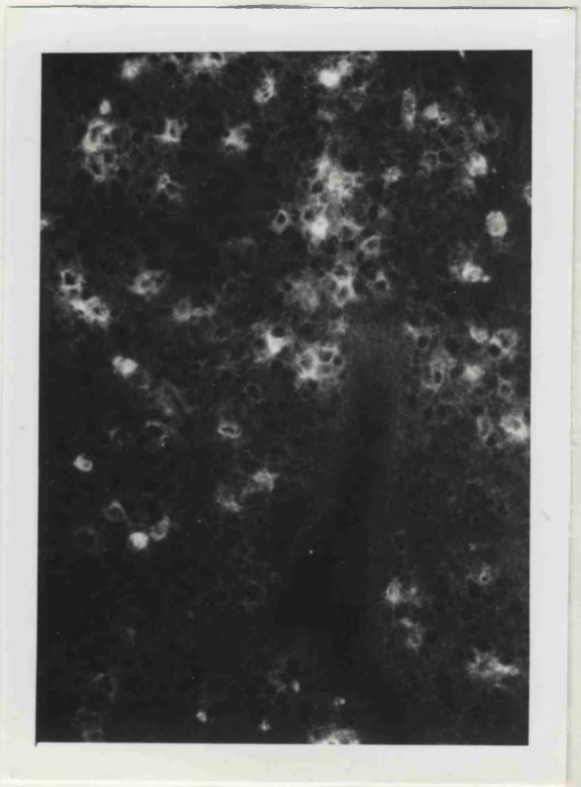

FIGURE 6.7-C, (x 250)

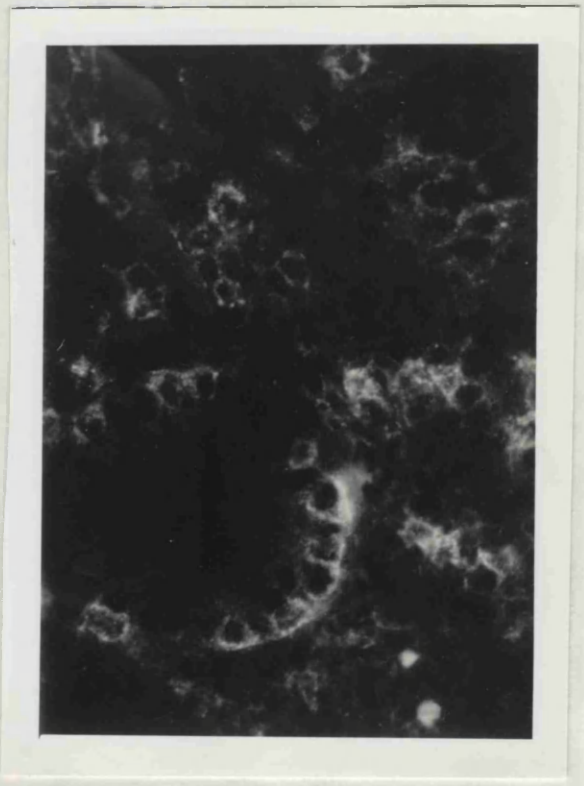

FIGURE 6.7-D (x 400)

FIGURE 6.7 IIF STAINING OF THE THYROID INFILTRATE IN AUG RAT. The pictures show the leukocyte subpopulations identified by ED.1 (A), OX-19 (B), OX-39 (C) and OX-8 (D).

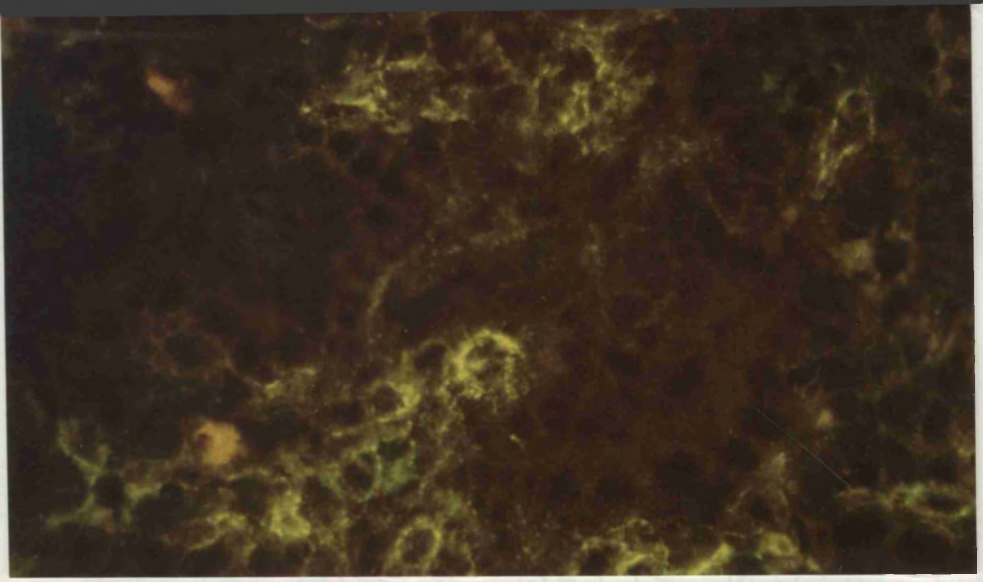

FIGURE 6.8-A:
OX-19

FIGURE 6.8-B:
OX-8

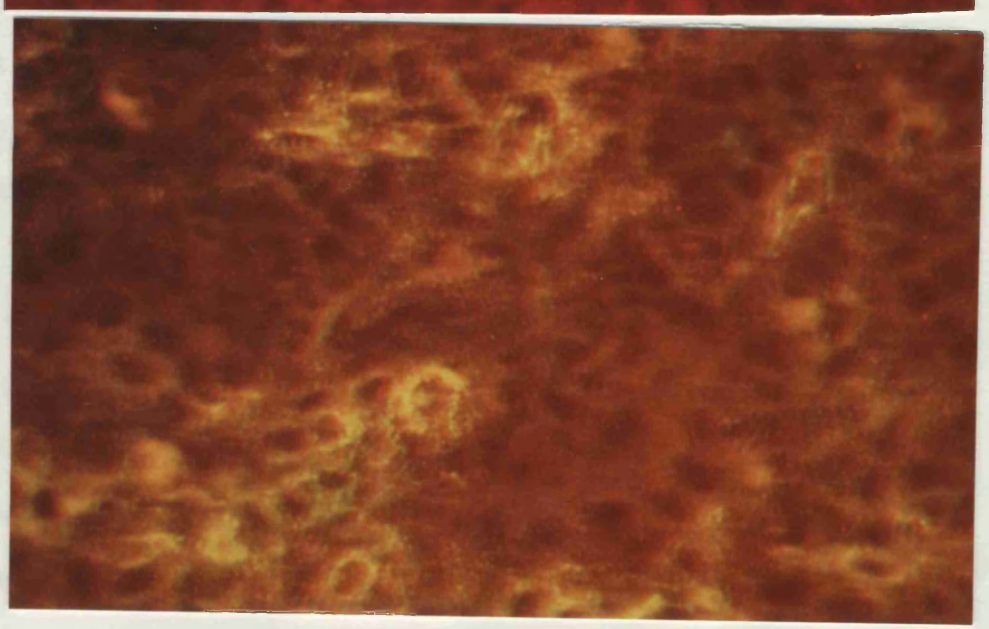

FIGURE 6.8-C:
OX-19/OX-8

FIGURE 6.8 DOUBLE IIF STAINING ON THE EAT INFILTRATE OF CDF USING OX-19 (GREEN) AND OX-8 (RED). A and B show single, C double exposure. Magnification x540.

PHENOTYPES		CELL PERCENTAGES			
		AUG	CDF	LEW	AO
Ox-8	a	21.1 ± 1.2	19.O± 1.O	18.9 ± 1.1	9.2 ± 2.6
Ox-19	a	38.3 ± 1.2	37.1 ± 1.3	36.7 ± 1.1	28.4 ± 3.6
Ox-39	a	35.6 ± 1.8	39.7 ± 1.2	36.6 ± 1.2	19.3 ± 4.O
Ox-42	a	46.8 ± 1.2	45.5 ± 1.8	46.1 ± 1.3	33.8 ± 3.2
W3/25	a	55.O ± 2.2	49.9 ± 1.4	52.5 ± 1.6	46.6 ± 3.7
ED.1	a	55.7 ± 3.5	56.5 ± 3.4	5O.4 ± 1.8	69.9 ± 5.3
ED.2	a	16.5 ± 1.6	13.O ± O.8	13.4 ± 1.4	67.O ± 3.7
N.K	b	7.7 ± 1.1	7.1 ± 1.2	7.8 ± 1.8	O.1 ± O.4
Tc/s	c	65.3 ± 2.4	58.2 ± 2.6	62.9 ± 2.4	26.1 ± 6.4

TABLE 6.1: EAT PHENOTYPES ON DIFFERENT INBRED STRAIN OF RATS. Cell percentages were calculated over 10 high power microscope fields (x630) and represent a) % of OX-6$^+$ cells, b) % of OX-8$^+$ cells and c) % of OX-19$^+$ cells. Data obtained from 3 animals are expressed as X±SE.

Cell subsets were also analysed in EAT of various inbred strains of rats. Similar proportions of macrophages and lymphocyte subpopulations were found in the three high-responder strains AUG (RT.1^c), LEW (RT.1^l) and CDF (RT.1^l). This was true for the percentages, and the spatial distribution of inflammatory cells within the thyroid was also similar in these strains.

In contrast, in the low-responder AO (RT.1^u) strain, the minimal inflammatory infiltrate showed a different cell composition with a predominance of stromal macrophages (ED.2$^+$) over infiltrating cells (Table 6.1).

6.3 DISCUSSION

The role of cell mediated immunity in the destruction of the thyroid follicle in autoimmune thyroiditis is not yet clarified. In this study the question was approached by examining the rat EAT infiltrates, induced by administration of rat-Tg plus CFA and B. pertussis. Applying a panel of mouse monoclonal antibodies specific for various leukocyte subpopulations with indirect immunofluorescence techniques it was demonstrated that these infiltrates are largely composed of macrophages and T lymphocytes.

In the early stages of the disease, W3/25$^+$ cells were the dominant phenotype accounting for approximately 90% of the infiltrate. Apart from T helper cells, the W3/25 marker is expressed on macrophages (Jefferies, Green and Williams, 1985) and on dendritic cells (Steueger, Klempnauer and Worngeit, 1984). The fact that very few OX-19$^+$ (pan-T) cells were detected in the infiltrate, indicates that antigen presenting cells (APC's) are the dominant phenotype in the initial phase of the process. Similar observations have been reported in the spontaneous thyroiditis of the BB rat (Kabel et al., 1987) and in EAT of the neonatally thymectomized BUF rats (Cohen, Dijkstra and Weetman, 1988). Later stages were associated with an increasing number of OX-19$^+$ cells and T cytotoxic/suppressor cells, as judged by the number of OX-8$^+$ cells. Taking into account the representation of OX-8$^+$ cells in the OX-19$^+$ population it is unlikely that cells from the T helper phenotype make a large contribution to the EAT infiltrate. This suggests that most of the W3/25$^+$ cells found throughout the disease process are macrophages or other APC's, indicating a

possible role for this subset in both the induction and the maintenance of the disease. Although what initially atracts antigen presenting cells into the gland remains to be resolved. However, it is interesting to note that in IFN-γ injected mice an increased accumulation of class II positive dendritic cells is found in thyroid and other tissues (Skoskiewicz et al., 1985). This raises the possibility that these cells are attracted by antigen specific T helper cells which pass through the gland.

The number of infiltrating cells correlated well with the thyroglobulin dose injected. These cells were mostly T lymphocytes and macrophages. Furthermore, the use of double staining techniques and of additional markers for macrophages and T-cells, allowed the identification of macrophages bearing the C3b receptor ($OX\text{-}42^+$) and of activated T lymphocytes bearing IL-2 receptors ($OX\text{-}39^+$) as the dominant subpopulations present in the EAT infiltrates of all high responder strains. The proportion of helper to cytotoxic/suppressor T cells was approximately 2:3. In contrast, in low responders (AO strain), tissue macrophages accounted for almost the entire Ia^+ cell population.

Cells with the NK phenotype ($OX\text{-}8^+$, $OX\text{-}19^-$) (Woda et al., 1984) accounted for only 1% of the infiltrate and in comparison with the findings in Buffalo rats immunized with Tg + CFA (Cohen and Weetman, 1987), the balance between T helper and T cytotoxic/suppressor (Tc/s) is shifted towards the Tc/s subset. No B lymphocytes were seen in the infiltrates at 21 days but it should be noted that in the spontaneous thyroiditis of the BB rat, such infiltration appears to be a late event (Voorbij, Kabel and Drexhage, 1986).

These data support the notion that in the Tg + CFA induced rat EAT cell mediated immunity plays a major role in the development of the disease. Since the inflammatory infiltrate in the thyroid gland of these animals is essentially composed of macrophages and T lymphocytes, readily able to produce interferon-gamma (IFN-γ) and tumor necrosis factor (TNFα), cytokine mediated cytotoxicity might be involved in the thyroid damage. A synergistic cytotoxic action of these two cytokines has been demonstrated on the FRTL-5 cells, in vitro (Taverne et al., 1987). Other mechanisms, for example antibody dependent cell cytotoxicity (ADCC) and class I restricted cell killing, cannot be excluded in the pathogenesis of EAT. Supporting a role for ADCC, immunogloblulin deposition was particularly strong within damaged and infiltrated follicles in high responder animals, whereas in poor responders where no thyroid damage could be seen, Ig deposition, although present, was more restricted to the thyroid interstitium. Finally, the large number of T c/s in the late stages, where the disease becomes more aggressive with severe follicle destruction, may point to class I restricted cytotoxicity as the effector mechanism of this phase. Creemers et al. (1984) have reported similar observations mice developing EAT.

CHAPTER 7

CLASS II MHC EXPRESSION ON RAT THYROID EPITHELIAL CELLS IN VIVO AND IN VITRO

7.1 INTRODUCTION

Under physiological circumstances thyroid epithelial cells (TEC) do not exhibit detectable levels of class II antigens. However, in pathological conditions, in both humans (Hanafusa et al., 1983) and animal models of autoimmune thyroiditis (Wick et al., 1984), epithelial class II expression is prominent. Since helper T-cell activation is, in general, class II MHC-restricted, this has been proposed as a means by which epithelial cells may communicate with T lymphocytes (Hart and Fabre, 1981). This would allow Ia-positive epithelial cells to present self antigens to the immune system thereby initiating (Bottazzo et al., 1983) or perpetuating (Dean et al., 1985; Wick et al., 1985) the autoimmune process. However, although presentation of defined antigen to T helper cells by thyroid epithelial cells (TEC) from experimental animals has not yet been directly demonstrated, primary T cell sensitization can occur on thyroid epithelial cell monolayers (Yeni and Charreire, 1981).

In the OS chickens epithelial class II expression was only seen in the presence of dense leukocyte infiltration (Wick et al., 1984). Moreover, sequential analysis of EAT in the neonatal thymectomized BUF rat indicated that the mononuclear infiltrate preceded TEC Ia-expression (Cohen, Dijkstra and Weetman, 1988).

This suggests that lymphocyte-mediated mechanisms might be responsible for the induction of Ia antigen in these animal models of autoimmune thyroiditis. It also indicates that lymphokines are likely to be triggering agents. The most favoured candidate for this is presently interferon-gamma (IFN-γ).

IFN-γ is a lymphokine secreted by T lymphocytes ($CD4^+$ and $CD8^+$ cells) and NK cells in response to both antigen-specific and non-specific stimuli (Trinchieri and Perussia, 1985). The presence of these cells in the inflammatory infiltrate of both spontaneous (Krömer et al., 1985) and induced autoimmune thyroiditis (Cohen and Weetman, 1987) suggests that class II antigen expression is probably a result of the release of IFN-γ which has been shown to induce Ia antigen on cultured cells from humans (Weetman et al., 1985; Todd et al., 1985) and experimental animals (Salamero et al., 1985). Furthermore, IFN-γ was detected in culture supernatants from leukoagglutinin-stimulated thyrocytes, confirming the notion that IFN-γ produced by contaminating T lymphocytes and NK cells under these conditions may be responsible for the induction of HLA-DR expression in this in vitro model as well (Iwatani et al., 1986). In summary, IFN-γ seems capable of amplifying both normal and pathological immune responses, and it could maintain inappropriate epithelial expression of class II MHC antigen which is a feature of both Graves' disease and Hashimoto's thyroiditis (Hanafusa et al., 1983).

In this chapter class II antigen expression on TEC was evaluated using in vivo and in vitro systems. In the former, thyroid

cryostat sections from rats, actively immunized with thyroglobulin as described in chapter 3, and also from BB/E rat spontaneously developing autoimmune thyroiditis, were immunostained for class II MHC determinants. Single and double immunofluorescence techniques were applied using an anti-I-A monoclonal antibody (OX-6) and an antiserum to the human microsomal antigen. For the in vitro study the well characterized rat thyroid epithelial cell line, FRTL-5, was cultured with recombinant IFN-γ either in the presence or absence of thyroid-active agents including dibutyryl cyclic AMP, calcium ionophore A23187 and TSH. Class II antigen induction on FRTL-5 was detected by immunofluorescence using anti I-A (OX-6) and anti I-E (OX-17) monoclonal antibodies and quantified by flowcytometric analysis.

7.2 RESULTS

Epithelial expression of class II MHC products in rat EAT and SAT

Using the same rats described in chapter 6 for phenotyping of the infiltrating cells, single and double staining protocols were used to detect inappropriate expression of class II MHC determinants on thyroid epithelial cells. The class II antigen identified by OX-6 monoclonal antibody (the rat homologue of mouse I-A) was quantified by comparison with a human anti-microsomal positive serum as a thyroid epithelium marker. OX-6 fluorescence was present on a minority (8.4 - 15.6%) of thyroid epithelial cells, and generally only in heavily infiltrated areas, where Ia^+ leukocytes were seen in close contact with these epithelial cells (Fig 7.1).

