CELL DEATH IN THE ~~MURINE~~ IMMUNE SYSTEM

Tessa Crompton

Imperial Cancer Research Fund
Tumour Immunology Unit
Department of Biology
University College London

A thesis submitted for the degree of PhD

University of London
1990

1

ProQuest Number: 10609764

ProQuest 10609764

ACKNOWLEDGEMENTS

ABSTRACT

A distinction based on morphological criteria has been made between two forms of mammalian cell death: necrosis, which is mediated by agents external to the cell, and apoptosis, which is believed to be the result of the triggering of an active suicide mechanism in a cell that dies as a result of a physiological, developmentally-regulated process. In the immune system, apoptosis has been implicated as a mechanism of thymic selection and of cytotoxic T lymphocyte (CTL) killing. DNA fragmentation is regarded as a hallmark of apoptosis.

This thesis examines three forms of cell death in the immune system. First, I show that the cell death induced by the removal of the specific growth factor from IL2- and IL3-dependent cell lines falls into the category of apoptosis, as defined morphologically. The DNA is cleaved into nucleosome-sized pieces and survival is enhanced by the nuclease inhibitor aurintricarboxylic acid and by protein synthesis inhibitors. The effect of the protein synthesis inhibitors does not seem to be due to partial cell cycle arrest, as synchronization of the cells does not alter the kinetics of death.

Second, in an attempt to confirm a report that acquisition of resistance to glucocorticoids correlates with acquisition of resistance to CTL killing, I isolated a series of dexamethasone (dex)-resistant mutants from a dex-sensitive clone of the mastocytoma P815 and tested them for susceptibility to killing by CTL. I have used Northern-blot analysis, with a probe to the glucocorticoid receptor, to see if dex resistance is due to a loss of receptor expression or to an increased ability to down-regulate receptor expression.

Third, I have developed an assay to measure the extent of apoptosis in a tissue *in vivo*, using radiolabelled DNA precursors. Apoptosis was shown to be induced in double positive thymocytes *in vivo* by injection of a monoclonal antibody anti-CD3.

3

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

5

6

ABBREVIATIONS

ADP	adenosine diphosphate
ATC	aurintricarboxylic acid
ATCC	American tissue culture centre
ATP	adenosine triphosphate
5azaC	5azacytidine
BDH	British drug house
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
$[Ca^{2+}]_i$	internal concentration of calcium ions
Ci	curie
CTL	cytotoxic T lymphocyte
CO_2	carbon dioxide
ConA	concavalin A
Cpm	counts per minute
CSF	colony stimulating factor
dex	dexamethasone
DMEM	Iscove's modified Dulbecco's medium
(c)DNA	(complementary) deoxyribonucleic acid
DTT	dithiothreitol
E20	embryonic day 20
EDTA	ethylenediamine tetra-acetic acid
EMS	ethylmethanesulfonate
E:T	effector:target
FCS	foetal calf serum
FDC	follicular dendritic cell
FL	fluorescence
FPLC	fast performance liquid chromatography
FSC	fluorescence side scatter
g	gravity (units of)
GR	glucocorticoid receptor
GRE	glucocorticoid response element
h	hour(s)
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane-sulphonic acid
HCl	hydrochloric acid
HS	horse serum
Hsp	heat shock protein
^{3}H-MNU	N-methyl-^{3}H-N-nitrosourea

HRF	homologous restriction factor
^{3}HTdR	[6^{3}H]-thymidine
ICRF	Imperial Cancer Research Fund
γIFN	γIFN
(s)Ig	(surface)immunoglobulin
IL2	interleukin 2
IL3	interleukin 3
i.p.	intra-peritoneal
^{125}IUdR	[5^{125}I]iodo-2'-deoxyuridine
k b	kilobase
m a b	monoclonal antibody
2-ME	2-mercaptoethanol
MeG	methylguanine
MEM(-H)	Minimum essential medium (buffered with 0.02M Hepes)
MHC	major histocompatability complex
MLR	mixed lymphocyte reaction
Mls	mouse lymphocyte stimulating
MOPS	morpholinopropanesulfonic acid
MTOC	microtubule organizing centre
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium salt
NAD	nicotinamide adenine dinucleotide
NGF	nerve growth factor
NaOAC	sodium acetate
PBS	phosphate buffered saline
P I	propidium iodide
PO	propylene oxide
(m/t)RNA	(messenger/transfer) ribonucleic acid
RPMI	Roswell Park Memorial Institute 1640 medium
SDS	sodium dodecyl sulphate
σ_n	standard deviation of mean
t	time
TCA	trichloroacetic acid
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TCR	T cell receptor
t k	thymidine kinase
TNF	tumour necrosis factor

CHAPTER ONE:

GENERAL INTRODUCTION

The death of a cell might be brought about by external damage, or might result from the triggering of an active suicide mechanism intrinsic to the cell. The latter possibility assumes that cell death is a physiologically controlled event, for which the cell has a genetically programmed capability, and its control might be considered to be analogous to the control of cell proliferation or cell differentiation. Evidence for programmed cell death in mammalian cells is still largely circumstantial, as the process has not been characterised at a molecular level. The existence of programmed cell death is, however, well established in the nematode, *Caenorhabditis elegans*. I shall, therefore, first review the evidence for programmed cell death in *C. elegans* development, before considering its possible roles in the development and maintenance of tissue homeostasis in vertebrates and, more specifically, its relevance to the murine immune system.