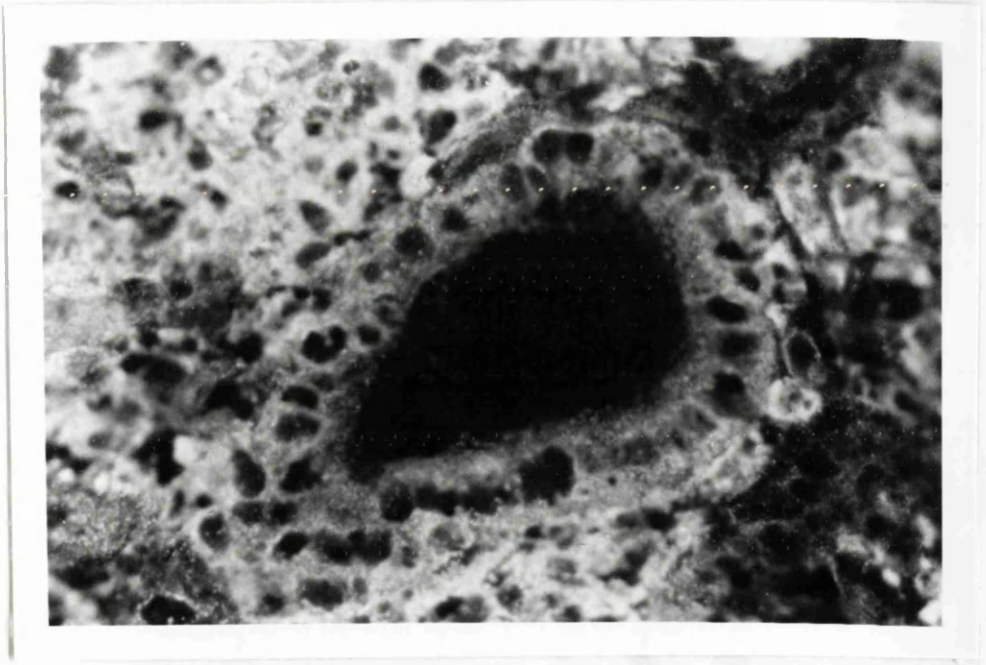

FIGURE 7.1: EPITHELIAL CLASS II MHC EXPRESSION IN RAT EAT. The photomicrograph illustrates a thyroid follicle with Ia-positive epithelial cells in a CDF rat cryostat section stained with OX-6 monoclonal antibody by IIF. Magnification x540.

The "inappropriate" epithelial expression of the OX-6 determinant was observed only in the high EAT-responder AUG, CDF and LEW strains and never in the resistant-EAT AO strain.

In the spontaneous autoimmune thyroiditis of the BB/E no thyroid epithelial cells appeared to bear class II antigen (Fig. 7.2). However, the leukocyte infiltration in these glands, as described in chapter 5, was not very dense and the cells did not generally express activation markers.

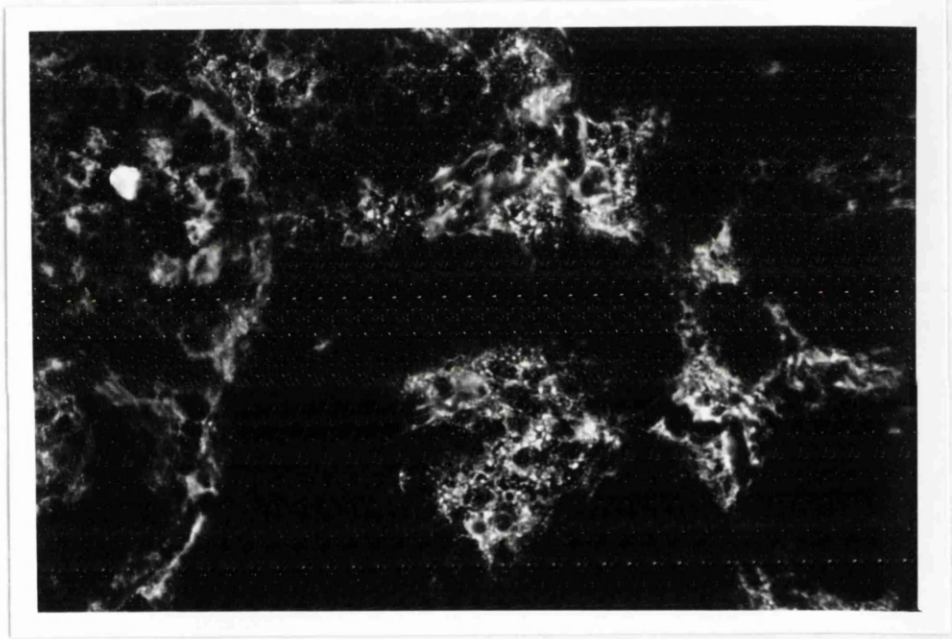

FIGURE 7.2 IIF STAINING WITH OX-6 ON THE SAT OF THE BB/E RAT. The picture shows a focus of Ia+ leukocytic infiltration surrounding thyroid follicles negative for the class II phenotype. Magnification x540.

Induction of class II MHC determinants on FRTL-5 cells <u>in vitro</u>.

FRTL-5 cells were cultured for 84 h in the absence of TSH with concentrations of rIFN-γ varying from 20-100 Uml^{-1} and stained with OX-6 and OX-17 monoclonal antibodies. OX-8 was used to control for nonspecific antibody binding by FRTL-5 cells.

Class II antigen expression was first detected less than 18 h

after exposure to 100 Uml^{-1} rIFN-γ. Plateau levels of class II expression (80-90% of the cells analysed) were seen in several experiments after 60-84 h. IFN-γ induced class II expression was dose dependent with maximal induction been achieved by 100 U ml^{-1} rIFN-γ, and this was unaffected by growth-sustaining levels of TSH. Other mediators such as LATS (1 mU ml^{-1}), dibutyryl cyclic AMP (1mM) and calcium ionophore (20 μg ml^{-1}) which are known to activate thyroid epithelial cell metabolism had no effect on class II antigen expression.

The induction of class II MHC antigen (both I-A and I-E) on TFEC by rIFN-γ was assessed by fluorescence microscopy (Fig 7.3). Control monolayers incubated in parallel without IFN-γ never showed detectable fluorescence. In some experiments quantitative flow cytometry was also used (Fig 7.4).

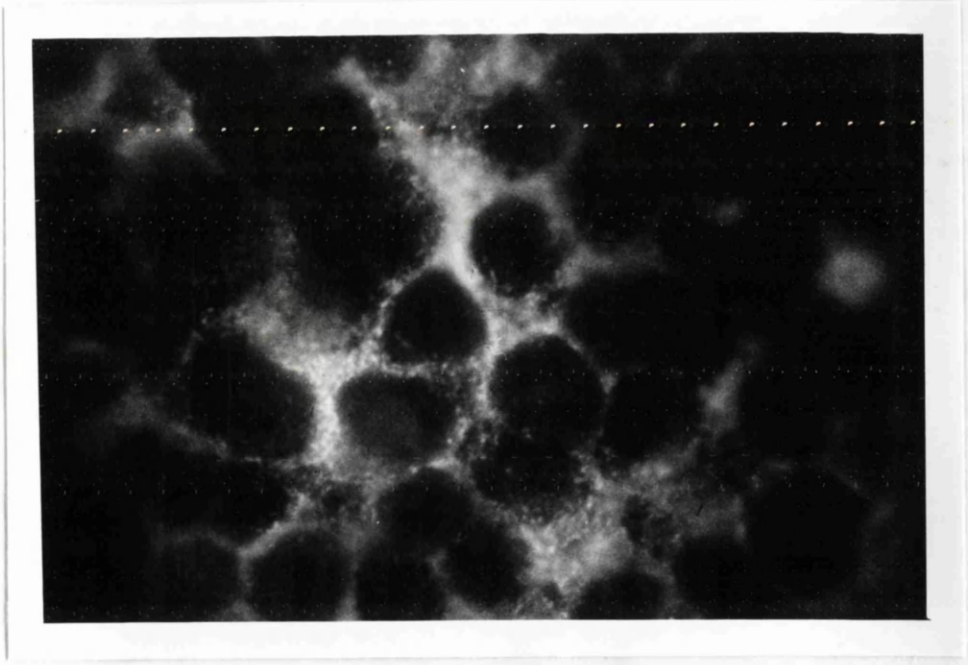

FIGURE 7.3: CLASS II MHC DETERMINANTS INDUCED IN VITRO ON FRTL-5 MONOLAYERS. Cells were incubated for 3 days with 90 Uml^{-1} rat rIFN-γ and stained by IIF with OX-6. Magnification x750.

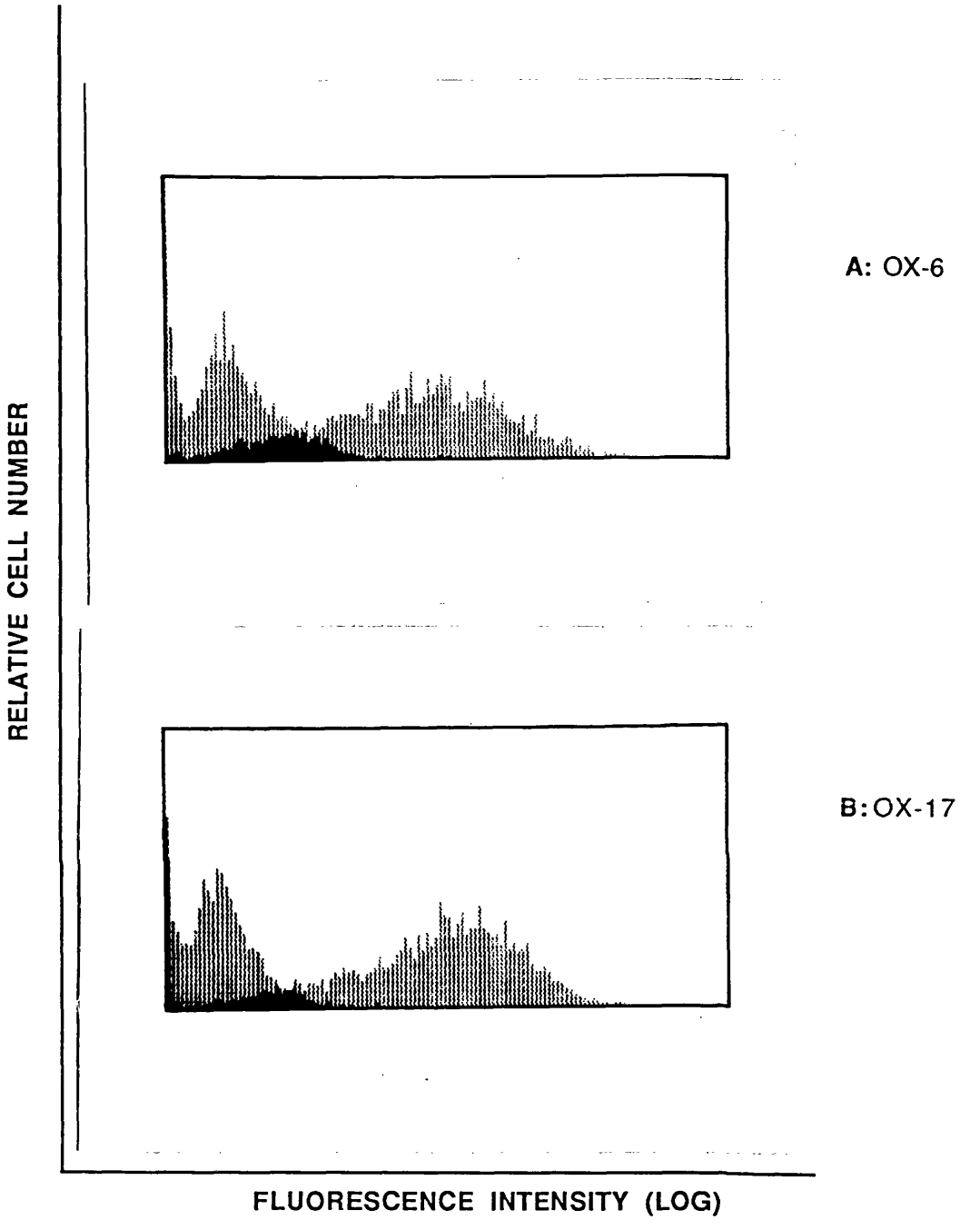

FIGURE 7.4: FLOW CYTOMETRIC ANALYSIS OF INDUCED MHC ANTIGENS ON FRTL-5 CELLS DETECTED BY A) OX-6 AND B) OX-17 MONOCLONAL ANTIBODIES. Fluorescence intensity is shown on a logarithmic scale on the horizontal axis. The relative cell numbers in each fluorescence channel are represented on the vertical axis. Basal fluorescence (following culture in IFN-γ free medium) is represented by the left-hand curve of each histogram.The superimposed right-hand curves shows identical samples cultured for 76 h with 100 Uml^{-1} rIFN-γ.

To confirm that the induced epithelial MHC antigen expression on FRTL-5 cells was directly attributable to IFN-γ, we tested the ability of DB-1 to block its induction. The addition of DB-1, a mouse monoclonal antibody with specific neutralizing capacity for rat and mouse-IFN-γ, to FRTL-5 cultures containing rIFN-γ inhibited class II MHC induction in a concentration-dependent manner.

It has been shown that IFN-γ induces Fc-receptors on macrophages. To exclude false positive results due to non-specific Fc-receptor binding in the FRTL-5 model parallel samples were stained with the irrelevant monoclonal antibody OX-8, which is of the same subclass (IgG1) as OX-6 and OX-17. One hundred units per millilitre rIFN-γ induced only a 2% increase in OX-8 (non-specific) binding, compared to 80% for OX-6 and 87% for OX-17. Similar slight increases in OX-8 binding were seen in most experiments. Clearly, however, this non-specific binding demonstrated in the control samples does not account for the dramatic increases in OX-6 and OX-17 fluorescence, which may therefore be regarded as specific, reflecting true de novo expression of class II antigens on FRTL-5 cells.

7.3 DISCUSSION

Inappropriate expression of class II MHC antigens has been observed in several pathological situations (Dean et al., 1985; De Waal et al., 1983; Jansson, Karlsson and Forsum, 1984; Mason, Dallman and Barclay, 1981) and such aberrant expression has been proposed as a means by which autoimmune disease may be initiated (Hanafusa et al., 1983; Jansson, Karlsson and Forsum, 1984) or alternatively by which pre-existing autoimmune processes may be amplified at the end organ level (Dean et al., 1985). However, the pathways by which epithelial class II expression evolves in vivo remain incompletely defined.

In this study expression of class II (I-A homologue) on thyroid follicular epithelial cells was occasionally seen in areas of severe infiltration but never in the absence of leukocytes. However, close contact with the infiltrate did not automatically lead to Ia expression since Ia negative epithelial cells were seen in follicles entirely surrounded by Ia^+ leukocytes. This also argues against the possibility that Ia antigens on epithelial cells are acquired by passive absorption. Despite the low number of follicles expressing Ia antigens in different rat strains, a clear correlation between such expression and the level of infiltration was noticed, high responder strains such as AUG, CDF and LEW showing some Ia^+ follicles whereas low responders, such as AO, were completely negative. Other studies performed on different rat strains in relation to the induction of experimental autoimmune encephalomyelitis (EAE), have shown that the amount of Ia induced by interferon-γ on the vascular endothelium (Male and Price, 1988 -

in press) and in astrocytes (Massa, Ter Meulen and Fontana, 1987) correlated with susceptibility to EAE. Together with our findings this strongly suggests that class II expression on thyroid epithelial cells is a secondary event in the pathogenesis of rat EAT, directly related to the release of cytokines such as IFN-γ by activated leukocytes. If this is true, the absence of epithelial class II expression in the SAT of the BB/E rats could be explained by the minor degree of leukocytic activation, as judged by the poor OX-6 staining and also by the negative expression of IL-2 receptor, in the scarse focal cell infiltrates (chapter 5).

Previous studies of lymphokine action on thyroid epithelial cells level have uniformly relied on thyroidectomy specimens as a source of cells for short-term tissue culture. Although these models using human material are appealing, inherent limitations exist which have obscured the interpretation of functional studies. Firstly, these preparations are heterogeneous with as many as 10% undefined non-epithelial cells (Davies, 1985). A second source of uncontrolled variability may cells in prior adjacent pathological lesions mixing with the supposedly normal cells being studied.

To overcome these limitations attempts were made to isolate and characterize four essential elements of this system by using: i) a functionally differentiated non-transformed thyroid epithelial cell line (described in chapter 2, materials and methods), ii) recombinant lymphokines, iii) monoclonal anti-IFN-γ neutralizing antibody, and iv) quantitative flow cytometry. It was demonstrated that rat and mouse IFN-γ are potent inducers of class II MHC determinants on FRTL-5 cells in a manner similar to the human model (Todd et al., 1985; Weetman et al., 1985). Other agents

which stimulate growth and iodide transport in FRTL-5 cells had no discernible effect under these conditions, suggesting that the pathways of metabolic activation and class II expression are largely separate.

While IFN-γ appears to be the major central modulator of thyroid epithelial class II expression, the data presented here do not address the question of whether other cytokines may act in synergy with IFN-γ. An enhancing effect of tumour necrosis factor (TNF) on IFN-γ induced epithelial class II expression was originally observed by Pujol-Borrell and colleagues (1987) on pancreas islet cells and has recently been demonstrated by Weetman and Rees for thyroid (1988). Direct cytotoxicity of lymphokines may also be relevant for follicular damage in autoimmune thyroiditis. TNF and lymphotoxin (TNF-β) have proved effective in cytotoxicity assays (Stone-Wolff et al., 1984; Taverne et al., 1987). It is likely that in the local environment within inflammed endocrine glands these interactions play a role in the pathogenesis of autoimmune diseases, since the inflammatory infiltrates seen in both rat SAT and EAT contain cells capable of secreting these cytokines.

Thyroid cells bearing class II (HLA-DR) antigens have been shown to stimulate proliferation of autologous T lymphocytes (Matsunaga et al., 1986), and are capable of presenting pre-processed antigens to T cells (Londei et al., 1984). However in other studies Ia-positive TEC induced a proliferative response of allogeneic T cells only in the presence of phorbol myristate acetate (PMA) (Stein and Stadecker, 1987). The authors interpret this as an effect of PMA on the TEC, however, it seems more likely that PMA preactivates the T cells mimicking additional factors derived from

APC's under physiological circumstances which Ia^+ TEC do not provide, thus rendering them inefficient as APC's. The FRTL-5 line was derived from an inbred rat strain, CDF, which is a high responder strain for experimental autoimmune thyroiditis (chapter 3). Since syngeneic CDF lymphocytes (and other cells) are readily available, FRTL-5 may represent a useful model system for the study of antigen presentation by epithelial cells in thyroid autoimmunity.

In summary, the in vitro model conclusively demonstrates that epithelial cells themselves are capable of expressing class II molecules. Data from the in vivo model support the view that epithelial class II expression in autoimmune thyroid disease may be secondary to the activation of autoimmune T lymphocytes.

CHAPTER 8

NON-OBESE DIABETIC MICE AS A MODEL FOR INDUCED TOLERANCE IN AUTOIMMUNE THYROIDITIS

8.1 INTRODUCTION

Non obese diabetic (NOD) mice provide a spontaneous animal model of insulin dependent diabetes mellitus (IDDM) (Makino et al.,1980). In this model 80% of females and about 10% of the males spontaneously develop IDDM with lymphocytic infiltration of the pancreas and selective destruction of the islet β-cells at about 150 days of age. The marked difference in incidence of IDDM has been shown to depend on sex hormones. Androgens have a protective effect since castrated males show the female disease pattern whereas female sex hormones may aggravate the disease (Makino et al., 1981). As in the other spontaneous model of IDDM, the BB rat, thyroiditis also develops spontaneously in some animals. The MHC of the NOD is unique being $K^{d}I\text{-}A^{nod}I\text{-}E^{0}D^{b}$. The unique I-A of the NOD has been shown to arise as a consequence of the unusual amino acid sequence in the first external domain of the Aβ chain, the Aα chain being $A\alpha^{d}$. The amino acid in position 57 of the Aβ chain has been shown to be a serine and by analogy with observations made on the relationship between human IDDM and the amino acid used at position 57 in DQβ this amino acid would be permissive for the development of IDDM (Todd, Bell and McDevitt, 1987). The non

-expression of I-E in this animal is due to a 650 bp deletion in the promoter region of the Eα gene exactly as is found in the C57Bl mouse (T. Lund, personal communication). Introduction of the $E\alpha^d$ gene into the NOD mouse has been shown to lead to the expression of I-E and to prevent the development of insulitis in these animals (Nishimoto et al.,1987). BALB/c ($H\text{-}2^d$) and C57Bl/6 ($H\text{-}2^b$) mice have been shown to be poor responders for induced autoimmune thyroid disease (Vladutiu and Rose, 1971). Therefore it was of interest to examine the responder status of the NOD mouse with regard to the experimental induction of thyroiditis.

It has been shown that mice injected intravenously with soluble mouse Tg become tolerant to subsequent immunization with Tg+CFA and that this tolerance could be transferred into syngeneic recipient with $Thy1^+$ spleen cells (Kong et al., 1982). Recently the cells responsible for induced tolerance have been phenotypically characterized in CBA/Ca and CBA/J mice and shown to be $CD5^+/4^+/8^-$. This supports the concept of suppressor cells but makes it unlikely that induced tolerance in this model is directly mediated by T suppressor cells of $CD8^+$ phenotype (Parish, Roitt and Cooke, 1988b). It was of interest to standardize the NOD mouse as a further model of autoimmune thyroiditis and tolerization since NOD mice spontaneously develop thyroiditis and thyroglobulin autoantibodies albeit at a low incidence.

8.2 RESULTS

Attempting to standardize this model of thyroiditis, TgAb titres in NOD and (NODxCBA)F1 were compared after injection of MTg with LPS or CFA as adjuvant. Groups of three male animals 3-4 month old were immunized with: 1) MTg(50μg i.v.)/LPS(20 μg i.p.) twice on day 0 and 7; 2) MTg+CFA (50 μg) administered i.p. once; 3) MTg+CFA (25μg) injected into each hind foot pad once. Individual sera were assayed on day 14 for TgAb titres by ELISA.

This experiment showed that TgAb responses tended to be lower in NOD as compared to (NODxCBA)F1 for all three protocols used. It also showed that NOD mice respond better to the priming with MTg+CFA in the foot pad injection than to the other two protocols. No difference between the immunization protocols was observed in (NODxCBA)F1 (Fig 8.1).

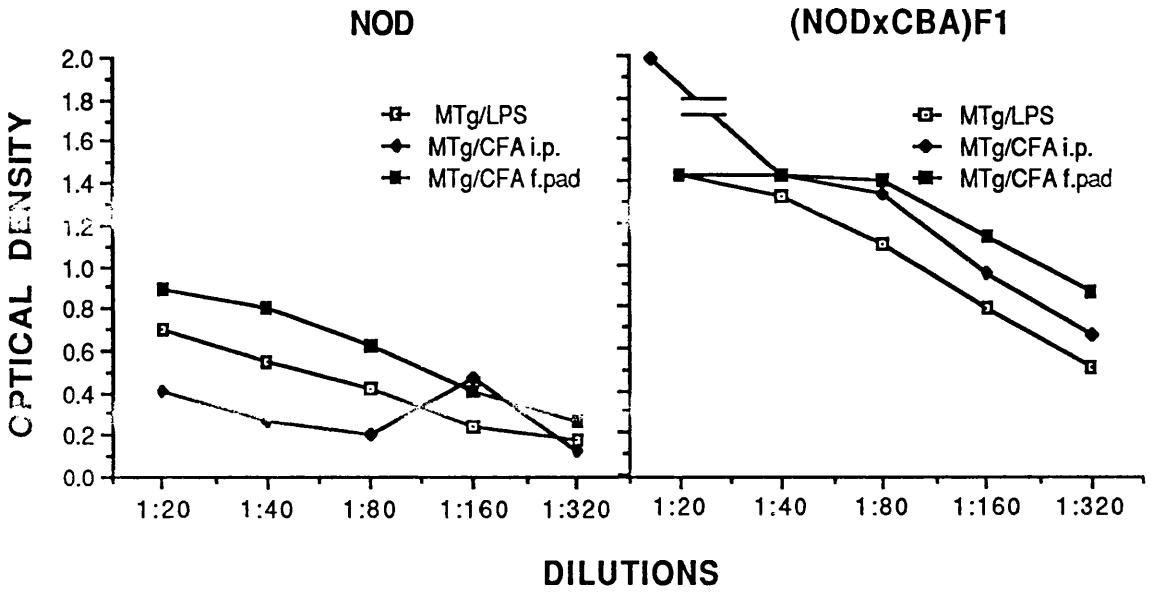

FIGURE 8.1: TGAB TITRES IN NOD AND (NODxCBA)F1 IMMUNIZED WITH MTG AND LPS OR CFA.

To further investigate the adjuvant effect, five male CBA/Ca mice and five female NOD were immunized with MTg(50 μg i.v.)/LPS(20μg i. p.) twice on day 0 and 7. Two groups of controls were included in this experiment, namely animals injected with LPS only and untreated mice (5 in each group). Sera were assayed on day 14 for TgAb titres by ELISA. On day 28 all animals were sacrificed and the thyroids were taken and snap frozen. Cryostat sections obtained from these glands were examined for the presence of mononuclear cell infiltration using Toluidine blue staining.

This experiment showed that LPS alone caused a slight increase in TgAb CBA mice ($p<0.05$) and also that CBA mice immunized with MTg and LPS developed higher TgAb titres ($p<0.05$)

than NOD (Figure 8.2). In some NOD mice treated with LPS only, thyroiditis was as severe as seen in the actively immunized animal. NOD mice immunized with MTg+LPS tended to develop higher degrees of thyroiditis than CBA mice similarly challenged (Figure 8.3), although this difference was not statistically significant ($p>0.05$).

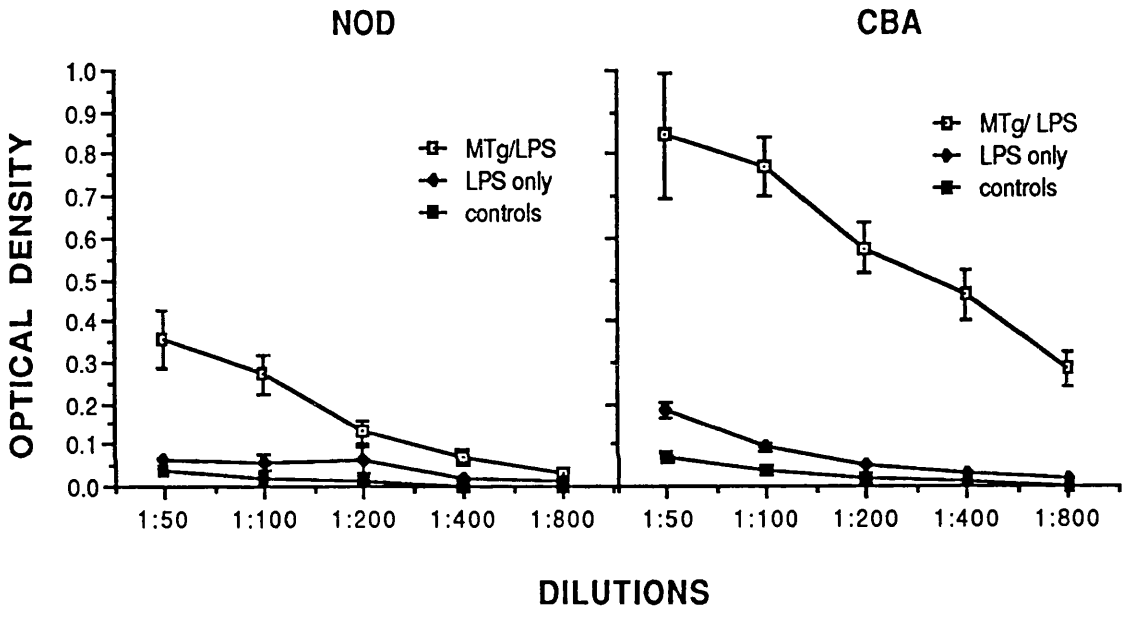

FIGURE 8.2: TgAB TITRES IN NOD AND CBA MICE IMMUNIZED WITH LPS EITHER ALONE OR SIMULTANEOUSLY WITH MTG.

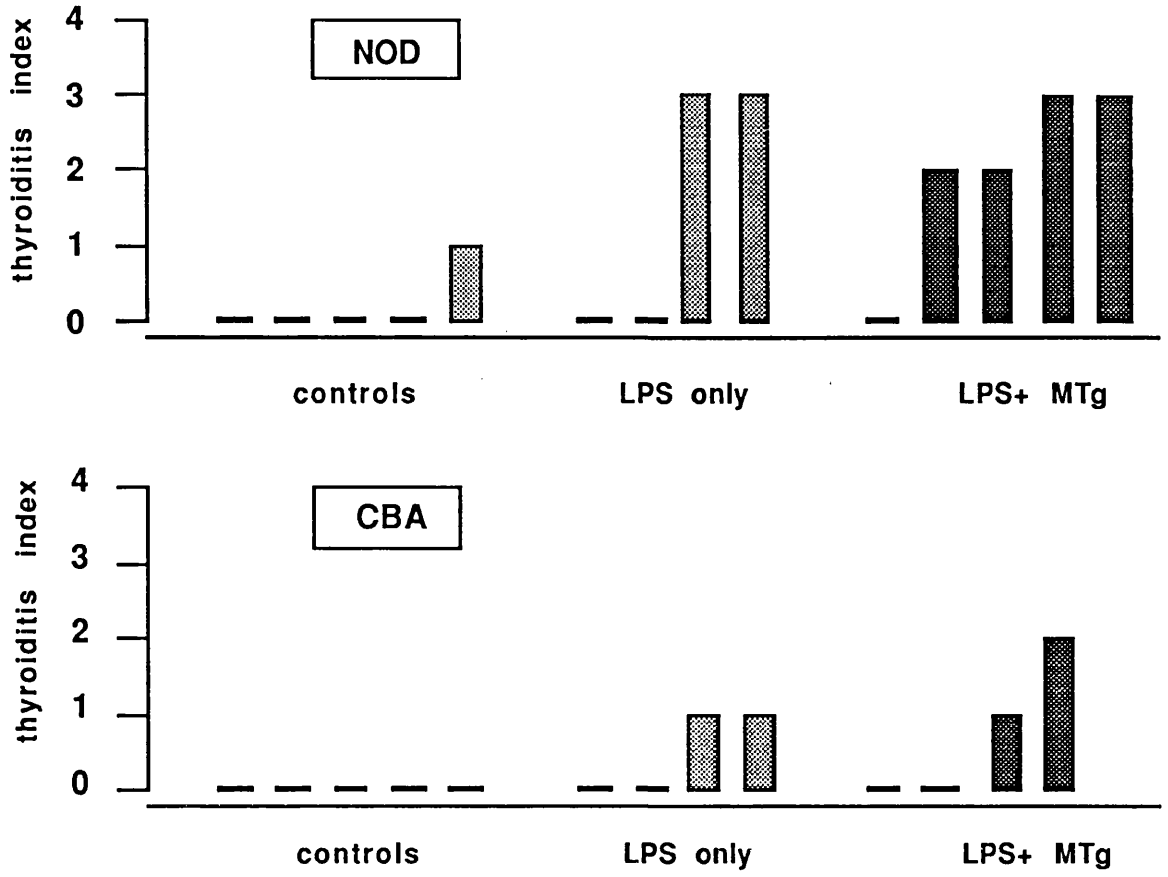

FIGURE 8.3: THYROIDITIS INDEX IN NOD AND CBA MICE USING VARIOUS IMMUNIZATION PROTOCOLS.

To assess the effect of a tolerizing dose of MTg on the incidence of thyroiditis and TgAb titres, five male NOD mice were tolerized by an injection of 200 μg of MTg in PBS intravenously on days 0 and 7. A control group was given PBS only. On day 10 both groups were challenged with 50 μg of MTg+CFA in the footpad. Mice were bled 14 days after immunization for quantitation of Tg-Ab by ELISA. On day 28 all animals were sacrificed and the thyroids snap frozen for cryostat sectioning and toluidine blue staining. Macroscopically, thyroids from the tolerized and non-TOLERIZED

groups were indistinguishable regarding size and colour.

Tg-Ab levels were significantly lower ($p<0.05$) in tolerized animals when compared to the group which was immunized only (Figure 8.4). Thyroiditis as focal infiltration was seen in only one out of five animals from the tolerized group. On the other hand, the group immunized without prior tolerization showed foci of lymphocytic infiltration in four out of five animals. The index of infiltration, scored from grade zero to four, was much lower in the tolerized animals but because of the small number of animals in this initial experiment the difference did not reach statistical significance ($p>0.05$)(Figure 8.5).

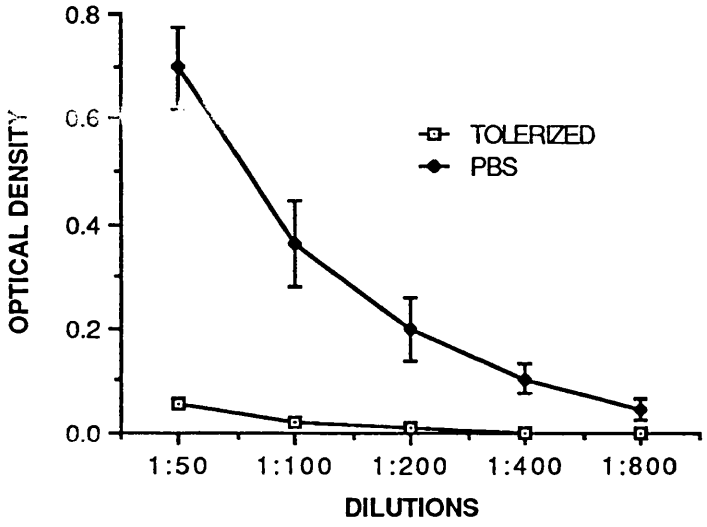

FIGURE 8.4: TGAB TITRES IN TOLERIZED AND NON-TOLERIZED NOD MICE AFTER IMMUNIZATION WITH MTG+CFA.

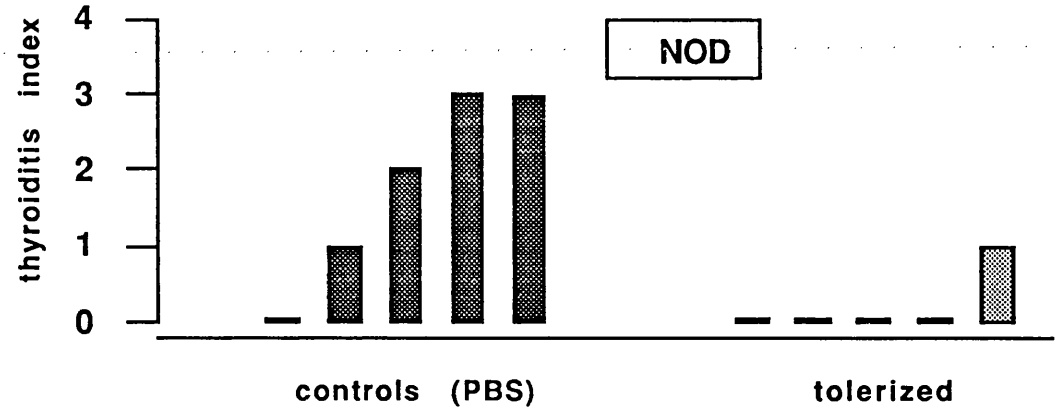

FIGURE 8.5: THYROIDITIS INDEX IN TOLERIZED AND NON-TOLERIZED NOD MICE AFTER IMMUNIZATION WITH MTG+CFA.

8.3 DISCUSSION

Preliminary studies in our laboratory have shown that some NOD mice spontaneously develop elevated TgAb in the serum. This did not correlate with sex or the development of diabetes and the animals remained healthy. Because NOD mice seem susceptible to various autoimmune diseases it was of interest to examine their susceptibility to the induction of thyroiditis. Therefore NOD mice were immunized with mouse thyroglobulin using different protocols, adjuvants and routes of immunization. The responses of NOD mice were compared with those of CBA mice ($H\text{-}2^k$) and the (CBAxNOD)F1 hybrids. Our preliminary studies suggest that although the induced levels of autoantibody to MTg appear to be lower in NOD compared to CBA (Figure 8.2) the extent of infiltration in the NOD thyroid appeared to be much greater (Figure 8.3). Thyroid infiltration in the NOD was markedly reduced following tolerization with high doses of Tg intravenously prior to immunization.

The fact that some NOD mice spontaneously develop thyroiditis will make it possible to compare this with the induced thyroiditis in the NOD and CBA with regard to those cell subsets involved in the thyroid infiltrates. Since $E\alpha^d$ transgenic NOD mice have now been developed (Nishimoto et al., 1987, Cooke et al., manuscript in preparation) the influence of the $E\alpha$ gene on susceptibility to induced and spontaneous thyroid autoimmunity can also be studied.

The serological and inflammatory response of the NOD to active immunization resembles that of high responder strains

despite the NOD mouse being of low responder haplotype (H-2^d and H-2^b) in the MHC. It has however unique sequences in the Aβ and the Eα subregions, the latter leading for example to the absence of expressed I-E molecules. Expression of I-E has been proposed to be of importance for the normal generation of suppression (Oliveira et al., 1985) and the prevention of autoimmune disease. Support for this proposal has come from the observed abrogation of IDDM in $E\alpha^d$ transgenic NOD mice (Nishimoto et al., 1987). It is possible that the $I\text{-}E^0$ NOD haplotype not only contributes to the development of diabetes but confers susceptibility to tolerance breakdown in these animals as demonstrated in these experiments by the facility of induction of thyroiditis. If this were true the $E\alpha^d$ transgenic mice will be of low responder status for EAT. However, the ability to induce tolerance to EAT in adult NOD argues against the hypothesis that non-expression of I-E may lead to an increased susceptibility to induced thyroid autoimmunity. An alternative explanation for the effect of I-E expression on IDDM in the NOD is that the expressed T cell repertoire is modified in the transgenic animal.

Future studies will aim at the mechanism underlying tolerance induction in NOD EAT. If it is similar to the CBA model (Parish, Roitt and Cooke 1988b) tolerance will be transferable by CD_4^+ T cells from tolerized animals. Since tolerance to EAT is readily induced in NOD mice which at the same time have a tendency to spontaneously develop thyroiditis, the question arises whether induced tolerance is a suitable model for the mechanisms preventing the spontaneous disease. Further investigations are therefore necessary to establish the effect of induced tolerance on

the spontaneous thyroiditis of the NOD mouse. If intravenous injection of thyroglobulin is able to abrogate SAT in these animals this could have important implications for our knowledge about the autoantigen involved in the thyroid autoimmunity of this animal.

CHAPTER 9

GENERAL DISCUSSION

Thyroglobulin from both endogenous and exogenous sources triggers humoral and cell mediated reactions in genetically susceptible animals which lead to thyroiditis. A major role of antibody mediated autoreactivity has been suggested from studies in the spontaneous models of autoimmune thyroiditis, the OS chickens (Wick et al., 1982) and BUF rats (Silverman and Rose, 1974), whereas cell mediated immunoreactivity is regarded to be essential in the experimental models in mice (Creemers et al., 1984) and rats (McGregor et al., 1983). However, full-blown disease is only found in the presence of both thyroid infiltrate and Tg autoantibodies, strongly suggesting cooperation of both humoral and cellular mechanisms in the pathogenesis of autoimmune thyroiditis.