Programmed cell death in C. elegans

C. elegans has a small, invariable number of cells, and all lineage relationships between these cells are known (reviewed by Kenyon 1988). During the development of the hermaphrodite, which has 959 somatic cells, 1090 somatic cells are generated of which 131 subsequently die (Sulston and Horvitz, 1977; Sulston et al, 1983; reviewed by Horvitz 1988). The dying cells all undergo the same sequence of morphological changes, which include the initial condensation of the nucleus followed by its disappearance (Sulston et al 1983), and in all cases, death requires the activities of the same genes (Hedgecock et al, 1983; Ellis and Horvitz 1986). Thus, undergoing programmed cell death can be regarded as a genetically-determined

specific cell fate, like any other fate in differentiation. Mutant animals, in which the predicted cells do not die on schedule, have enabled the genes that control both the decision to die (determination) and the expression of that decision (ie. death), to be defined. *C. elegans* is therefore a powerful system in which to study programmed cell death, and it may provide a way into the molecular analysis of mammalian programmed cell death, as there are similarities between both the morphology and the occurrence of death between phyla. As in mammals (Cowan et al, 1984), programmed cell death in *C. elegans* is particularly common in the cells of the nervous system, and about 20% of presumptive neurons die (Sulston et al, 1983; Horvitz et al 1982).

Cell death determination genes have been recognized on the basis of mutations which alter the pattern of death without affecting the machinery of death per se. One mutation (*ces-1*) has been defined which causes some cells that would normally die to survive (Horvitz 1988) and the genes *egl-1* and *lin-39* are defined by mutations which cause cells that would normally become motor neurons to undergo programmed cell death (Trent et al, 1983; Ellis and Horvitz 1986).

Five genes involved in the mechanism of death have been identified by mutations. Two genes, *ced-3* and *ced-4*, are necessary for the onset of programmed cell death (Ellis and Horvitz 1986): in *ced-3* and *ced-4* mutants, cells that would normally die survive and differentiate, and in some cases function (Ellis and Horvitz, 1986; Avery and Horvitz 1987). Surprisingly, animals which are mutants in *ced-3* and *ced-4* do not display any gross behavioural abnormalities, indicating that programmed cell death is not an essential part of *C. elegans* development (Ellis and Horvitz 1986). Two genes, *ced-1* and *ced-2*, are required for engulfment of dying cells by their neighbours, and in mutants of these genes, cells destined to die undergo the initial

11

morphological changes associated with death, but they are not phagocytosed (Hedgecock et al 1983). The gene *nuc-1* controls a nuclease which degrades the DNA of dying cells and in mutants of this gene the remains of cells that have died can be seen as pycnotic nuclei containing undegraded DNA (Sulston 1976, Albertson et al 1978; Hedgecock et al 1983). Complementation studies, in which animals with multiple mutations were constructed, have indicated the order of gene action to be (*ced-3, ced-4*), (*ced-1, ced-2*), *nuc-1* (Ellis and Horvitz 1986).

The question of whether these genes act cell autonomously or in neighbouring cells to those which die (suicide vs murder) has been addressed using mosaic analysis, and has revealed that both *ced-3* and *ced-4* act cell autonomously (Horvitz 1988). Thus programmed cell death in *C. elegans* can be regarded as suicide. Whether *ced-1, ced-2* and *nuc-1* also act within the dying cells rather than in the cells that engulf them has not yet been determined. When engulfment is blocked by mutations in *ced-1* or *ced-2* alone, only two male-specific cell deaths fail to occur (Hedgecock et al 1983), and so these two deaths apparently result from murder, rather than suicide, a view that is supported by the finding that they can be prevented by ablation of the cells that would engulf them (Sulston and White 1980).

The cDNA for *ced-4* has been cloned and sequenced, and has no homology to previously cloned genes (H.R.Horvitz, personal communication). The other cell death genes are as yet uncloned.

Cell death in vertebrates

Necrosis and apoptosis

A morphological distinction has been made in vertebrates between cell death due to external damage (necrosis) and cell death which is presumed to be the result of suicide (apoptosis) (reviewed by

Wyllie 1981). Necrosis is characterized by plasma membrane damage and eventual lysis due to the cell swelling. In apoptosis the earliest changes are in the cell nucleus, where the chromatin becomes condensed, while the plasma membrane and other cellular organelles remain intact; eventually the cell breaks up into membrane-bound apoptotic bodies, which often contain pieces of condensed nucleus (Kerr 1971, reviewed by Duvall and Wyllie 1986). Apoptotic cells and apoptotic bodies are rapidly phagocytosed *in vivo* (Duvall et al 1985, Wyllie et al 1980, Ijiri and Potten 1983), and it has been shown that there are changes in cell-surface carbohydrate which make them particularly susceptible to phagocytosis by macrophages (Morris et al 1984, Wyllie 1985). In apoptosis DNA is cleaved into nucleosome-sized fragments by a endonuclease(s) (Cohen and Duke 1984) and this is thought to account for the nuclear morphology (Wyllie et al 1984). Apoptosis has been regarded as cellular suicide because it has been shown to be inhibited or delayed by protein synthesis inhibitors (eg Duke and Cohen 1986, Shi et al 1989, Pratt and Greene 1976). It tends to occur when death is a physiologically or developmentally regulated process (Wyllie et al 1980, Wyllie 1987), such as in the disappearance of the tadpole's tail during morphogenesis Kerr et al 1974).