The work described in this thesis approached the relative contribution of these mechanisms using a four-step programme. Initially, the protocol for the induction of EAT in rats was standardized in terms of antigen dose, time course, adjuvants and strains of rats, looking at inbred, congenic and intra-RT.1 recombinant animals. In this step, the locus of the putative immune-response gene to Tg (Ir-Tg) was mapped in the rat MHC (RT.1). Secondly, the binding profiles to Tg from different species were defined for Tg autoantibodies both spontaneously arising (SAT) and induced (EAT) in order to characterize their epitope fine specificity. Thirdly, the thyroid infiltrates were phenotyped in both induced and spontaneous models using monoclonal antibodies,

particularly directed to leukocyte markers. Finally, epithelial class II (Ia) antigen expression was investigated in both rat SAT and EAT using immunohistology and studied in the rat thyroid cell line FRTL-5, in vitro, inducing epithelial class II expression with interferon-γ.

9.1 MAPPING OF THE Ir-Tg IN THE RAT MHC (RT.1) COMPLEX

Since the Tg plus CFA immunization-induced EAT was established as a model for human autoimmune thyroiditis, efforts have been concentrated on the localization of the immune response gene to Tg (Ir-Tg). The first evidence that high and low responder status was conferred by genes mapping to the MHC region came from the mouse model (Vladutiu and Rose, (1971). In the rat the genetic linkage of the Ir-Tg with the MHC (RT.1) complex has not been possible because of the lack of intra-RT.1 recombinant strains of rats (Lillehoj, Beisel and Rose, 1981). The recent availability of PVG rat strains congenic for different haplotypes at the RT.1 locus and, particularly, of the PVG.r1 strain intra-RT.1 recombinant for the haplotypes 'a' and 'c', has permitted mapping of the gene controlling high susceptibity to rat induced EAT (haplotype c) to the right side of RT.1 complex (diagram Figure 8.1).

The genotype of the PVG.r1 strain ($A^aB^cD^cC^c$) suggests that high susceptibility is a trait mainly controlled by class II genes although further experiments are necessary to fully define the influence of other regions, especially a possible role for class I genes on the right hand side of the RT.1 complex. It is interesting to

note that in mice the severity of thyroid cellular infiltration determined by the I-A region is modulated by the D-end encoding class I genes (Kong et al., 1979).

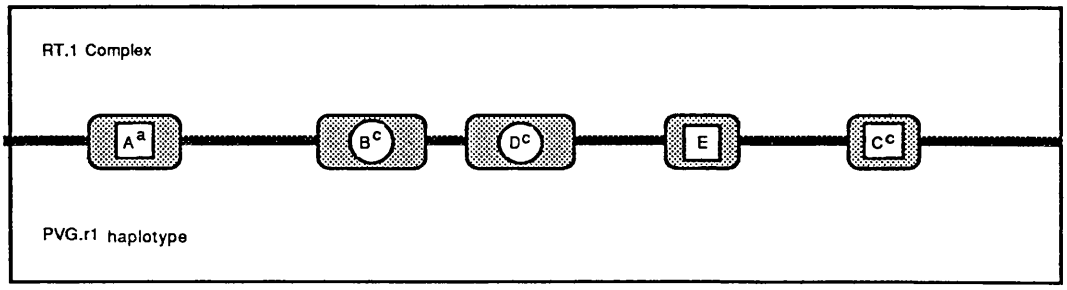

FIGURE 8.1: Schematic representation of the intra-RT.1 recombinant haplotype of the PVG.r1 strain. A^a, B^c, D^c and C^c indicate the allotypes at the different MHC subregions in this strain.

From a panel of inbred rat strains carrying different RT.1 haplotypes it was possible to classify the susceptibility to disease induction according to the haplotype. Thus, strains of $RT.1^c$ and $RT.1^l$ are high responders whereas those of $RT.1^u$ are low responders. This genetic pattern of susceptibility to EAT, as already reported in mice (Rose et al., 1977), is autosomally dominant, since F1 animals from the cross of congenic PVG-$RT.1^a$ and PVG-$RT.1^c$, low and high responder respectively, are generally high responders. Responsiveness could not be predicted based on Tg antibody titres as already reported in the mouse EAT (Tomazic and Rose, 1977). The dissociation of the humoral and cellular arms of the immune response was most clearly seen in the poor responder strain AO. In

this strain (RT.1^{u} haplotype) no thyroid infiltrate was seen even though high Tg autoantibody levels were attained.

The TNF gene has recently been mapped to the MHC complex (Muller et al., 1987). Genetic analysis using restriction length fragment polymorphisms have indicated that there are allotypic variants of the TNF gene which have been shown to correlate with levels of TNF secretion (Jacob and McDevitt, 1988). Because of the location of the TNF gene within the MHC it could be expected to be in linkage disequilibrium with MHC. Therefore, high and low responses thus far attributed to the MHC haplotype of the animal, might in fact be due to genetic differences in the TNF gene which in turn influence TNF secretion, thus shifting the balance of the immune system towards hyperreactivity.

The fact that RT.1^{u} haplotype in SAT is associated with susceptibility to the development of thyroiditis indicates that the RT.1 locus is not alone responsible for the responder status of the animal but that other genes also play a role.

9.2 DIFFERENCES IN FINE-SPECIFICITIES OF Tg AUTOANTIBODIES BETWEEN EAT AND SAT IN THE RAT

Although thyroglobulin is a major autoantigen implicated in thyroid autoimmunity the molecular identity of the autoimmunogenic determinants causing the autoimmune response is not yet known. Using a large panel of Tg extracted from various species the specificities of induced and spontaneous Tg autoantibodies were characterized. Induced autoantibodies from high and low EAT responder rats reacted with all Tg species

whereas the spontaneously arising Tg autoantibodies showed three different patterns of recognition. The fact that most of the autoantibodies studied recognized rat as well as other heterologous Tgs indicates that highly conserved epitopes constitute the Tg antigenic domains. This is in agreement with the findings obtained with Tg autoantibodies from mice (Champion et al., 1988) and humans (Kohno, Nakajima and Tarutani, 1985).

Displacement studies have demonstrated that Tg autoantibodies arising in humans (Pearce et al., 1981) and in OS chickens (Brown, Bagchi and Sundick, 1985) recognize T3 and T4 sites in the thyroglobulin molecule. Using a small panel of human Tg with different molar concentrations of iodine a clear dependence of the binding capacity of Tg autoantibodies on the degree of Tg iodination was demonstrated. This indicates that hormonogenic regions of the molecule are recognized by both spontaneous and induced Tg autoantibodies. Confirming this, thyroxine inhibited the binding of spontaneous and induced Tg autoantibodies to highly iodinated thyroglobulin.

Demonstration of thyroid hormone autoantibodies in patients with autoimmune thyroiditis and evidence that thyroglobulin is the autoimmunogen for the synthesis of such autoantibodies (Himsworth, Byfield and Copping, 1983) support the view that thyroid hormones are important antigenic determinants on the thyroglobulin molecule. However, on a molar basis Tg has been reported to be more efficient than the free hormones in displacing labelled hormones from thyroid hormone autoantibodies. This suggests that amino acids surrounding the iodothyronine residues on the Tg primary structure also contribute to the binding sites of

thyroid hormone autoantibodies (Pearce et al., 1981). The idea that residues surrounding an epitope take part in the antigen-combining site has also been drawn from experiments with digested thyroglobulin. Male et al. (1985) have shown that fragments of human Tg, produced with staphylococcal V8 protease, bound Tg autoantibodies less effectively than the undigested protein. Using peptic fragments of human Tg, Stylos and Rose (1977) observed that larger fragments had the same reactivity with Tg autoantibodies as native Tg whereas smaller fragments had reduced reactivity. All these observations support the concept that conformational epitopes depending on the tertiary structure of the Tg are recognized by Tg autoantibodies.

In summary the data presented here indicate that spontaneous and induced Tg autoantibodies recognize different epitopes on Tg, although the conserved hormonogenic regions are important binding sites for Tg autoantibodies in both cases.

9.3 PHENOTYPIC CHARACTERIZATION OF THE THYROID INFILTRATES IN RAT EAT AND SAT

The majority of leukocytes infiltrating the thyroid in both SAT and EAT were T lymphocytes and macrophages. In contrast to the SAT, the T cell population in the EAT expressed class II antigens at high density and also IL-2 receptors suggesting a state of T cell hyperreactivity in the EAT infiltrate. Among the T lymphocytes, the $CD4^+$ subset predominated in SAT whereas $CD8^+$ cells were more frequent in the EAT infiltrate. A dominant role for $CD8^+$ cells in generating thyroid lesions has also been demonstrated

in mouse EAT (Creemers et al., 1983; Simon et al., 1986). In the infiltrates of both Graves' disease and Hashimoto's thyroiditis $CD4^+$ cells are more frequent than $CD8^+$ cells, this bias being particularly pronounced in Graves' disease. In this respect rat SAT more closely resembles the human forms of autoimmune thyroiditis. The validity of induced thyroiditis in the rat as a model of the human disease thus remains an open question.

Macrophages of different subpopulations as well as dendritic cells were found in both EAT and SAT infiltrates in significant numbers. These cells seem to play an important role in the initiation and maintenance of the disease. Kabel et al. (1987) demonstrated that dendritic cells were the first phenotype present in the thyroid interstitium in the initial phase of the disease in BB rats. A contribution of macrophages in the perpetuation of the disease was recently demonstrated by Cohen, Dijkstra and Weetman (1988). They observed in BUF rats treated with silica that both Tg autoantibody titres and thyroid damage ameliorated simultaneously with the clearance of tissue macrophages.

A fundamental difference observed between the SAT and EAT infiltrates is the presence of B lymphocytes in SAT as opposed to their absence in the EAT. This confirms the importance of the thyroid as a site of production of Tg autoantibodies in the spontaneous disease (Wick et al., 1982) and emphasizes the participation of antibody mediated mechanisms in the expression of the disease. Despite the absence of B cells from the gland the characteristic deposits of immunoglobulin in the thyroid interstitium and in damaged follicles in EAT infiltrates indicate that humoral mechanisms operate in both animal models.

9.4 INDUCTION OF CLASS II EXPRESSION BY IMMUNE MEDIATORS

Inappropriate class II expression on thyroid epithelial cells has been reported in SAT (Wick et al., 1984) and EAT (Cohen, Dijkstra and Weetman, 1988) in the presence of a T cell infiltrate. Potentially, therefore, autoantigens bound to the thyroid cell surface could be recognized by the immune system in conjunction with class II gene products, which may be relevant for the pathogenesis fo autoimmune thyroiditis. It has been reported that Ia-positive thyroid epithelial cells are indeed capable of presenting foreign peptide antigens which do not need further processing (Londei et al., 1984). In this context, the characterization of the cell infiltrate associated with epithelial class II expression as well as the nature of possible inducing agents and the conditions modulating its effects is important for the understanding and future therapeutic control of the autoimmune process.

In the rat EAT, epithelial class II expression, although not very common, was always restricted to areas of dense leukocyte infiltration. In contrast, the rat SAT did not show any expression even in areas of focal infiltration. The fact that cells in the SAT infiltrates are poorly activated supports a role for activated cells in the EAT infiltrate in the induction of Ia molecules on the thyroid epithelium. It is therefore likely that such Ia expression is cytokine-mediated. The EAT infiltrate was rich in $CD4^+$ and $CD8^+$ cells which are capable of producing interferon-γ (IFN-γ) (Trinchieri and Perussia, 1985). The possible role for interferon-γ as trigger

for epithelial class II expression was tested in a rat thyroid epithelial cell line (FRTL-5) in vitro, using rat recombinant IFN-γ as the immune mediator. Class II expression was induced with this cytokine and this effect could be inhibited by simultaneous incubation with a monoclonal anti-IFN-γ antibody (Rayner et al., 1987). Attempts to mimic interferon-γ activity by using thyroid mediators such as dibutyryl cyclic AMP and TSH suggested that the pathways through which IFN-γ induces class II expression on FRTL-5 do not involve the conventional thyrocyte function. However, evidence has been provided that the respective activities of these mediators and IFN-γ are not totally independent (Todd et al., 1987). This is in agreement with the reported enhancement of membrane thyroglobulin expression after stimulation of thyrocytes with IFN-γ in culture (Champion et al., 1988).

Although the mechanisms by which IFN-γ exerts its effect on the thyrocyte are not completely understood, the available evidence indicates that the observed epithelial class II expression in vivo might be secondary to leukocytic infiltration. Nevertheless, this mechanism could play an important role in the enhancement or maintainance of the autoimmune reaction, particularly if it occurs in conjunction with enhanced expression of membrane Tg, leading to aberrant autoantigen presentation to the immune system.

9.5 OUTLINE OF FUTURE PROJECTS

To further investigate the relevance of the rat models of EAT and SAT with human autoimmune thyroid disease, the disease in the animals should be followed up in long term studies with continual assessment of their endocrine and metabolic status.

In this study the humoral arm of the immune response to thyroglobulin has been analysed at the serum level. To study the B cell repertoire at the cellular level hybridomas could be generated from spleen cells and cells from regional lymphnodes to obtain panels of monoclonal antibodies from three groups of rats: non-immunized control animals, aimals immunized with Tg (EAT) and animals prone to develop thyroiditis spontaneously (SAT). This approach could address the question of differential epitope recognition at the level of single antibody specificities.

B cells capable of secreting TgAb may be present in the normal repertoire (natural autoantibodies) but secrete only small amounts of antibodies not detectable at the serum level. A panel of hybridomas generated from non-immunized animals could help to identify such autoreactive B cells which may be the precursors of B cells secreting the pathogenic autoantibodies in thyroiditis.

In a second step anti-idiotypic antibodies may be used to look for the presence of cross reactive idiotopes which may be regulatory and to analyse V-gene usage in TgAb.

Thirdly, B cells could be analysed at the molecular level by probing RNA on Northern blots for different V-region families as well as by cloning and sequencing the Ig genes.

These investigations would reveal any restrictions in Ig-gene rearrangements in TgAb and identify somatic mutations. By comparing natural autoantibodies with those derived from the immunized group it may become possible to determine whether the autoimmune repertoire is really derived from naturally autoreactive cells. It is still an open question if in spontaneous autoimmune disease breakage of B cell tolerance is due to polyclonal activation or if it is antigen driven. Since EAT is clearly antigen-induced, comparison of monoclonal TgAb derived from these animals with those generated in the process of SAT will be of particular interest.

To complement the histological analysis of the cellular arm of the immune response to Tg, T cell cloning tecniques can be applied. Using limiting dilution studies with thyroglobulin, thyrocytes or thyrocyte extracts as antigens, precursor frequencies of autoreactive T cells in the thyroid infiltrates and spleen could be determined and compared between animals with high and low responder haplotype.

The clones could be further investigated with regard to

a) epitope specificity
b) phenotype (flow cytometry)
c) MHC class I or class II restriction
d) MHC haplotype restriction
e) activation requirements (cytokines)
f) cytokine production
g) <u>in vitro</u> cytotoxicity
h) B cell help
i) T cell receptor gene use (Vα and Vβ families)

This will lead to a better understanding of the function of the cells which have been demonstrated in the thyroid infiltrates by phenotypic analysis.

To identify antigens other than Tg which might play a role in rat autoimmune thyroiditis extracts from thyroids and from FRTL-5 cells (possibly after exposure to cytokines) should be analysed.

B cell epitopes may be identified by Western blotting using immune sera and monoclonal antibodies, while proliferation assays with poly- and monoclonal T cells will detect T cell antigens in the fractions of the extracts.

Finally, the functional importance of the different components of the immune response should be assessed in transfer experiments. Immune serum, monoclonal antibodies, different cell populations and T cell clones should be tested for their ability to induce, aggravate, maintain, or on the other hand, alleviate or prevent autoimmune thyroiditis in the rat.

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Interferon-Mediated Enhancement of Thyroid Major Histocompatibility Complex Antigen Expression

A Flow Cytometric Analysis

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Rayner, D.C., Lydyard, P.M., de Assis-Paiva, H.J., Bidey, S., van der Meide, P., Varey, A.-M. & Cooke, A. Interferon-Mediated Enhancement of Thyroid Major Histocompatibility Complex Antigen Expression. A Flow Cytometric Analysis. *Scand. J. Immunol.* 25, 621–628, 1987

Epithelial expression of class II antigens encoded by the major histocompatibility complex (MHC) has been proposed as a means by which autoimmune thyroid disease may be initiated and maintained. We studied a rat thyroid epithelial cell line (FRTL-5), which constitutively expresses class I (OX18) but not class II (OX6 or OX17) determinants to quantify in vitro MHC antigen induction using flow cytometry. Recombinant rat γ interferon (rIFN-γ) induced dose-dependent expression of OX6 (I-A) antigen at >48 h (maximum 80–90% of cells in culture at 100 U/ml), which was abrogated by DB-1, a monoclonal antibody to rat IFN-γ. OX17 antigen (I-E) was also induced (86%) and OX18 (class I) markedly increased under these conditions. Other thyroid-active agents including the calcium ionophore A23187, dibutyryl cyclic AMP, thyroid-stimulating autoantibodies from Graves' disease patients (LATS), and TSH, caused no I-A induction. Supernatants from spleen cells stimulated with plant lectins (concanavalin A or phytohaemagglutinin), but not lectin alone, evoked substantial class II induction, which was inhibited by DB-1. These findings suggest that IFN-γ is the central mediator of thyroid epithelial class II expression. FRTL-5 provides a powerful model for the analysis of thyroid MHC class II dynamics and a potential means of analysing the role of epithelial class II in autoimmune pathogenesis.

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Antigens encoded by the major histocompatibility complex (MHC) show marked differences in their tissue distributions: class I MHC antigens, which include the classic tissue transplantation antigens, are expressed in varying densities on virtually all nucleated cells. In contrast, class II antigen expression is largely restricted to leucocytes, including B lymphocytes and some activated T lymphocytes, macrophages, and other antigen-presenting cells [15].

With certain exceptions [13, 16, 20], epithelial cells do not exhibit detectable levels of class II antigens under normal physiological conditions. However, in numerous clinical and experimental disease states, class II expression is dramatically increased [2, 6, 9, 10, 12, 14, 18, 28]. Since helper T-cell activation is, in general, class II MHC-restricted, this has been proposed as a means by which epithelial cells may communicate with T lymphocytes [13], potentially presenting self antigens and thereby initiating [7] or perpetuating [9, 29] autoimmune diseases.

Gamma interferon (IFN-γ) is a lymphokine produced by T cells and NK cells in response to both antigen-specific and nonspecific stimuli. While IFN-γ acts on a variety of target tissues,

one of its best defined effects is the enhancement of class II MHC antigen expression on both leucocytic and non-leucocytic cells [30].

In this way, IFN-γ provides central amplification for normal and pathological immune responses, and may maintain the abnormal epithelial expression of class II MHC antigens that is a feature of both Graves' disease and Hashimoto's thyroiditis [12].

In the last few years, the availability of recombinant lymphokines such as IFN-γ (and monoclonal antibodies specific for them) has permitted a striking increase in our understanding of the biochemistry and biological actions of individual lymphokines. In contrast, in vitro studies of thyroid epithelial class II expression have in general relied on primary cultures of clinical material, and have consequently been limited by interspecimen variability and lack of cellular uniformity.

In this study we describe the use of a well-characterized rat thyroid epithelial cell line, FRTL-5, as a new model for the analysis of fluctuations in thyroid MHC antigen levels.

MATERIALS AND METHODS

Animals. Fischer (F344) rats were obtained from Olac, Bicester, UK. Wistar rats were obtained from a breeding colony maintained at the Middlesex Hospital Medical School.

FRTL-5 culture. The cultured FRTL-5 cells used in this investigation were a non-transformed continuous line of epithelital cells derived from the Fischer rat thyroid. The morphology, TSH-dependent growth characteristics, and functional responses of this cell line have been described previously [1, 3, 25]. The culture conditions and incubation procedures adopted in the present study were essentially identical to those in previous reports [3, 4, 5]. For studies of MHC antigen induction, cells were plated at a density of 2×10^5/ml in 24-well tissue culture plates (0.5 ml cell suspension per well) and exposed to test agents for 60 to 84 h before detachment and staining. MHC antigen expression was assessed as described below.

Test stimuli. Recombinant IFN-γ (rIFN-γ) was produced in monolayer cell cultures of Chinese hamster ovary (CHO) cells. These cells were transformed with a plasmid containing the chromosomal rat IFN-γ gene, which was placed under the control of the simian virus 40 (SV40) early promoter [11]. The lymphokine was purified by antibody affinity chromatography as described by Van der Meide *et al.* [24] and was more than 98% pure, as shown by sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis and western blot analysis.

Recombinant human IFN-α_1 was the generous gift of Wellcome Biotechnology Ltd, Beckenham, UK. Recombinant murine IFN-γ and human TNF, produced by Genentech, Inc., were kindly provided by Boehringer Ingelheim, Vienna, Austria.

DB-1, a monoclonal antibody specific for rat and mouse IFN-γ, was prepared as described previously [24]. The antibody was obtained from ascites-bearing BALB/c mice and had antiviral neutralizing activity equivalent to 4×10^4 U/ml of IFN-γ.

Lymphokine-containing supernatants were prepared by exposing F344 or Wistar rat spleen cells to crude phytohaemagglutinin (PHA) (M form, Gibco, Paisley, Scotland) at a dilution of 1:50 from stock solution or to concanavalin A (Con A), 2.5 μg/ml (Miles-Yeda, Rehovot, Israel), for 48–54 h under standard tissue culture conditions [26], followed by centrifugation and ultrafiltration. Other test agents included calcium ionophore A23187 (Sigma, Poole, UK) at 20 μg/ml and N^6,2′-O-dibutyryladenosine 3′:5′-cyclic monophosphate ($(Bu)_2$cAMP) (Sigma) at 1 mM final concentration. Long-acting thyroid stimulator, used at 1 mU/ml, was a lyophilized standard preparation (LATS-B, coded 65/122) supplied by the National Institute for Biological Standards and Control, London, England. TSH (Armour Pharmaceuticals, Eastbourne, UK) was added to some cultures at 5.6 mU/ml.

Flow cytometry. The IgG_1 monoclonal antibodies OX 6, OX 8, OX 17, and OX 18, with the specificities shown in Table 1, were the gift of Dr D. W. Mason, MRC Cellular Immunology Unit, Sir William Dunn School of Pathology, University of Oxford. Fluoresceinated rabbit anti-mouse Ig was obtained from Dako, High Wycombe, England.

Cell monolayers were detached from 24-well plates by a 15-min exposure at 37°C to phosphate-buffered saline (PBS), pH 7.4, containing 1 mg/ml bovine albumin (Sigma) and 0.2 mg/ml disodium EDTA. Washed cell suspensions were incubated for 45 min at 0°C with 1:2 dilutions of monoclonal antibodies in PBS containing 0.05% sodium azide and 1 mg/ml bovine albumin. Cells were then washed prior to a second 45-min incubation with the fluoresceinated conjugate. After two final washes, the cells were resuspended in 100–200 μl PBS containing sodium azide and albumin as above prior to flow cytometry (EPICS C, Coulter Electronics, Luton, UK). The fluorescence distribution was measured as log green fluorescence (LGFL), gated on the forward angle light scatter signal. The change in fluorescence intensity induced by the various treatments was evaluated by comparison with the LGFL of unstained control cells that had not been exposed to IFN (or alternately control cells stained with the irrelevant IgG_1 monoclonal antibody OX 8). All measurements were made at 250 mW laser power

TABLE 1. Monoclonal antibodies used for flow cytometry

Antibody	Specificity
OX 6	Class II MHC product (I–A homologue)
OX 17	Class II MHC product (I–E homologue)
OX 18	Class I MHC product
OX 8	T_c surface antigen (negative control)

and at least 5000 cells were analysed in each determination.

Fluorescence microscopy. In some experiments, cells were grown and stimulated on sterile 13-mm glass cover slips in 24-well plates. The cell monolayers were stained in situ without being detached from the cover slips. Specific cytofluorescence was visualized by means of a Zeiss Photomicroscope III fitted with an epi-fluoresence condenser and Zeiss filter set 48 77 10.

RESULTS

Modulation of MHC antigen expression by rIFN-γ

FRTL-5 cells were cultured for 76 h in the absence of TSH with 100 U/ml rIFN-γ and stained with OX 6, OX 17, and OX 18 IgG monoclonal antibodies. OX 8, an IgG monoclonal antibody specific for the rat CD8 molecule (homologous with human T8 or mouse Ly 2), was used to control for nonspecific antibody binding by FRTL-5.

Class II MHC antigen expression (both I-A and I-E) was induced on FRTL-5 by rIFN-γ, and this observation was verified by both conventional fluorescence microscopy (Fig. 1) and quantitative flow cytometry (Fig. 2a and b).

A trivial explanation would be that rIFN-γ induced Fc receptor expression on FRTL-5 cells, as has been shown for murine macrophages. To test this hypothesis, we stained parallel samples with the irrelevant monoclonal antibody OX 8, which is of the same subclass (IgG_1) as OX 6 and OX 17. One hundred units per millilitre rIFN-γ induced only a 2% increase in OX 8 (non-

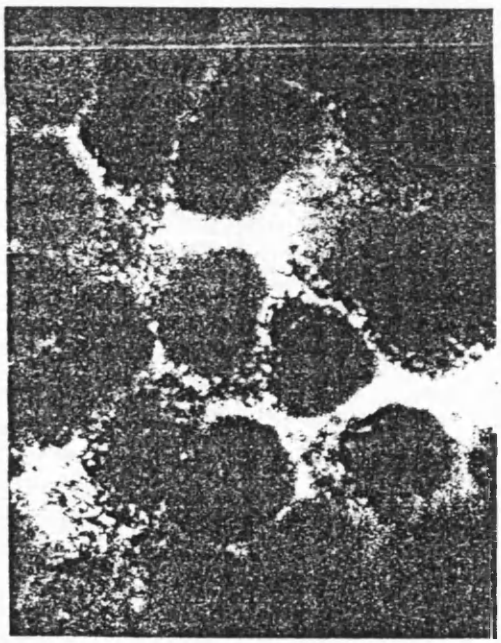

FIG. 1. Class II MHC determinants induced in vitro on FRTL-5 monolayers. Cells were incubated for 3 days with 90 U/ml rat rIFN-γ. Control monolayers incubated in parallel with no IFN-γ showed no detectable fluorescence. OX 6, indirect immunofluorescence. ×630.

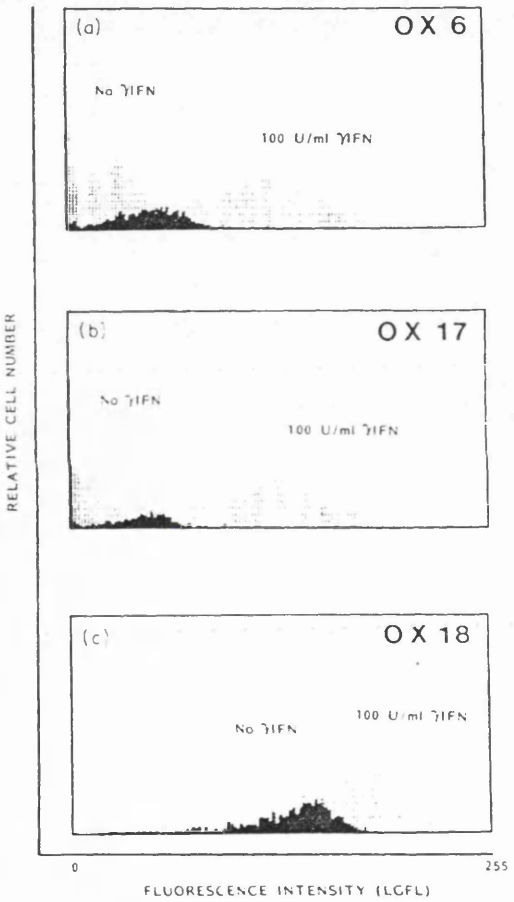

FIG. 2. Flow cytometric analysis of induced MHC antigens detected by (a) OX 6, (b) OX 17, and (c) OX 18 monoclonal antibodies. Fluorescence intensity is shown on a logarithmic scale (LGFL) from 0 to 255 on the horizontal axis; the relative cell numbers in each fluorescence channel are represented on the vertical axis. Basal fluorescence (following culture in IFN-free medium) is indicated by the left-hand curve of each histogram. The superimposed right-hand curves denote identical samples cultured for 76 h with 100 U/ml rat rIFN-γ.

specific) binding, compared with 80% for OX 6 and 87% for OX 17. Although similar slight increases in nonspecific binding were seen in most experiments, the specific development of OX 6 and OX 17 fluorescence on nearly all cells clearly reflects actual expression of new class II antigens.

Class I determinants are constitutively expressed by FRTL-5, but this expression was enhanced by incubation with rIFN-γ (Fig. 2c). Expressed quantitatively, 89% of control FRTL-5 cells (exposed to medium alone) exceeded an arbitrary gate value. Exposure to 100 U/ml rIFN-γ increased this proportion to 96%, and there was a corresponding shift in the peak channel from 116 to 172.

Effect of DB-1 on induced Class II MHC antigen expression

DB-1 is a mouse monoclonal antibody with specific neutralizing capacity for rat and mouse-IFNγ. To confirm that these increases in epithelial MHC antigen expression were directly attributable to IFN-γ, we tested the ability of DB-1 to block their induction. Addition of DB-1 to FRTL-5 cultures containing rIFN-γ inhibited class II MHC induction in a manner dependent on the concentration of DB-1 (Table II). DB-1 also inhibited the rIFN-γ-induced increase in class I MHC expression to baseline level (data not shown).

TABLE II. DB-1 inhibits IFN-γ mediated class II induction

rIFN-γ concentration	DB-1 concentration	OX 6 expression (%)
50 U/ml	0	82±2
50 U/ml	1:20,000	72±3
50 U/ml	1:2000	53±3
50 U/ml	1:200	4±1
0 U/ml	0	3±1

*Results are means±standard errors of the mean.

Time- and dose-dependence of IFN-γ action

Class II antigen expression was first detected less than 18 h after exposure to 100 U/ml rIFN-γ (Fig. 3). Plateau levels of class II expression (80–90% of the cells analysed) were seen in several experiments after 60–84 h. IFN-γ-induced class II expression was dose-dependent; maximal induction was achieved by 100 U/ml

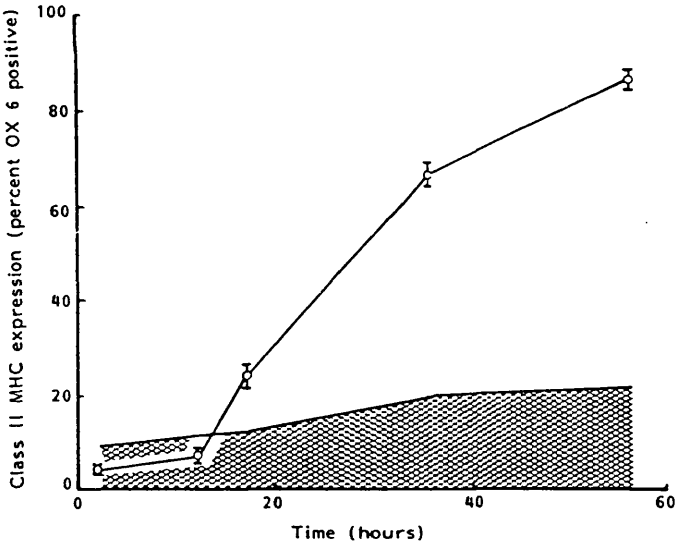

FIG. 3. Time course of class II antigen induction quantified by flow cytometry. Triplicate wells containing subconfluent FRTL-5 monolayers were exposed to 100 U/ml rat rIFN-γ for the times shown prior to detachment and staining with OX 6 monoclonal antibody. The shaded area denotes non-specific background staining measured using OX 8 monoclonal antibody.

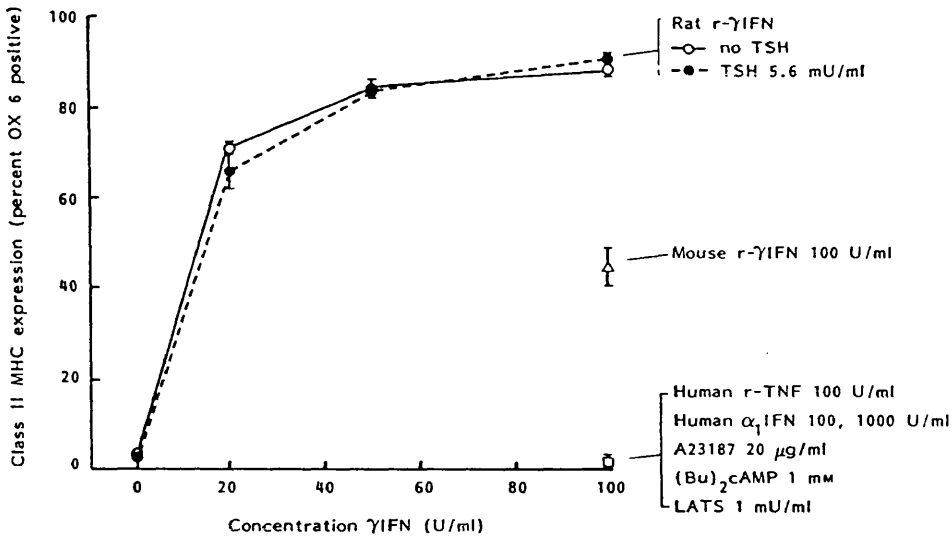

FIG. 4. Dose-dependence of class II antigen induction. FRTL-5 monolayers were cultured for three days in the presence of rat rIFN-γ or the other test agents shown prior to detachment, OX 6 staining, and flow cytometry.

rIFN-γ, and this was unaffected by growth-sustaining levels of TSH (Fig. 4). In other experiments, no further increase was effected by higher doses of rIFN-γ up to 1000 U/ml (data not shown). Murine rIFN-γ induced substantially less class II expression than rat rIFN-γ, while recombinant human IFN-α_1 and recombinant human TNF had no effect. Other mediators such as LATS [4], $(Bu)_2$cAMP, and A23187, which are known to activate thyroid epithelial cell metabolism, had no effect on class II antigen expression.

Effect of lectin-conditioned spleen cell supernatants

Supernatants from Con A- or PHA-stimulated Wistar rat splenic lymphocytes used at a final concentration of 80% caused an increase in class II (OX 6) antigen expression (Table III).

TABLE III. Induction of class II determinants by lectin-conditioned supernatants*

Stimulus	Inhibitor	OX 6 expression (%)
—	—	1.5±0.2
PHA (M form, 2%)	—	6±1†
PHA-supt (80%)‡	—	26±5
Con A 2.5 μg/ml	—	1±0
Con A-supt (80%)‡	—	20±2
Con A-supt (80%)‡	Db-1 1:100,000	23±2
Con A-supt (80%)‡	DB-1 1:10,000	11±1
Con A-supt (80%)‡	DB-1 1:1000	4±1
Con A-supt (80%)‡	DB-1 1:100	3±1

*Results are means±standard errors of the mean.

†PHA alone caused a parallel increase in OX 8 antibody binding from 5 to 13%, indicative of non-specific binding.

‡PHA- and Con A-conditioned supernatants were prepared by exposing Wistar spleen cells for 48 h to lectin in RPMI 1640 containing 5% newborn calf serum. Supernatants were centrifuged, dialysed into FRTL-5 culture medium (Coon's modification of Ham's F-12) and ultrafiltered.

Con A alone had no significant effect. PHA alone induced only a slight increase in non-specific binding, shown by comparable increments in OX 6 and OX 8 control fluorescence. Addition of 1:1000 DB-1 to Con A-stimulated spleen cell supernatant abrogated the ability of the supernatant to support class II induction, indicating that such induction was critically dependent on the presence of IFN-γ in the Con A-conditioned medium.

DISCUSSION

Inappropriate expression of class II MHC antigens has been observed in several pathological situations [2, 9, 10, 14, 18], and such aberrant expression has been proposed as a means by which autoimmune diseases may be initiated [12, 14], or alternatively by which pre-existing autoimmune processes may be amplified at the end organ level [9]. However, the pathways by which epithelial class II expression evolves in vivo remain incompletely defined.

Previous studies of lymphokine action at the thyroid epithelial cell level have uniformly relied on thyroidectomy specimens as a source of cells. These models have had inherent appeal on the basis of being human material in short-term tissue culture, but have had attendant limitations which in particular have obscured the interpretation of functional studies. First, these preparations may suffer from substantial cellular heterogeneity (with as much as 10% undefined nonepithelial elements) [8]. A second source of uncontrolled variability may be the effects of adjacent pathological lesions.

We used the techniques of modern cell biology to isolate the four essential elements of this system: (a) a functionally differentiated non-transformed thyroid epithelial cell line, (b) recombinant lymphokines, (c) monoclonal anti-IFN-γ-neutralizing antibody, and (d) quantitative flow cytometry. Our findings indicate that rat and mouse IFN-γ are potent inducers of class II (and class I) MHC determinants on FRTL-5 cells, in a manner similar to the human model [23, 27]. Other agents that stimulate growth and iodide transport in FRTL-5 cells had no discernible effect under these conditions, suggesting that the pathways of metabolic and immunological activation are largely separate.

Whether other agents such as plant lectins can act directly to stimulate class II expression [8, 21] has been a controversial point [27], which has been extremely difficult to evaluate using inhomogeneous cell populations. Our highly defined model shows clearly that Con A and PHA are only able to induce class II expression through other intermediary cells. Since this induction is sensitive to DB^{-1}, these intermediary cells can be reasonably identified as T lymphocytes (or NK cells) acting through IFN-γ. This finding supports the view that class II expression in autoimmune thyroid disease is secondary to activation of autoimmune T lymphocytes. Moreover, our model demonstrates conclusively that class II molecules are a product of the epithelial cells themselves, and not acquired secondarily from 'passenger' leucocytes.