Clearly there are striking functional similarities between programmed cell death in *C. elegans*, which has been shown to be genetically programmed cell suicide, and apoptosis. Both processes involve nuclear condensation due to cleavage of DNA, both seem to be cell autonomous (in *C. elegans* from cell ablation and complementation studies, and in vertebrate apoptosis from inhibitor studies), and both involve changes which lead to rapid phagocytosis of the dying cells. It would seem surprising if these correspondences were the result of convergent evolution, and not due to evolutionary conservation,

1 3

particularly given that the cell death occurs during the development of the nervous system in nematodes (Ellis and Horvitz 1986), insects (Goodman and Bate 1981), birds (Pittman and Oppenheim 1979), and mammals (Cowan et al 1984). This adds weight to the contention that apoptosis is cell suicide and also suggests that cloning the cDNAs for the nematode cell death genes may enable one to identify mammalian homologues involved in apoptosis.

Cell death in the development of the vertebrate nervous system

Cell death is a major feature of the development of the nervous system (Collin 1906; Hamburger and Levi-Montalcini, 1949; reviewed by Cowan et al, 1984). In many regions of the nervous system, about 50% of the neurons produced die. This over production seems extremely wasteful, especially given the observation that both in *C. elegans* (Ellis and Horvitz, 1986; Avery and Horvitz 1987) and in rats (Zamenhof 1942) the animal seems phenotypically normal even if the predicted cell death does not occur. What then are the selective pressures that favour neuronal death? Williams and Herrup (1988) have argued that for a small animal such as *C. elegans*, where the nervous system takes up such a large proportion of the body, the primary consideration will be energetic, particularly as neurons have high energy requirements relative to other cell types (Kety and Schmidt 1948, Kreisman et al 1986). *Ced-3* and *ced-4* mutant animals, which have 20% increase in the normal neuron number, have a brood size of 15-30% below normal (Ellis and Horvitz 1986). Thus, as metabolic constraints in a small species seem to limit brain size (Martin, 1981), one would expect every neuron to be necessary. In larger species these constraints seem not to exist, and excess neurons may increase evolutionary plasticity.

14

In vertebrate development the phase of cell death is usually confined to a well-defined period that is distinctive for each neuronal population, and which seems to be the time at which the majority of axons of that neuronal subpopulation are beginning to establish connections with their targets. Studies in which target fields were removed (eg Hamburger 1958, Cowan and Wenger, 1967; Oppenheim et al 1978), partially ablated (eg Hamburger 1975, Landmesser and Pilar, 1974), or artificially extended (eg Hollyday and Hamburger 1976; Boyston and Sohal, 1979), have shown that the normal target field can support a smaller number of neurons than are initially produced and that cell death provides a means of matching the size of each neuronal population to the magnitude of its target field.

The mechanism of matching seems to be the competition by neurons for limiting amounts of survival (trophic) factors produced by the target cells. The best characterised neurotrophic factor, nerve growth factor (NGF), is required for the survival of sympathetic and some sensory neurons (reveiwed by Levi-Moltalcini 1987, Davies 1988). Loss of sympathetic and sensory neurons occurs when endogenous NGF is neutralized by administration of anti-serum against NGF (Levi-Montalcini and Brooker 1969), or by making the animal autoimmune to NGF (Johnson et al 1980). Conversely, exogenously applied NGF can increase the survival of these neurons during development (Hendry and Cambell 1976; Hamburger et al 1981) and after injury (Rich et al 1987). NGF is taken up by the axon terminals at their targets and retrogradedly transported back to the cell body (Claude et al 1982; Palmatier et al 1984), where it exerts its survival effect. It is assumed that there are other neurotrophic factors which are specific for the survival of other populations of neurons (Johnson and Yip 1985, Oppenheim et al 1988); one candidate is a brain-derived neurotrophic

15

factor (BDNF), whose cDNA has recently been cloned (Leibrock et al 1989).

How does NGF exert its effect? Intuitively one might expect that a survival factor would allow a cell to survive by providing it with something that it needs, so that in its absence, the cell would gradually decline and die. This does not seem to be the case. In the absence of NGF, rat sympathetic neurons die actively, and their survival can be enhanced by inhibitors of protein synthesis and RNA synthesis (Martin et al 1988; Oppenheim and Prevette 1988). In culture, on NGF deprivation, a minority of dying neurons display the morphology of apoptosis (Martin et al 1988). Apoptosis has been observed *in vivo* in the cerebellar cortex of homozygous staggerer mice (Kerr et al 1987).