While IFN-γ appears to be the major central modulator of thyroid epithelial class II expression, our data do not address the question of whether other cytokines may act in synergy with IFN-γ to produce effects at the epithelial cell level. Such synergistic actions between IFN-γ and tumour necrosis factor or lymphotoxin have been observed in cytotoxicity assays (ref. 22 and J. Taverne, unpublished observations) and it is likely that in the local environment within endocrine glands these interactions may play a role in the cellular pathogenesis of autoimmune diseases.

Thyroid cells bearing class II (HLA-DR) antigens have been shown to stimulate proliferation of autologous T lymphocytes [19], and are capable of presenting pre-processed antigen to T cells [17]. The FRTL-5 line was derived from an inbred rat strain, F344, which is a high responder strain for experimental autoimmune thyroiditis. Since syngeneic F344 lymphocytes (and other cells) are readily available, FRTL-5 may represent a useful model system for the study of antigen presentation by epithelial cells in thyroid autoimmunity. In preliminary experiments, we found no evidence for secretion of interleukin 1 (IL-1) by FRTL-5 (data not shown), and a similar observation has been made using human thyroid cultures [27]. Whether this limits the capacity of thyroid cells to present antigen in vivo is unknown.

The FRTL-5 cell line has been closely studied as a model of normal thyroid cellular function [1, 4]; here we show that it provides a model for analysing at least one facet of the pathogenesis

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Paper 390

Epitope specificity of spontaneous and induced thyroglobulin autoantibodies in the rat

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SUMMARY

We have investigated the epitope specificities of rat thyroglobulin (Tg) autoantibodies arising either spontaneously in BB hybrid and BB rats or following induction in normal rats with thyroglobulin and adjuvant. Using a panel of thyroglobulins from different animal species it was possible to identify three different patterns of reactivity. These were: 1) recognition of all species of thyroglobulin; (2) recognition restricted to rat and mouse thyroglobulins and 3) recognition biased towards dog, rat and mouse thyroglobulins. Furthermore, using human thyroglobulin manifesting different levels of iodination, it was possible to show that sera with recognition pattern 1 recognized the iodination site of thyroglobulin and was inhibitable by thyroxine. Taken together these data provide evidence of restricted epitope recognition by Tg autoantibodies in the rat.

Keywords ???????

INTRODUCTION

Spontaneously arising human autoantibodies against thyroglobulin (Tg) have been shown to recognize a restricted number of epitopes (Ruf *et al.*, 1983; Chan *et al.*, 1987; Piechaczyk *et al.*, 1987). Autoantibodies in sera from patients with Graves' disease and Hashimoto's thyroiditis recognize non-species-specific epitopes on thyroglobulin (Kohno, Nakajima & Tarutani, 1985) suggesting that the autoantigenic determinants involve conserved regions of the thyroglobulin molecule. Autoantibodies to Tg also arise spontaneously in the diabetic BB and Buffalo (BUF) rat (Noble *et al.*, 1976; Sternhal *et al.*, 1981) but little is known of their binding specificity. This also applies to Tg autoantibodies induced by immunizing rats with thyroglobulin and complete Freund's adjuvant (CFA) (Jones & Roitt, 1961).

Dietary iodine has been shown to influence the spontaneous incidence of thyroiditis in humans, chickens and rats (Boukis *et al.*, 1983; Bagchi *et al.*, 1985; Allen, Appel & Braverman, 1986; Sundick *et al.*, 1986) suggesting that iodinated *tyrosines* or thyroid hormone residues (thyroxine (T_4) or tri-iodothyronine (T_3) within the Tg molecule may be important recognition elements for autoreactive T or B cells. In an attempt to investigate this hypothesis with respect to the B cell we have compared the cross-reactivity patterns of spontaneously arising and induced rat thyroglobulin autoantibodies and examined their ability to recognize thyroxine.

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MATERIALS AND METHODS

Animals

CDF, AUG, AO and LEW rats were purchased from OLAC, Bicester. The BB/E rats bear the rat MHC allele RT.1^u. The hybrids which resulted from an original cross between either a BB male and a female BUF or PVG.r8 were used to generate lines by brother/sister mating of the F_1 progeny. Lines of rats generated by brother/sister mating of these F_1 animals may be either RT.1$^{u/u}$, RT.1^{u,b}, or RT.1$^{b/b}$. The RT.1 genotype and the derivation of these lines of spontaneously autoimmune rats has been described in detail elsewhere (Colle, Guttmann & Seemayer, 1985; Varey *et al.*, 1987).

Sera

Sera were obtained from BB/E rats and from hybrid BUF and BB rats. A monoclonal antibody to human thyroglobulin was kindly provided by Dr P. Shepherd.

Antigens

Thyroglobulin was extracted and purified from the thyroids of different species as described previously (Champion *et al.*, 1985). The characterization of human Tgs with different iodine content

has been described elsewhere (Byfield *et al.*, 1982). The degree of iodination is exemplified by their T_4 content: TgA = 4·0, TgB = 0·9, TgC = 0·08, TgD = 1·02 and TgE = 1·0 T_4 residues/mole. These human Tg preparations were generously provided by Peter Byfield, Clinical Research Centre, Harrow. Thyroxine was obtained from Sigma Chemical Co., St. Louis, Mo.

Induction of thyroglobulin autoantibodies (TgAb)

Rat TgAb Female rats (4–6 weeks old) of the appropriate strains were immunized twice with 2 mg Rat-Tg emulsified in CFA by footpad injection with a 1 week interval between the two injections. An alternative immunization schedule was employed in which Pertussis vaccine (containing 8×10^8 bacilli) was administered in the footpad as a co-adjuvant at the time of the first injection. Sera were obtained by cardiac puncture 28 days after the first imunization.

Mouse TgAb Female CBA/J mice were immunized with mouse Tg emulsified in CFA as previously described (Male *et al.*, 1983) with or without Pertussis vaccine as described above for rat TgAb.

Immunoradiometric assay (IRA) for thyroglobulin autoantibodies

Assays were performed on Titertex 96-well microtitre plates previously coated with 100 μl of the appropriate thyroglobulin preparation at 10 μg/ml in PBS at 4 °C for 24 h. After washing off unbound Tg, plates were blocked with 5 mg/ml bovine serum albumin (BSA). Test antisera were diluted in PBS containing 0·5% BSA and 0·05% Tween 20 (PBS/BT). After a 2 h incubation at room temperature, the plates were washed three times with PBS containing 0·05% Tween (PBS/T) and the bound antibodies detected following a 2 h incubation with ^{125}I-labelled, affinity purified rabbit anti-rat $F(ab')_2$. The plates were washed three times with PBS/T before being cut up and counted in a gamma counter.

Competitive binding inhibition assay

To characterize the fine binding specificity of induced (TgAb) and spontaneous (S-TgAb) autoantibodies a competition assay was used to examine the ability of T_4 or Tg to inhibit antibody binding to Tg on the solid phase.

Competitive inhibition by thyroxine: Rat sera containing high Tg antibody titres were appropriately diluted and incubated overnight at 4°C with different concentrations of T_4 (1 mg/ml to 0·1 μg/ml) or with rat Tg (0·5 mg/ml to 0·1 μg/ml). The pre-incubated sera were then tested by IRA as described above for their ability to bind at room temperature to plates coated with human TgA (T_4 = 4·0 residues/mole) or human TgC (T_4 = 0·08 residues/mole).

Normal rat serum at the same dilution served as a control in these experiments. The percent inhibition was calculated as follows:

$$\% \text{ inhibition} = 1 - \left[\frac{\text{ct/min with competitor} - \text{ct/min control}}{\text{ct/min with no competitor} - \text{ct/min control}}\right] \times 100$$

RESULTS

Species binding specificities of induced (TgAb) and spontaneously (S-TgAb) arising Tg autoantibodies

Autoantibodies to thyroglobulin have been detected in both BB and BUF rat strains as well as in hybrids between them. We initially screened 75 sera from BB hybrids and 90 sera from BB/E rats for the presence of autoantibodies to rat Tg. Of these, 28 BB hybrids and 30 BB/E sera were positive for S-TgAb, giving at least twice the background binding of normal rat serum. Sera giving the highest binding to rat Tg were then selected for further analysis.

TgAb were induced in CDF, AUG, LEW and AO rats. All strains of rats produced high titres of autoantibodies against rat Tg.

The ability of TgAb and S-TgAb sera to bind to a panel of Tgs obtained from 12 different species was investigated. Three discrete patterns of binding emerged. The first group consisted of all the TgAb sera and some sera from the BB hybrids (Fig. 1a). The sera in this first group manifest the ability to bind to thyroglobulin of many species. The second group which included all of the BB/E S-TgAb and some of the BB hybrid sera (Fig. 1b and 1c) was similar to mouse TgAb (Table 1 and Champion *et al.*, 1988) in having a preference for binding to rat and mouse Tg with little reactivity with other species Tg. The last group contained the remaining BB hybrid S-TgAb sera and showed a higher level of binding to dog Tg than to rat Tg of other species (Fig. 1d). These findings have been summarized in Table 1.

Relationship between species cross-reactivity and thyroxine related epitopes

Although group 1 sera were able to bind to thyroglobulin from most species, they were relatively poor at recognizing human thyroglobulin used in these experiments. Since this human Tg was prepared from thyroids of thyrotoxic patients, which have abnormally low iodine content, it seemed possible that some of the antibodies in this group 1 sera were recognizing iodination related epitopes on the Tg molecule. To investigate this possibility we studied the binding to a small panel of human Tg with different iodine contents. Using a monoclonal antibody to human Tg we could show that the different human Tgs varying in iodine content bound to the plates to a comparable extent (data not shown). Some representative results are shown in Fig. 2 from which it can be seen that both TgAb (Fig. 2a) and S-TgAb (Fig. 2b) sera from this group bind best to human Tg with the higher iodine content, TgA (T_4 = 4·0 residues/mole). Thyroglobulin containing the lowest molar ratio of iodine, TgC (T_4 = 0·08 residues/mole) being least well recognized. BB/E sera (group 2) were retested for their ability to bind human Tg but still failed to recognize human Tg irrespective of the degree of iodination (Fig. 2c). This indicates that a major proportion of antibodies in group 1 sera recognize a site on human Tg which is dependent upon the degree of iodination.

The ability of group 1 sera to recognize Tg from many different species and the effect of iodine content on the binding to human Tg suggested that they might recognize thyroxine (T_4) residues within the Tg molecule. Therefore we examined the ability of T_4 to inhibit the binding of group 1 sera to human Tg with either a low (TgC) or a high (TgA) iodine content. Thyroxine clearly inhibited the binding of both Tg-Ab and S-TgAb to bind to TgA but had little effect on their binding to TgC (Fig. 3a). As might be expected, the binding of TgAb sera to both TgA and TgC was completely inhibitable by rat Tg. However, rat Tg inhibited the binding of S-TgAb to TgA but not

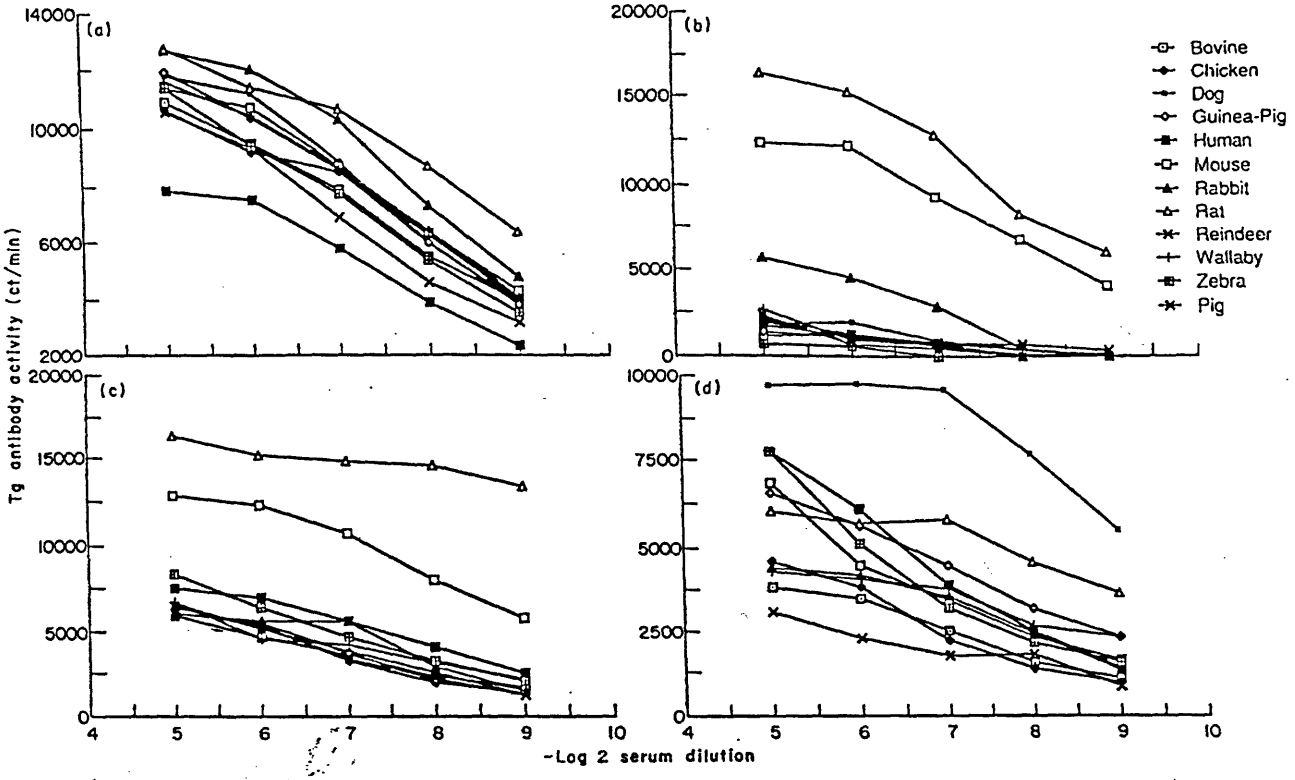

Fig. 1. Ability of various spontaneously arising Tg autoantibodies (S-TgAb) to bind to a panel of thyroglobulin from 12 different species: a representative pattern of binding observed with a) BB hybrid ($RT.1^{u/u}$) serum from group 1, b) BB/E ($RT.1^{u/u}$) serum from group 2, c) BB hybrid ($RT.1^{u/u}$) serum from group 2 and d) BB hybrid ($RT.1^{b/b}$) serum from group 3.

Table 1. Binding of anti-Tg antibodies from different Induced (TgAb) or Spontaneous (S-TgAb) autoimmune sera to various thyroglobulin species.

	Group 1 S-TgAb		Group 2 S-TgAb		Group 3 S-TgAb
Thyroglobulins	BB hybrids ($n=5$)	Rat TgAb ($n=11$)	BB hybrids ($n=3$)	BB/E ($n=8$)	BB hybrids ($n=2$)
Bovine	+++	++/+++	+	±	+
Chicken	+++	++/+++	+	±	+
Dog	+++	++/+++	+	±	+++
Guinea-pig	+++	++/+++	+	±	+
Human	++	++	+	±	+
Mouse	+++	++/+++	+++	+++	++
Pig	+++	++/+++	+	±	+
Rabbit	+++	++/+++	+	±	+
Rat	+++	++/+++	+++	+++	++/+++
Reindeer	+++	++/+++	+	±	+
Wallaby	+++	++/+++	+	±	+
Zebra	+++	++/+++	+	±	+

Sera from BB hybrids (BUF × BB) and (PVG.8 × BB) as described by Colle, Guttmann & Seemayer, 1985. All BB hybrids in groups 1 and 2 including BB/E are $RT.1^{u}$ at the class II MHC loci; animals in group 3 are $RT.1^{b}$ at the class II loci. ± 50% over the background (bg = 2500 ct/min at 1:64 dilution); + 2–3 × bg; ++ 3–5 × bg and +++ > 5 × bg.

the binding to TgC (Fig. 3b). This observation suggests that some of the spontaneous antibody activity against thyroglobulin recognizes human Tg and does not cross-react with rat Tg.

DISCUSSION

Thyroglobulin is a 660 kD glycoprotein, the complete amino acid sequence of which has only recently become available for the bovine (Mercken *et al.*, 1985) and the human (Malthiéry & Lissitzky, 1987) molecules. One approach to examining the epitope specificity of induced or spontaneously arising Tg autoantibodies is to investigate their ability to bind to Tgs isolated from different animal species. The availability of sera from rats which spontaneously develop thyroglobulin autoantibodies together with the relative ease of induction of these antibodies in normal rats allowed us to carry out a comparative study of the epitope specificity of Tg autoantibodies.

Initial examination of sera from spontaneously autoimmune BB/E or BB hybrid rats revealed that approximately 30% had Tg autoantibodies. The presence of these autoantibodies was not related to either the development of diabetes or the development of thyroid lesions in these rats (data not shown). Since the BB hybrid lines are of $RT.1^{u}$, $RT.1^{b}$ or mixed haplotypes (Colle, Guttmann & Semmayer, 1985) it was possible to establish that the presence of these autoantibodies was also not related to MHC haplotype. The high titred sera which were further examined could be divided into three distinct groups according to their reactivity with the panel of Tg species variants. The BB/E and some BB hybrid sera (group 2) bound

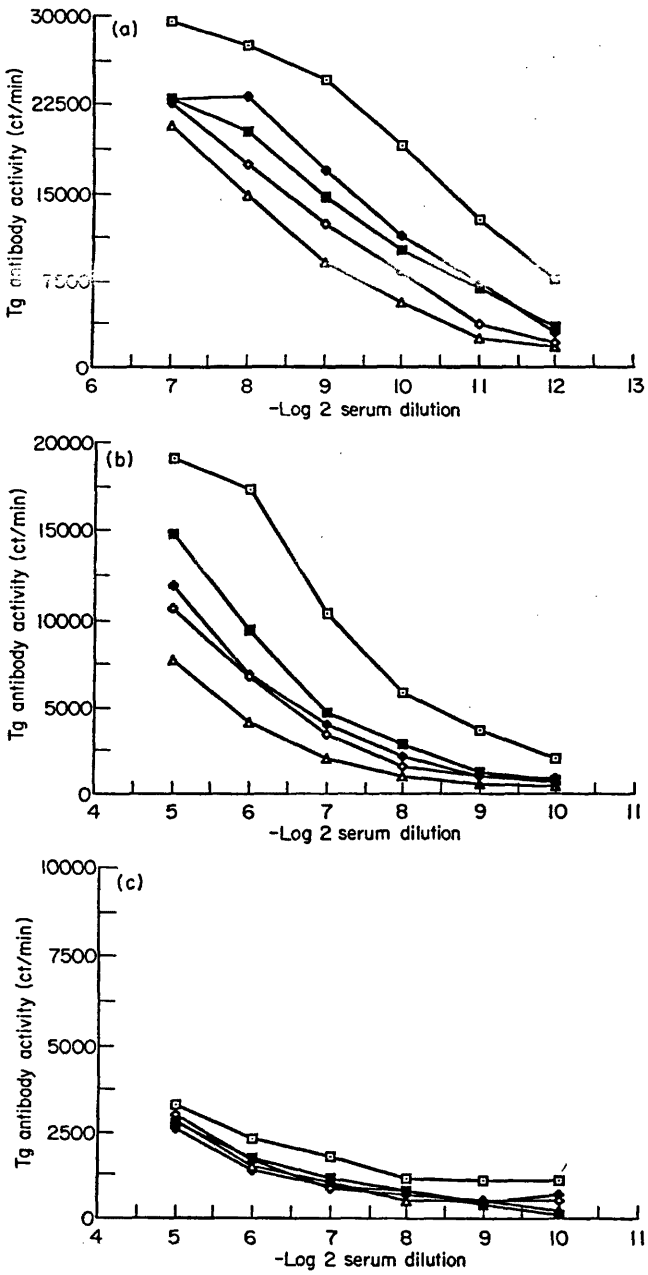

Fig. 2. Cross-reactivity of induced (TgAb) and spontaneously arising (S-TgAb) Tg autoantibodies with preparations of human thyroglobulin containing different molar ratios of iodine: HTG-A (T_4=4·0) (□), HTg-B (T_4=0·9) ♦, HTg-C (T_4=0·08 (△), HTg-D (T_4=1·02) (◊) and HTg-E (T_4=1·0) (■). This figure shows the binding profiles of a) induced CDF rat TgAb, b) BB hybrid S-TgAb and c) BB/E S-TgAb.