All neurons seem to be destined to commit suicide at a fixed point in their development, unless their neurotrophic factor rescues them. Thus, a mechanism that evolved in simpler organisms to limit neuron numbers (ie for efficiency of metabolism) may have been adapted to fine tune the development of more complex nervous systems, and so increase fitness by improving efficiency of information processing. The strategy of neuronal overproduction followed by death ensures that all target cells get approximately innervated and also has the advantage of facilitating evolution: if a mutation alters the size of part of the body, its innervation is automatically adjusted, without the need for a corresponding mutation that changes the number of innervating neurons.

Cell death in the control of cell life-span

Mammalian somatic cells have a finite life-span and proliferative potential in culture (Hayflick 1965), that is partly dependent on the age and type of tissue from which the cells are derived (Martin et al 1970).

This phenomenon of senescence cannot simply be due to accumulation of errors, as has frequently been proposed (Orgel 1973, Holliday, 1984, 1986): sister cells do not always exhibit the same loss of proliferative potential (Smith and Whitney 1988); in fusions between senescent and cycling cells, senescence is often dominant (Norwood et al 1974); Proteins (Drescher-Lincoln and Smith 1984) and polyA$^+$ RNA (Lumpkin et al 1986) from senescent cells, when injected into proliferating cells, can cause them to drop out of cycle. Senescence, therefore, seems to be a state of differentiation (Bell et al 1978), which is distinct morphologically (Kerr et al 1987) and biochemically (Bulmer et al 1984) from quiescence of young cells.

Most studies of senescence have concentrated on the loss of proliferative potential of fibroblasts, and not on their actual death, and it has been reported that non-cycling senescent fibroblasts can be maintained for several months in culture (Pereira-Smith et al 1985), whereas senescent T cells have been reported to die rapidly on becoming quiescent (Walford et al 1981). An ultrastructural comparison of fibroblasts from young and senescent cultures, however, showed an increase in the proportion of non-viable cells in senescent cultures, and that the dying cells had the morphology of apoptosis (Brock and Hay 1971). It is therefore tempting to speculate that in addition to the very specific role of matching target-neuron interactions in nervous system development, apoptosis may play a more general part in the determination of life-span of maturing cells (Umansky 1982). Unfortunately, cells from *C. elegans*, have not yet been cultured and so the participation of the *ced* genes in senescence *in vitro* cannot be assessed.

Growth factors appear to influence the onset of senescence in vertebrate cells. Maintaining rodent fibroblasts in low-serum medium

17

delays senescence (Loo et al 1987). Fibroblasts of patients with Werner's syndrome become senescent more rapidly than normal fibroblasts, and have a diminished response to PDGF (Bauer et al 1986). Transfection of a single oncogene can be sufficient to confer immortality on somatic cells (Spandidos and Wilkie 1984a and b, Wilkie and Spandidos 1984) and many oncogenes are known to encode for growth factors (eg *sis*), growth factor receptors (eg *mas, erbB, neu, frus*), tyrosine protein kinases (eg *src, fps, abr*), GTP-binding proteins (eg *ras, abl*) and nuclear localized proteins that are cell cycle regulated (eg *myc, myb, fos, jun*) (reviewed by Sigal and Gibbs 1989). Transfection of non-growth-factor-encoding oncogenes (eg *ras*) can induce cells to release growth factors and become autonomous for cell proliferation (Spandidos 1985). Thus, many oncogenes encode products involved in growth factor signal transduction, implicating these pathways in the control of cell life-span. Immortalization of diploid cells by oncogenes is associated with reduction in growth factor dependence (Rassoulzadegan et al 1983, Mougneau et al 1982), and it has been suggested that normal cells differ from immortalized cells only in having a higher requirement for growth factors (Brooks and Riddle 1988).

In the case of developing sympathetic neurons, NGF is necessary for survival because it prevents programmed cell death, but since these cells are post-mitotic, the growth factor does not immortalize them in the sense that a fibroblast can become immortal; and as they mature their NGF dependency is diminished (Koike et al 1989). However, if one considers growth factor dependence of proliferating cells, such as IL2-dependent T cell clones, then providing the cells with a constant supply of growth factor might be sufficient to immortalize them. For example, cytotoxic T cell clones that have lost their antigen-dependence, (and so may have already undergone a genetic change), can be maintained for

years in culture by addition of IL2 (Smith and Gillis 1977). On removal of IL2 the cells do not become quiescent, but die by apoptosis (Duke and Cohen 1986). Spontaneous loss of IL2-dependence in rat x mouse IL2-dependent CTL hybrids does not correlate with loss of CTL-derived chromosomes, but is accompanied by acquisition of glucocorticoid-sensitivity, vicia villosa lectin-resistance, loss of cytolytic activity and morphological changes (Samoza et al 1989). IL-2 dependence, therefore, seems to be determined by the state of differentiation of the cell, and the transition to independence seems to involve a stable, pleiotropic change in gene expression.

Thus, it seems that a cell might have three possible fates, which are dependent on its stage of differentiation, growth factor environment, and probably also a stochastic element (Brooks 1985): (1) to progress through the cell cycle; (2) to become quiescent; or (3) to commit suicide. Interestingly, the same sequence of transient nuclear protein expression (ie myc, fos, Hsp70) that was seen when quiescent cells were stimulated with growth factors (Lau et al 1986; Milarski and Morimoto 1986) has also been reported in prostate cells dying by apoptosis (Buttyan et al 1988). Just as genes involved in the regulation of proliferation have been found to be implicated in oncogenesis, one might predict that a mutant gene in the pathway of cell suicide might be potentially oncogenic, though it would presumably be recessive.

Further evidence in favour of the viewpoint that suicide is a physiological option for a cell, controlled by the same kinds of signals as control proliferation and differentiation, comes from studies in which primary cultures of endometrial cells were grown on collagen (Lynch et al 1986). In this system homeostasis in total culture mass is achieved by positive feedback mechanisms in which soluble factors control both the rate of proliferation and the rate of apoptosis.

Evolutionary arguments against genetic control of cell life-span have often rested on the assumption that *in vitro* senescence directly correlates with aging of the organism as a whole. Most organisms die in a natural environment from disease, predation, or starvation, and so an organism will tend to benefit from diverting metabolic resources into efficient reproduction rather than avoidance of aging (Medawar 1952). (This may not always hold, as genes that control the life-span of the whole organism in *C. elegans* do not exert their influence until after reproductive maturity is reached, Johnson and Wood 1982, Johnson 1987). It has been argued that the continuity of the germ-line is best achieved by investment of resources in proof reading and processes that preserve the integrity of macromolecules, and not in indefinite maintenance of the soma (Kirkwood and Holliday, 1979). These arguments might hold for the control of life-span of post-mitotic cells but do not seem relevant to the life-span of proliferating cells of hierarchical tissues, such as the cells of the epithelium and the haemopoietic system, where limits on proliferative capacity and cell life-span presumably reduce the risk of the accumulation of mutations leading to cancer.

Apoptosis in the murine immune system

Apoptosis has been implicated in the development, homeostasis, and as an effector mechanism of the murine immune system. As in the development of the nervous system, there is a vast and seemingly wasteful overproduction of cells during the development of the immune system, followed by their subsequent deaths. This overproduction may facilitate the selection of a repertoire that can distinguish non-self from self (Burnet 1961). In addition, cell death seems to be a means of limiting the numbers of effector cells which expand during an immune

20

response (Duvall and Wyllie 1986). This thesis examines whether the cell death in T cell development in the thymus (Chapter 5) and in the growth factor-dependent determination of life-span of effector T cells and of haemopoietic progenitor cells (Chapter 3) fall into the category of apoptosis. It also considers the hypothesis that one mechanism by which CTL kill is by triggering the target to commit suicide (Golstein 1987), (see Chapter 4). Since apoptosis in relation to T cells in discussed in detail later, here I shall only review cell death of B cells.

Cell death in B cell development

Precursors of B cells arise early during development in the yolk-sac , although the earliest cell identifiable as B cells by their expression of surface immunoglobulin (sIg) appear later in the fetal liver (Owen et al 1977). Shortly after birth, the bone marrow becomes the sole site of B cell development in the mouse (Verlardi et al 1984). It contains about 3×10^7 pre-B cells which give rise to 2×10^7 virgin B cells each day (Opstelten and Osmond 1983), and as the total number of B cells remains roughly constant, an equivalent number must die each day.

B cells were once thought to have a short half-life of 1-2 weeks (Sprent and Basten 1973), but experiments in which partially irradiated rats were reconstituted with thoracic duct lymphocytes of previously immunized rats, and then were themselves challenged with the same antigen, have demonstrated the existence of a population of short-lived host virgin B cells, and a population of donor derived memory B cells whose response was still apparent 3 weeks later (Gray et al 1986). There are, therefore, thought to be two populations of B cells: the short-lived virgin B cells present in the lymphoid organs, and the recirculating

memory B cells which survive for long periods in a quiescent state between each antigen encounter.

The first identifiable stage of B cell development is the progenitor B cell, which has rearranged its V_H genes by recombination of VDJ segments (Alt et al 1986) but does not express μ chains (McKearn and Rosenberg 1985). The pro B cell develops into a pre-B cell (Raff et al 1976), a large rapidly dividing cell in which μ chains are expressed in the cytoplasm. After several divisions the pre-B cell drops out of cycle, decreases in size, rearranges its V_L genes with resultant expression of sIg, and becomes a virgin B cell.