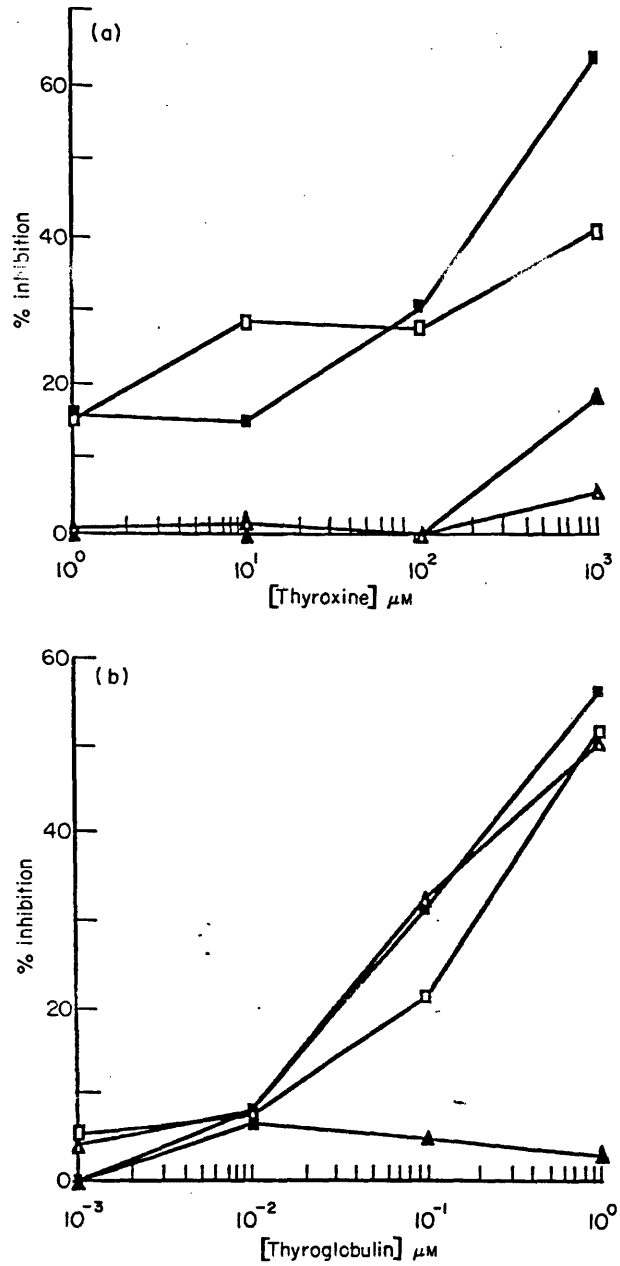

Fig. 3. Inhibiton of binding of group 1 sera to human thyroiglobulins with high (TgA) and low (TgC) iodine content by a) thyroxine and b) rat thyroglobulin. BUF/BB-IgA; □EAT-TgA; ▲BUF/BB-TgC; △EAT-TgC.

strongly to rat and mouse Tg, but only weakly to other Tg species tested. Two BB hybrid sera (group 3) also reacted strongly to rat and mouse Tg, but bound dog Tg even better.

The remainder of the BB hybrid sera (group 1) were found to be highly cross-reactive, binding all twelve Tg species tested including the evolutionary distinct chicken Tg. We did not have any high titred BUF sera available to examine the epitopic specificities of Tg autoantibodies in this inbred strain.

Although the presence of Tg autoantibodies in the spontaneously autoimmune rats did not show any relationship with MHC haplotype, the cross-reactivity profiles of the ten BB hybrid sera examined showed a good correlation with MHC haplotype. Animals giving group 1 profiles all had the RT.1^u haplotype at the class II MHC loci, even though some had a recombinant haplotype (PVG.r8) in which the class 1 A locus carries the RT.1^a allele. Those with group 2 profiles were also RT.1$^{u/u}$ whereas those in group 3 were homozygous for the RT.1^b haplotype of the Buffalo rat. The numbers are quite small but the correlation was supported by the BB/E sera which are RT.1$^{u/u}$ and also give a group 2 pattern of binding. Since B cell recognition of antigen does not involve MHC molecules, it seems likely that the basis of the observed association of epitope specificity with MHC haplotype is related to recognition of Tg by T cells. In breeding studies the presence of at least one RT.1^b

haplotype favours the occurence of a more severe thyroiditis than that seen in animals without an RT.1^b haplotype (Colle, Guttmann & Seemayer, 1985).

TgAb were also generated in several rat strains of different MHC haplotypes and although the amounts of antibody elicited was strain-dependent, the patterns of binding specificity were identical and fit with the group 1 sera from spontaneously autoimmune rats. As expected this induced antibody activity against other species Tg was wholly inhibited by rat Tg. Because of the apparent dominance of highly cross-reactive antibodies, we further analysed the nature of the epitope(s) recognized by group 1 sera. Binding to a small panel of human Tgs of different iodine content revealed a dependence on Tg iodination for high titre activity of both TgAb and S-TgAb in group 1 sera. Inhibition experiments showed that thyroxine could block the binding of these sera to Tg with a high iodine content but could not block binding to human Tg with a low iodine content. Thus, group 1 sera therefore contain a dominant set of antibodies which recognize thyroxine, which accounts for the high cross-reactivity and lower levels of antibodies which recognize determinants unrelated to iodination. Interestingly, rat Tg could not inhibit the binding of some group 1 S-TgAb sera to poorly iodinated human Tg. Although this could mean that these sera see human specific epitopes which are only revealed in the absence of iodination it could also mean that there is some determinant which is only seen on poorly iodinated Tg, rat or human. The latter possibility could be tested by competition assays utilizing poorly iodinated thyroglobulin obtained from rats treated with aminotriazole.

The very restricted pattern of binding specificity of group 2 and 3 rat sera also suggested a limited epitope recognition. In all cases group 2 sera bound rat and mouse Tg more effectively than many other species Tg. Group 3 sera were particularly interesting in that they bound dog Tg better than mouse and rat Tg.

The fact that both spontaneous and induced Tg autoantibodies are against conserved sites suggests that B cell tolerance is not always directed toward conserved regions of the autoantigen as originally deduced from studies on the autoantigenic determinants of haemoglobin and cytochrome C (Reichlein, 1972; Jemmerson, Morrow & Klinman, 1982).

Taken together this data provides evidence for recognition of a very restricted number of epitopes on Tg by autoantibodies arising spontaneously in rats prone to develop autoimmune thyroid lesions. Further analysis of the sera may provide some clues to their mode of induction.

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Cellular infiltration in induced rat thyroiditis: phenotypic analysis and relationship to genetic restriction

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SUMMARY

We have investigated the responsiveness to thyroglobulin (Tg) plus complete Freund's adjuvant (CFA) and *B. pertussis* in a variety of inbred and MHC congenic strains of rats in terms of both Tg-autoantibody titres and histological thyroiditis index. Severity of thyroiditis was strongly Tg-dependent and closely related to the RT.1-MHC haplotype. Phenotypic examination of the inflammatory thyroid infiltrate using single and double indirect immunofluorescence techniques revealed a high proportion of macrophages and T lymphocytes, mainly of the cytotoxic/suppressor subset, in the high responder strains. Thyroid epithelial class II MHC expression although not prominent was strain-restricted and related to the amount of Ia^+ leukocyte infiltrate.

Keywords ???? LYMPHOCYTIC INFILTRATION, THYROID, AUTOIMMUNITY

INTRODUCTION

Experimental autoimmune thyroiditis (EAT) in rats, induced by inoculation with rat-thyroglobulin (rat-Tg) and CFA (Jones & Roitt, 1961), constitutes a powerful experimental model for the analysis of thyroid autoimmunity. Although the model has attracted substantial experimental attention, a number of questions regarding its pathogenesis remain unsolved. In particular, the role of genetic influences governing response status are unclear, with different groups favouring either MHC-associated (Penhale *et al.*, 1975; Kotani *et al.*, 1981) or non-associated genes (Lillehoj, Beisel & Rose, 1981) as the major determinants. Moreover, the pathogenic effector mechanisms remain largely uncharacterized at the cellular level.

We compared the differential induction of EAT in a variety of inbred and MHC congenic strains, and characterized the inflammatory thyroid infiltrate by immunofluorescence. The results presented here are consistent with the view that responder status in rat-EAT is largely determined by MHC haplotype. Moreover, the predominance of activated T cells and macrophages in the inflammatory infiltrate suggests a central pathogenic role for T-cell-dependent mechanisms.

MATERIALS AND METHODS

Animals

Female inbred rats of the CDF ($RT.1^l$), AUG ($RT.1^c$), AO ($RT.1^a$) and LEW ($RT.1^l$) strains were obtained from OLAC, Bicester, UK. Rat strains PVG^a, PVG^c and PVG^u, congenic for different haplotypes at the RT.1 locus were also obtained from OLAC. All animals were 4–6 weeks of age at the start of the experiments and were acclimatized to our animal holding facilities for at least 1 week before immunization.

Sprague-Dawley (outbred) rats for thyroglobulin (Tg) preparation were from a breeding colony maintained at the Middlesex Hospital Medical School.

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Thyroglobulin

Rat-Tg was prepared exactly as described previously (Champion *et al.*, 1985) and stored as frozen 1 ml aliquots at 10 mg/ml.

Induction of EAT

EAT was induced by a modification of the method of Lillehoj, Beisel & Rose (1981). Tg in PBS (phosphate-buffered solution pH 7·2) was emulsified with an equal volume of CFA (Difco, Detroit, Michigan, USA) and a 200 μl volume of emulsion containing 2 mg of rat-Tg was injected as divided doses in the four footpads on each of days 0 and 7. On day 0 only, 8×10^8 killed pertussis bacilli (Wellcome, Beckenham, UK) were also injected but in the dorsal side of the footpad. Animals were generally killed at day 21. In some experiments as noted, the day of killing and the Tg dose were varied.

At the time of killing the thyroid lobes were separated without crushing, and one lobe was snap-frozen in isopentane at −70°C for immunohistochemical staining. The other lobe was processed for routine histology: haematoxylin/eosin stained sections at six levels were scored blindly for EAT according to the protocol: 0 = no lesions; 1^+ = 1–2 foci < 500 μm; 2^+ = any lesion > 500 μm or more than two discrete lesions; 3^+ = 40–70% of the section infiltrated and 4^+ = > 70% infiltrated.

Table 1. Mouse monoclonal antibodies

Clone	Isotype	Working dilution	Specificity
Ox-6	IgG1	1:2	Class II (I-A homologue)
Ox-8	IgG1	1:2	T-cytotoxic/suppressor, NK
Ox-12	IgG2a	1:2	Ig Kappa light chain
Ox-19	IgG1	1:2	T lymphocyte
Ox-39	IgG1	1:8	IL-2 receptor
Ox-42	IgG2a	1:30	C3b receptor
W3/25	IgG1	1:2	T helper, macrophages
ED.1	IgG1	1:500	Monocyte and macrophages
ED.2	IgG2a	1:500	Tissue macrophage

Table 2. Thyroglobulin dose dependence for the induction of EAT in CDF inbred rats

Rat-Tg dose mg	Anti-Tg titre	Thyroiditis index	Proportion with infiltrates
0·5	11·6±0·2	0·3±0·3[a]	1/4
1·0	11·7±0·2	1·2±0·4[b]	4/5
2·0	11·7±0·1	3·0±0·5[c]	5/5
4·0	12·1±0·3	4·0±0·0[d]	4/4

Anti-Tg titres and thyroiditis index are expressed as X±s.e. $P<0·05$ compared to (a) 2 mg and 4 mg, (b) 4 mg, (c) 0·5 mg, (d) 0·5 and 1·0 mg.

Assay for Anti-Tg autoantibodies

Tg autoantibody titres were assessed using a solid phase radioimmunoassay as detailed in Male *et al.* (1985). Briefly, rat-Tg was bound to 96-well polyvinyl chloride microtitre plates using 100 μl per well of a 10 μg/ml solution in PBS. Unbound sites were blocked with 5 mg/ml bovine serum albumin before incubation with thyroglobulin antibody dilutions. Anti-Tg antibody binding was detected with ^{125}I-labelled affinity-purified rabbit anti-rat F(ab′)$_2$. Anti-Tg titres were expressed as the dilution ($-\log_2$) giving 50% of the binding of a control EAT antiserum.

Indirect immunofluorescence (IIF)

To characterize the inflammatory infiltrate in EAT, consecutive 4 μm cryostat sections were stained with a panel of mouse monoclonal antibodies the specificities of which are shown in Table 1. These antibodies apart from the ED.1 and ED.2 (Serotec Ltd, Oxford, UK) were a kind gift of Dr D. W. Mason (Sir William Dunn School of Pathology, Oxford). The properties of all these antibodies have been described by Mason *et al.* (1983) and Dijkstra *et al.* (1985).

Single-specificity staining

Air-dried unfixed sections wer incubated with single monoclonal antibodies and the cellular distribution of binding was visualized using a fluoresceinated rabbit anti-mouse IgG antiserum (Dako, High Wycombe, Bucks, UK). The appropriate

Table 3. Strain-difference in the response to induced rat-EAT

Strains-haplotype	Anti-Tg titre	Thyroiditis index	Proportion with infiltrates
CDF-RT.1[l]	11·4±0·2[b]	4·0±0·0[a]	5/5
AUG-RT.1[c]	11·2±0·3[b]	4·0±0·0[a]	5/5
LEW-RT.1[l]	9·9±0·4[a]	3·4±0·2[a]	5/5
AO-RT.1[a]	7·1±0·7[c]	0·8±0·5[c]	2/5

Anti-Tg titres and thyroiditis index are expressed as X±s.e. $P<0·05$ compared to (a) AO, (b) AO and LEW, (c) LEW, CDF and AUG.

dilutions of both primary and secondary antibodies were determined by standard titration curves. Mouse monoclonal antibodies were diluted in 10% normal rabbit serum and the FITC-conjugate, previously adsorbed with rat liver acetone powder (Sigma, Poole, Dorset, UK), was diluted in PBS. All incubations were performed at room temperature for 25 min with a 20 min washing period in PBS after each incubation. Sections were mounted in 70% glycine buffered glycerol containing 2·5 g% of DABCO (Aldrich, Gillingham, Dorset, UK). Fluorescence was examined and recorded using a Zeiss Photomicroscope II equipped with epifluorescence illumination and Ektachrome 200 exposed at an exposure index of 400 to 800 ASA with normal development.

Dual-specificity staining

We used double-labelling methods to quantify the expression of more than one leukocyte antigen simultaneously and also to relate this to class II MHC antigen expression on the thyroid epithelial cells. This method relied on staining with a first monoclonal antibody which was then detected and blocked by addition of biotinylated horse anti-mouse IgG (Vector, Burlingame, California, USA) and developed with rhodaminated avidin D (Vector, Burlingame). Antibody with a second specificity was then added and visualized with FITC rabbit anti-mouse IgG (for mouse monoclonals) and FITC sheep anti-human IgG (for human anti-microsomal antibody) (Wellcome, Dartford, UK).

Using, dual-specificity staining leukocyte subsets could be expressed as a proportion of Ox-6 positive leukocytes. This approach was valid since essentially the entire leukocyte infiltrate in EAT was consistently Ox-6$^+$. Additionally the Ox-8$^+$ (Tc/s) subset could be expressed as a proportion of total T cell and also the NK cells (Ox-8$^+$, Ox-19$^-$) (Woda *et al.*, 1984).

For lymphocyte subpopulation analysis, rhodamine-labelled cells were expressed as the percentage of fluorescein-labelled (Ox-6 Ox-19) cells. Counts were made in representative ed areas over ten 63Ox high power fields.

Controls

The specificity of this double staining procedure was confirmed in consecutive sections (both in spleen and thyroid) by incubating the secondary antibodies in the absence of the primary antibodies. In addition, cross-reactivity between two layers was verified by omitting one of the primary antibodies in the presence of the second antibody (conjugate). Further negative controls included application of the anti-mouse FITC conjugate

Table 4. Susceptibility to induced EAT in a PVG congenic strain of rats

Strains-haplotype	Anti-Tg titre	Thyroiditis index	Proportion with infiltrates
PVG^u	$13{\cdot}2 \pm 0{\cdot}2^a$	$1{\cdot}4 + 0{\cdot}4^d$	4/5
PVG^c	$13{\cdot}3 \pm 0{\cdot}2^a$	$3{\cdot}4 + 0{\cdot}2^e$	5/5
PVG^a	$11{\cdot}3 \pm 0{\cdot}2^b$	$0{\cdot}6 + 0{\cdot}4^d$	2/5
AUG-RT.1^c	$11{\cdot}9 \pm 0{\cdot}5^b$	$4{\cdot}0 + 0{\cdot}0^e$	4/4
AO-RT.1^u	$6{\cdot}8 \pm 1{\cdot}8^c$	$0{\cdot}2 + 0{\cdot}2^d$	1/5

Anti-Tg titres and thyroiditis index are represented as X + s.e. $P < 0{\cdot}05$ compared to (a) AO and PVG^a, (b) AO, (c) PVG^u, PVG^c, and AUG, (d) PVG^c and AUG, (e) AO, PVG^a and PVG^u.

on sections stained with the human anti-microsomal antibody and also incubation of the anti-human FITC as secondary antibody on sections with mouse monoclonals as primary antibodies.

Statistical analysis

The significance of differences between groups was determined using the non-parametric Mann–Whitney *U* test.

RESULTS

Thyroglobulin dose dependence for the induction of Rat-EAT

The dose of rat thyroglobulin required for induction of EAT was established initially by immunizing CDF rats with a range of doses of rat-Tg (0·5, 1, 2 and 4 mg) given as two single injections 1 week apart. Animals were killed 21 days after the first immunization since our preliminary studies had shown histological evidence of thyroiditis at this time point (data not shown).