On the leaving the bone-marrow, the virgin B cell with phenotype $IgM^+IgD^+MEL-14^+$ (homing receptor, Gallitin et al 1983) either moves to the spleen, where it (1) is induced by antigen to differentiate into a plasma cell or memory B cell, and then migrates to the spleen, or (2) dies. In the follicular region of the lymph nodes antigen is presented to virgin B cells, followed by cognate T-B interactions with T_H (Van Ewijk and Van der Kwast 1980). The antigen activated B cell is then thought to enter the germinal centres, which play a key role in memory cell formation (Cioco et al 1983). Follicular dendritic cells (FDC) retain large amounts of antigen in the form of complexes held via Fc and C3B receptors (Heinen et al 1985) and stimulate survival and proliferation, but inhibit differentiation into plasma cells. B cells divide rapidly in the germinal centres, but there is also much cell death, and surviving B cells either enter the light zone of the follicle, where they tend to develop into memory B cells, or leave via the opposite pole into the medullary zone, where they develop into plasma cells, which die after several days.

Thus, there is extensive cell death at all stages of B cell development. Death may result from (i) failure to rearrange VDJ

memory B cells which survive for long periods in a quiescent state between each antigen encounter.

The first identifiable stage of B cell development is the progenitor B cell, which has rearranged its V_H genes by recombination of VDJ segments (Alt et al 1986) but does not express μ chains (McKearn and Rosenberg 1985). The pro B cell develops into a pre-B cell (Raff et al 1976), a large rapidly dividing cell in which μ chains are expressed in the cytoplasm. After several divisions the pre-B cell drops out of cycle, decreases in size, rearranges its V_L genes with resultant expression of sIg, and becomes a virgin B cell.

On the leaving the bone-marrow, the virgin B cell with phenotype $IgM^+IgD^+MEL-14^+$ (homing receptor, Gallitin et al 1983) either moves to the spleen, where it (1) is induced by antigen to differentiate into a plasma cell or memory B cell, and then migrates to the spleen, or (2) dies. In the follicular region of the lymph nodes antigen is presented to virgin B cells, followed by cognate T-B interactions with T_H (Van Ewijk and Van der Kwast 1980). The antigen activated B cell is then thought to enter the germinal centres, which play a key role in memory cell formation (Cioco et al 1983). Follicular dendritic cells (FDC) retain large amounts of antigen in the form of complexes held via Fc and C3B receptors (Heinen et al 1985) and stimulate survival and proliferation, but inhibit differentiation into plasma cells. B cells divide rapidly in the germinal centres, but there is also much cell death, and surviving B cells either enter the light zone of the follicle, where they tend to develop into memory B cells, or leave via the opposite pole into the medullary zone, where they develop into plasma cells, which die after several days.

Thus, there is extensive cell death at all stages of B cell development. Death may result from (i) failure to rearrange VDJ

segments correctly (reviewed by Foerster et al, 1989a), (ii) arguably as a mechanism of tolerance induction (Nossal 1983) or (iii) during affinity maturation in the germinal centres (reviewed by MacLennan et al 1989). Most of the evidence for this cell death comes from 'balance-sheet' arguments, but BrdU labelling studies have recently demonstrated that most $B220^{dull}\mu^{+}\delta^{-}$ cells die *in situ* in the bone marrow (Foerster et al 1989b); in addition cell death has been demonstrated in the spleen and lymph nodes by labelling studies (McPhee et al 1979, see chapter 5), and apoptosis has been observed in the spleen (Schwartzendruber and Congdon 1963). Little is known about what controls the onset of death in these tissues, and the issue of B cell life-span remains controversial, but considerably more attention has been devoted to the question of whether clonal deletion is a mechanism for tolerance induction in B cells, as has been shown to be the case for T cells (Kappler et al 1987), and to the conditions favouring affinity maturation and memory cell survival in the germinal centres.

B cell tolerance induction

Studies of B cell tolerance have suggested three alternatives: (i) B cell tolerance does not exist, but autoantibody production is prevented by the absence of autoantigen-specific T cell help (Rolink et al 1987); (ii) autoreactive B cells are functionally inactivated, but still persist in the animal and can still bind antigen (Ventkataraman et al 1977); (iii) autoreactive B cells are eliminated early in development (Sidman and Unanue 1975; Metcalf and Klinman 1977). The third possibility is attractive because it mirrors what is thought to happen in T cell development, where autoreactive cells are deleted in the thymus, perhaps by signaling them to commit suicide by engaging their T cell receptors (Smith et al 1989, Shi et al 1989). One might envisage that at a

stage in B cell development, ligand binding to sIg might trigger the cell to die.

Systems using immunoglobulin transgenic mice which express light and heavy chains specific for model autoantigens have provided an approach to distinguish between these possibilities (Goodnow et al 1988; Nemazee and Buerki 1989a). In Nemazee's mice, which expressed an IgM which binds H-2K molecules of all haplotypes but d and f, the presence of the antigen led to deletion of all idiotype positive B cells (Nemazee and Buerki 1989a) and in bone-marrow chimeras, where the recipient expressed the antigen, all donor autoreactive B cells were deleted in the bone marrow (Nemazee and Buerki 1989b). This work indicates that autoreactive cells are deleted if they see autoantigen in the bone marrow, and that the B cells need not express the antigen themselves in order to be deleted.

Goodnow's experiments, however, provided evidence in favour of clonal silencing, not deletion (Goodnow et al 1988). . Here mice that were transgenic for hen egg lysozyme, were crossed with mice, transgenic for rearranged light and heavy chains of a high affinity anti-lysozyme antibody. The double transgenic mice had mature peripheral B cells in a silenced state, which correlated with down-regulation of membrane IgM with no change in IgD (Basten et al 1989).

One explanation for this discrepancy might lie in the stage at which the B cells first see the autoantigen, so that deletion only occurs if the self-antigen is present in the bone marrow (Nemazee and Buerki 1989c). The tolerance sensitive stage of B cell development is believed to be that of a virgin B cell (Lawton and Cooper, 1974; Nossal 1983), whereas in the double transgenic model the B cells see the autoantigen in the periphery. An alternative explanation ascribes the discrepancy to the nature of the antigen (Basten et al 1989). It is argued that in the

H-2k model the antigen is a highly expressed integral membrane protein, whereas lysozyme is a monomeric soluble molecule, and that there may be physiological reasons for deleting high avidity self-reactive cells, but allowing lower avidity cells to persist in an anergic state. It seems, then, that both clonal deletion and clonal anergy play a part in the induction of B cell tolerance. Both mechanisms may also operate in the case of T cells, as though clonal deletion is well established to occur in the thymus, auto-reactive anergic T cells have been recently demonstrated to persist in the periphery (Rammensee et al 1989). The hypothesis that apoptosis induced by binding sIg is the mechanism of clonal deletion of auto-reactive B cells in the bone marrow remains untested, but has a precedent in the deletion of self-reactive T cells (see Chapter 5).

Apoptosis in germinal centres

Apoptosis of B cells has been demonstrated in germinal centres (MacLennan et al 1989) and this is believed to be the result of the production and selection of memory B cells in the follicles.

Memory B cell clones appear to be able to survive throughout the life of mice, rats and humans (Askonas et al 1970; Gray et al 1986; Jerne 1966). They can be transferred to syngeneic or congeneic hosts, and will initiate an antibody response if antigen is administered (Askonas and Williamson 1972; Siekevitz et al 1987), but in the absence of antigen the transferred B cell memory can only last for about one month. It seems, then, that antigen is required for the survival of memory clones, and that as memory can last a lifetime, but the life-span of quiescent B cells is unlikely to be more than a month (Strober 1975, Lui et al 1988), there must be a proliferating pool of potential memory B cells.

MacLennan et al (1989) have suggested that B cell follicles are the most likely site for this B cell proliferation necessary to sustain memory B cell clones. They postulate that the high death rate in the germinal centres is associated with selection of sIg-expressing B cells on the basis of their affinity for antigen localised on the FDC. *In vitro* apoptosis of germinal centre B cells isolated from human tonsils can be prevented by cross-linking their sIg with antibodies (Lui et al 1990). These cells seem to conform to the NGF paradigm: at a particular stage in their development the withdrawal of a signal (antigen in this case) induces them to commit suicide.

Affinity maturation of the antibody response by hypermutation (Koch and Rajewsky 1988) is believed to occur in the germinal centres (MacLennan and Gray 1989), and so antigen-dependence at this stage may be a means of selection of cells expressing high-affinity antibodies. There seems, however, to be a problem with this theory, analogous to the problem posed by the need for both positive and negative selection of the T cell repertoire in the thymus. During affinity maturation autoreactive clones can be generated (Siekevitz et al 1987), and if the self-antigen is present in the follicles, these clones would presumably be positively selected. In fact, B cells of the germinal centres seem to be particularly sensitive to tolerance induction (Linton and Klinman 1989). Perhaps the two mechanisms of B cell tolerance induction demonstrated by the experiments with transgenic mice partly explain this conflict. Clonal anergy may play a more important role in B cell tolerance induction than in T cell tolerance induction because of the absence of somatic mutation in the generation of the T cell repertoire.

CHAPTER TWO:

MATERIALS AND METHODS

MATERIALS AND METHODS

Materials

Chemical reagents were purchased from Fisons laboratories or from British Drug House, unless otherwise stated. Restriction enzymes were purchased from Bethesda Research laboratories. Radioactive isotopes were obtained from Amersham.

Mice

The following strains were bred and supplied by the Imperial Cancer Research Fund (ICRF) Central Animal Breeding Facility at Clare Hall, South Mimms : BALB/c, CBA, AKR, and B10.BR mice and AO rats.

Media

Eagle's minimum essential medium buffered with 0.02 M HEPES (MEM-H) and Roswell Park Memorial Institute 1640 medium (RPMI) were supplied by ICRF, Lincoln's Inn Fields, London. Iscove's modified Dulbecco's medium (DMEM) (Gibco) supplemented with 0.03% sodium bicarbonate, 5×10^{-5} M 2-mercaptoethanol (2-ME), 100 U/ml penicillin-streptomycin (Gibco), 5ng/ml human transferrin, and 10% heat inactivated Foetal Calf Serum (FCS) was used for culture of thymocytes and cytotoxic T cell clones. All other tissue culture was carried out in RPMI. For growth of FDCP-2, A4 and Wehi-3b 5% Horse serum (HS) was added; for all other cell lines 10% FCS was added. Tissue culture was carried out in a 5% CO_2 incubator at 37°C.