High titres of anti-thyroglobulin (anti-Tg) autoantibodies were shown to develop in all groups. Any differences in titres between groups were of no statistical significance ($P > 0{\cdot}05$). However, a positive correlation was observed between the dose of thyroglobulin administered and the index of thyroiditis as defined by the numbers of mononuclear cells present in the thyroid infiltrate. As shown in Table 2 are reliable thyroiditis was only seen following the injection of a total dose of 4 to 8 mg thyroglobulin ($P < 0{\cdot}05$). We therefore used a total final dose of 4 mg in all subsequent experiments for the induction of rat EAT.

Strain differences in the response to induced rat EAT

In order to examine strain differences in the rat response to immunization with rat thyroglobulin plus adjuvants, we used inbred strains of rats differing in MHC haplotype. Autoantibody titres and thyroiditis indices induced by rat thryoglobulin in AO (RT.1^u), CDF (RT.1^l), AUG (RT.1^c) and LEW (RT.1^l) are shown in Table 3. Significantly higher titres of thyroglobulin autoantibodies were induced in AUG and CDF than in LEW and AO rats ($P < 0{\cdot}05$). LEW developed significantly higher titres of autoantibodies than AO.

All AUG, CDF and LEW rats developed severe thyroiditis whereas in the AO strain the few animals which developed thyroiditis showed minimal infiltration. No significant differences in the indices of thyroiditis were seen between CDF, LEW and AUG rats. Again there was no direct relationship between the titres of autoantibodies and the severity of the thyroiditis developed by the animals.

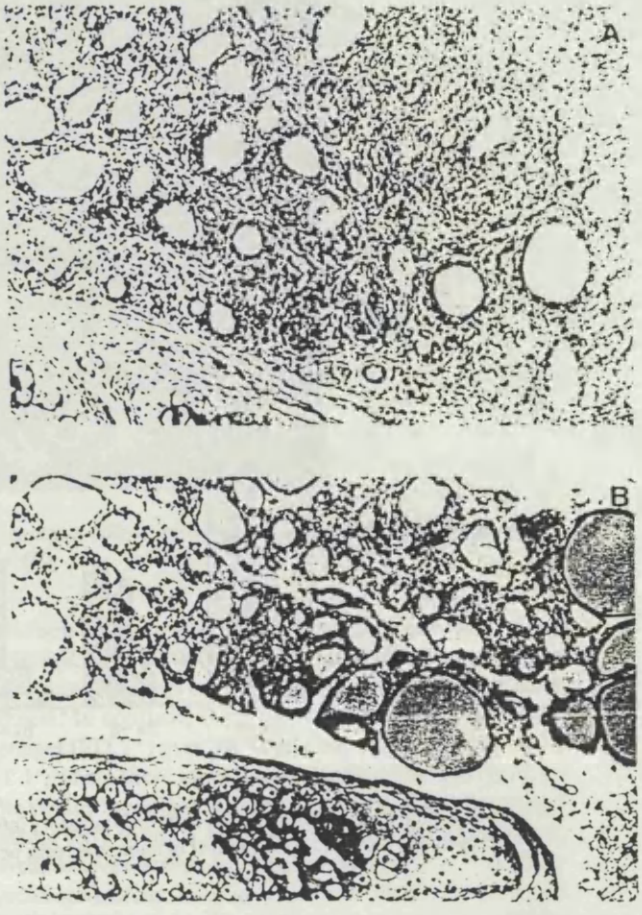

Fig. 1. Photomicrographs of thyroids from rats immunized with 2 mg Tg + CFA twice and *B. pertussis*: A. Thyroiditis in the high responder PVG^c strain, illustrating diffuse lymphocytic infiltration with reduction in the number of thyroid follicles. B. Normal thyroid in the poor responder AO strain. H & E staining. Original magnification × 160.

Genetic control of EAT

To further analyse the MHC-associated genetic factors influencing the induction of rat EAT we used congenic PVG rats with differing MHC haplotypes (RT.1^c, RT.1^a and RT.1^u). We also induced AUG and AO rats in this study as controls. As shown in Table 4 animals sharing the RT.1^c haplotype (PVG^c, AUG) developed the most severe thryroiditis and the highest titres of thyrogloblin autoantibodies ($P < 0{\cdot}05$). On the other hand, strains with the RT.1^u haplotype (PVG^u, AO) developed poor thyroiditis despite the presence of high titres of thyroglobulin autoantibodies in the PVG^u strain (Fig. 1, A–B).

These results indicate that genes located in the RT.1 complex must influence the response to thyroglobulin, animals with the RT.1^c haplotype being high responders whereas animals of RT.1^u and RT. 1^a haplotypes are poor responders with respect to thyroiditis induction.

Cellular phenotypes in the rat EAT

Thyroglobulin dose dependence The inflammatory infiltrate in this induced EAT was characterized using monoclonal

Fig. 2. Cell phenotypes identified in the inflammatory infiltrate of CDF rats immunized with various doses of thyroglobulin. Data are expressed as X ± s.e. of the number of fluorescent cells per high power field (× 630). Tg-dose: ■ 0·5 mg, □ 1·0 mg, □ 2·0 mg, □ 4·0 mg.

correct shading, according to original graph.

Table 5. EAT phenotypes on different inbred strain of rats

	Cell percentages			
Phenotypes	AUG	CDF	LEW	AO
Ox-8	21·1 ± 1·2	19·0 ± 1·0	18·9 ± 1·1	9·2 ± 2·6
Ox-19	38·3 ± 1·2	37·1 ± 1·3	36·7 ± 1·1	28·4 ± 3·6
Ox-39	35·6 ± 1·8	39·7 ± 1·2	36·6 ± 1·2	19·3 ± 4·0
Ox-42[a]	46·8 ± 1·2	45·5 ± 1·8	46·1 ± 1·3	33·8 ± 3·2
W3/25	55·0 ± 2·2	49·9 ± 1·4	52·2 ± 1·6	46·6 ± 3·7
ED.1	55·7 ± 3·5	56·5 ± 3·4	50·4 ± 1·8	69·9 ± 5·3
ED.2	16·5 ± 1·6	13·0 ± 0·8	13·4 ± 1·4	67·0 ± 3·7
N.K[b]	7·7 ± 1·1	7·1 ± 1·2	7·8 ± 1·8	0·1 ± 0·4
Tc/s[c]	65·3 ± 2·4	58·2 ± 2·6	62·9 ± 2·4	26·1 ± 6·4
Ia[+] TFEC[d]	15·6 ± 2·7	10·3 ± 2·1	8·4 ± 4·1	0·0 ± 0·0

Cell percentages were calculated over 10 high power microscope fields (630×) and represent (a) percentage of Ox-6[+] cells, (b) percentage of Ox-8[+], (c) percentage of Ox-19[+] and (d) Percentage of follicles anti-microsomal positive. TFEC, thyroid follicular epithelial cells. Data obtained from three animals are expressed as X ± s.e.

antibodies with different leukocyte surface markers and indirect immunofluorescence techniques. In the CDF rats immunized with various doses of thyroglobulin we detected a linear correlation between the amount of antigen injected and the total number of mononuclear cells infiltrating the thyroid gland. These cells were mainly T lymphocytes (Ox-19[+], W3/25[+]) and macrophages (Ox-42[+], W3/25[+]) and as seen in Fig. 2, their number increased in parallel with the Ox-6[+] cells. In contrast, the number of cells bearing the ED.2 determinant which is expressed on resident tissue macrophages (Dijkstra *et al.*, 1985) did not differ among the four experimental groups. These data imply that the vast majority of leukocyte subpopulations present in the thyroid infiltrate coexpress the class II determinant recognized by the Ox-6 monoclonal antibody suggesting a state of activation. In addition, no B lymphocytes (Ox-12[+]) were detected in the infiltrates irrespective of the immunization protocol adopted.

Strain differences in the rat E. ellular phenotypes. Since Ox-6[+] cells are present in the inflat tory infiltrate the relative contribution of macrophages and T lymphocytes to the infiltrate were assessed. Using the double labelling immunofluorescence technique we could analyse the Ox-6[+] infiltrate and also identify the different subpopulations of T lymphocytes. Additionally we could determine whether or not the thyroid epithelial cells actually expressed class II antigens.

These phenotypes were assessed by examining thyroid

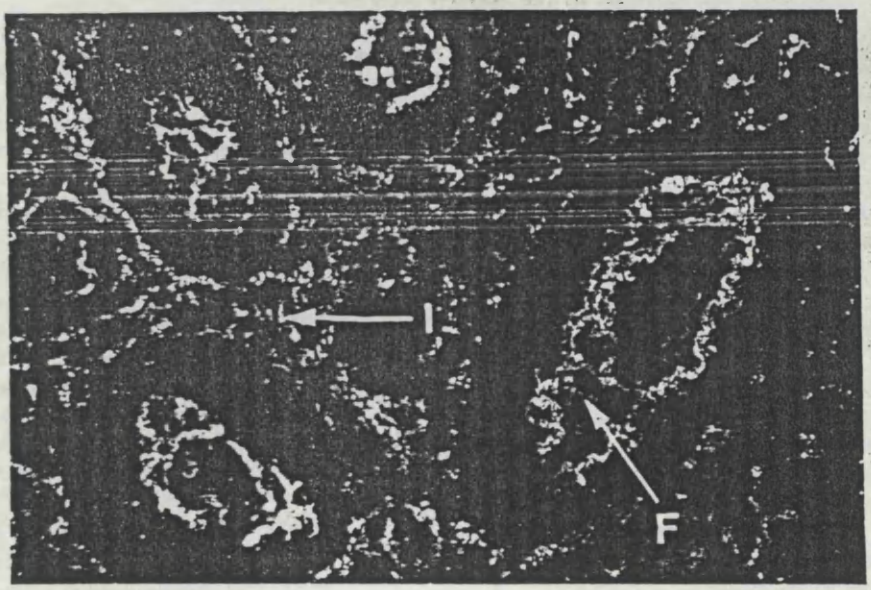

Fig. 3. IIF staining of EAT infiltrate in CDF rats with Ox-12. The picture shows a diffuse deposition of immunoglobulin in the interstitium (I) and within the damaged follicles (F). Original magnification ×480.

cryostat sections from CDF rats immunized with 2 mg twice of rat-Tg, which was defined as the threshold dose for inducing a reliable EAT either by histological (H/E) or immunofluorescence (IIF) criteria. Sections were incubated sequentially with two monoclonal specificities and the first layer developed with rhodamine and the second layer with fluorescein-conjugated antibody.

Comparing the relative percentages of Ox-6$^+$ subpopulations identified by this procedure it was unquestionable that macrophages (Ox-42$^+$ and ED.1$^+$) and T lymphocytes (Ox-19$^+$) were the most abundant cell populations comprising the infiltrate. In addition, the high proportion of cells expressing the IL-2 receptor (Ox-39$^+$) strengthened the supposition that these T cells were in an activated state. Taking together the number of T cytotoxic/suppressor (Ox-8$^+$) cells and total T-cell number (Ox-19$^+$), the T cytotoxic/suppressor subset would appear to represent approximately 60% of the Ox-19$^+$ population, the T-helper subpopulation presumably consituting the remainder. This possibility was investigated using the Ox-8/Ox-19 dual-specificity staining which demonstrated that almost 60% of the Ox-19$^+$ population belonged to the T cytotoxic/suppressor phenotype (Ox-8$^+$, Ox-19$^+$). It also indicated that NK cells (Ox-8$^+$, Ox-19$^-$) were a fairly rare phenotype in the EAT infiltrate of CDF rats, accounting only for 7% of the total Ox-8$^+$ population (or approximately 1% of the total leukocyte infiltrate) (Table 5).

To further analyse the distribution of the Ox-6$^+$ subsets in the infiltrate and their relationship with the thyroid epithelium, particularly with regard to its expression of class II gene products, we carried out double-staining using an anti-human microsomal positive serum as a thyroid epithelium marker. using this approach we identified 'inappropriate' expression of Ia antigen on thyroid follicle epithelial cells. This phenomenon although not very frequent (8·4–15·6%), occurred in extensively infiltrated areas, where Ia$^+$ leukocytes were seen in close contact with these epithelial cells.

These cell subsets were also analysed in EAT in various inbred strains of rats to see if they differed. We found a similar representation of macrophages and lymphoid subpopulations in the AUG (RT.1^c), LEW (RT.1^l) and CDF (RT.1^l) not only in their percentage numbers but also in their spatial distribution in the infiltrate. In contrast, there was a predominance of tissue macrophages (ED.2$^+$) over the T-lymphocyte population in the AO (RT.1^a) where there was a minimal infiltration (Table 5). In all of the strains investigated most of the cells which composed the infiltrate were found scattered throughout the interstitium, but in areas of severe infiltration they were frequently seen lining the thyroid epithelium. In some situations mainly in the AUG and CDF strains where the infiltration was most striking, peripolesis was also observed. No particular cell phenotype was regularly associated with this observation.

The 'inappropriate' expression of class II MHC antigen on the thyroid epithelium also differed among the strains studied. This was more frequent in the EAT induced in AUG, CDF and LEW rats although any differences in percentages were of no statistical significance ($P > 0{\cdot}05$). However, they strongly differed from the AO ($P < 0{\cdot}05$) which did not show any epithelial class II expression in any of the glands examined.

We also investigated the presence of B lymphocytes in these infiltrates. Despite the absence of Ox-12$^+$ cells, this anti-kappa antibody revealed a diffused interstitial deposition of immunoglobulin, particularly within the damaged follicles present in heavily infiltrated areas (Fig. 3). Again, no difference was found in this regard among the four inbred strains of rats studied.

DISCUSSION

In accord with general experience, the amount of thyroglobulin injected markedly affected the severity of the thyroid lesions. Although some thyroiditis could be induced with 1 mg of Tg, reliable lesions were only obtained following the administration of 2 or 4 mg of Tg. It is known that some strains of rat are more susceptible to EAT than others (Rose, 1975), and among inbred strains of rats that we immunized with Tg plus CFA and pertussis, the incidence and severity of the disease were consis-

tently higher in the August and CDF strains with respect to both anti-Tg titres and thyroid damage. Albino Oxford rats albeit defective in the cell-mediated response, developed high titres of anti-Tg autoantibodies suggesting that in rat EAT as in the mouse (Kotani *et al.*, 1981), Tg autoantibody levels are not a reliable indicator of EAT susceptibility.

The genetic basis for high or low responsiveness to Tg remains unclear. Some experiments suggest that genes associated with the major histocompatibility complex (MHC) govern the response to Tg in the mouse (Vladutiu & Rose, 1971). Although there does not seem to be concordance between levels of Tg autoantibodies and throiditis, Tomazic, Rose & Shreffler (1974) suggested that an immune response gene (Ir-Tg) located in the I-A subregion of the H-2 complex governed the development of thyroiditis. In the rat, variations in Tg responsiveness among different strains has been related to the MHC haplotype of the strain (Penhale *et al.*, 1975). However, attempts to map the Ir-Tg to the rat MHC (RT.1) have failed. Lillehoj, Beisel & Rose (1981) using an F_1 and backcross progeny of a poor and a high responder strain of rats, were unable to establish any linkage of the so-called Ir-Tg tothe RT.1 complex either with regard to Tg autoantibody levels or thyroid damage. Furthermore, animals with the same MHC haplotype (PVG^c and AUG) showed considerable differences in their responsiveness to Tg, on the bases of both anti-Tg titres and the thyroiditis intensity (Lillehoj & Rose, 1982).

However, comparing the differential induction of thyroiditis among inbred and congenic strains of rats with different MHC-RT.1 haplotypes, we observed in two separate experiments that animals with the $RT.1^c$ haplotype (AUG and PVG^c) developed the most severe disease which correlated strongly with the anti-Tg autoantibody titres. On the other hand, rats with the haplotype $RT.1^u$ (AO and PVG^u) developed the lowest thyroiditis index in spite of high circulatory anti-Tg levels. This was particularly clear with rats on the PVG^u background. These results with recombinant strains, show that the $RT.1^c$ and $RT.1^u$ haplotype MHC genes on identical genetic backgrounds, are directly associated with high and low responses to EAT induction respectively. The lack of such a clear relationship in other studies, using non-congenic strains (Lillehoj & Rose, 1982) implies that genes outside the MHC also influence the response to Tg. To further delineate this system experiments are now being carried out using intra-RT.1 recombinant rat strains with the proposed high and low responder MHC-RT.1 haplotypes.

The number of infiltrating cells correlated well with the thyroglobulin dose injected. These cells were essentially T lymphocytes and macrophages. Furthermore, the use of double-staining techniques and of additional markers for macrophages and T cells, allowed us to identify macrophages bearing the C3b receptor (Ox-42) and activated T lymphocytes bearig IL-2 receptors ($Ox\text{-}39^+$) as the predominant subpopulations present in the EAT infiltrate of all high responder strains. The proportion of helper to cytotoxic/suppressor T cells was approximately 2:3. In contrast, in low responders (AO strain), tissue macrophages acounted for almost the entire Ia^+ cell population.

NK cells ($Ox\text{-}8^+$, $Ox\text{-}19^-$) accounted for only 1% of the infiltrate and in comparison with the findings in Buffalo rats immunized with Tg+CFA (Cohen & Weetman, 1987), the balance between T helper and T cytotoxic/suppressor (Tc/s) is shifted towards the Tc/s subset. We did not observe any B lymphocytes in the infiltrate at 21 days but it should be noted that in the spontaneous thyroiditis of the BB rat, such infiltration appears to be a late event (Voorbij, Kabel & Drexhage, 1986).

Expression of the class II I-A homologue on thyroid follicular epithelial cells was occasionally seen in areas of severe infiltration but never in the absence of leukocytes. However, close contact with the infiltrate did not automatically confer Ia expression since Ia negative epithelial cells were seen in follicles entirely surrounded by Ia^+ leukocytes; this also argues against the possibility that Ia antigens on epithelial cells are acquired by passive absorption. Despite the low number of follicles expressing Ia antigens among different rat strains, a clear correlation between such expression and the level of infiltration was noticed, high responder strains such as AUG, CDF and LEW having some Ia^+ follicles whereas low responders such as AO were completely negative. Studies on different rat strains in relation to the induction of experimental autoimmune encephalomyelitis (EAE), showed that the amount of Ia induced by interferon-gamma (IFN-γ) on the vascular endothelium (Male & Pryce, 1988—submitted) and in astrocytes (Massa, Ter Meulen & Fontana, 1987) correlated with higher susceptibility to EAE. Taken together with our finding of a massive Ia^+ thyroid mononuclear cell infiltrate in high responder strains, it seems likely that class II expression on thyroid follicular epithelial cells is a secondary event in the pathogenesis of rat EAT and directly related to the release of cytokines such as IFN-γ by activated leukocytes. Our experiments on a rat thyroid epithelial cell line demonstrated that IFN-γ readily induces class II MHC antigen expression n these cells (Rayner *et al.*, 1987) and furthermore makes them highly susceptible to damage by tumour necrosis factor (TNF) and lymphotoxin (Taverne *et al.*, 1987). The inflammatory infiltrate seen in these rats contains cells capable of making such cytokines and this may contribute to the pathology of EAT. Alternatively, we cannot exclude the possibility that other mechanisms for example class I restricted cell killing and ADCC also play a role in the pathogenesis.

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