Monoclonal antibodies (Mabs), tumours and cell lines

MAR-18, GK1.5, YTA 156, 2.43, Wehi-3b, MLA-144, CTLL and 2C11 were supplied by ATCC. FDCP-2 and A4 were a gift from Michael Dexter. P815

and CTL-P35.10 were a gift from Jacqueline Marvel.   Directly conjugated

Mabs against CD4, CD8 and Bromodeoxyuridine (Brd-U) were obtained

from Becton   Dickinson.

Protein A purification of antibodies (Mishell and Shiigi 1980)

1.5g Protein sepharose CL 4B (Pharmacia) was swollen in Tris-buffered

saline, pH8.6, and the resin was packed into a column of bed volume of 5

ml.   The culture supernatant of the hybridoma producing the antibody

to be purified was harvested, by centrifugation, filtered, and adjusted to

pH8.6 by addition of dilute NaOH.   The culture supernatant was applied to

the protein A column and the column washed with Tris-buffered saline,

pH8.6.   The hybrid cell antibody was eluted by step elution with buffered

saline at pH7.0, 5.5, 4.3 and 2.3, avoiding low pH buffers where possible.

Antibody elution was detected with a UV monitor.   The fractions

containing antibody were pooled and dialyzed using Tris-buffered

saline, pH8.1.   The column was regenerated by washing with the

glycine-HCl-buffered saline, pH8.6 (0.02%azide) and stored at $4^{o}C$.

Buffers	pH8.6	0.05M Tris 0.15 M NaCl 0.02 % azide (to pH8.6 with 10 M HCl)
	pH7.0 For 1 litre	0.05 M phosphate 0.15 M NaCl 4.34 g Na_2HPO_4 2.7 g NaH_2PO_4 monohydrate 8.76 g NaCl
	pH5.5 For 1 litre	0.05 M citrate 0.15 M NaCl 2.68 g citrate acid monohydrate 10.96 g trisodium citrate dihydrate 8.76 g NaCl
	pH4.3	0.05 M acetate 0.15 M NaCl (to pH 4.3 with acetic acid)

0.05 M glycine-hydrochloride
                  0.15 M NaCl
For 1 litre   5.6 g glycine-HCl
                  8.76 g NaCl
                  (to pH2.3 with 10 M HCl)

Cell Culture

Unless otherwise stated methods were taken from "Selected Methods in ·
Cellular Immunology" edited by Mishell and Shiigi (1980)

Storage of cell lines

Frozen stocks of cell lines were stored under liquid nitrogen in DMEM +
10% FCS + 10% dimethylsulphoxide at a density of 10^6/ml in round
bottomed cryotubes.

Cell suspensions, preparation and counting

Spleens, thymuses and lymph nodes were removed from animals which
had been killed by cervical dislocation and cell suspensions were
prepared by pulverising the organs through a tea sieve with a syringe
barrel in MEM-H. Cells were then washed twice in MEM-H. Gey's
treatment of cell suspensions to lyse erythrocytes (Gey and Gey 1936)
was carried out by resuspending the cells at 10^8 per ml in MEM-H, with
3 ml Geys for every ml of MEM-H. After 5 min on ice, the cells were
washed twice in MEM-H, and viability assessed by trypan blue exclusion.

Growth of CTL:P35:10

CTL:P35:10 was grown in DMEM, supplemented with 10% FCS, 1%
glutamine, 1% penicillin/streptomycin, 1% sodium pyruvate, 1% non-
essential amino acids, 5×10^{-5} M 2-ME and 30 % T cell growth factor. Cells
were restimulated every 3-4 days. 10^5 ml^{-1} CTL:P35:10 were cultured
5×10^6 ml^{-1} syngeneic irradiated spleen feeders and 10^5 ml^{-1} irradiated
P815 cells. Spleen cells were irradiated with 2000 rads and P815 cells

were irradiated with 5000 rads. After irradiation cells were washed 2x with MEM-H.

Mixed Lymphocyte Reaction (MLR) for generation of CTL

BALB/c spleen cells were irradiated with 2000 rads and washed 2X with MEM-H. They were placed in 10 ml cultures with CBA spleen cells at a concentration of 10^6 of each cell type per ml for 5 days. The medium was RPMI supplemented with 50 μM 2-ME, 2mM L-glutamine, 10mM HEPES and 5%FCS (tested previously for its ability to generate CTL).

Chromium release assay for cytotoxicity

Targets were labelled with ^{51}Cr (sodium chromate, Amersham) by incubating $2\text{-}10\times10^6$ cells with 100-200 μCi ^{51}Cr for 1 h at 37 °C in a total volume of 0.2-0.5 ml MEM-H + 5% FCS . Excess isotope was removed by washing 3x with MEM-H and the labelled targets were adjusted to a final concentration of $1\text{-}2 \times 10^5$/ml in RPMI + 5% FCS. Graded numbers of effector cells in a total volume of 0.1 ml were mixed in round-bottomed microplates with a fixed number of ^{51}Cr labelled targets (5×10^3 or 10^4) to make a total volume of 0.2 ml. Plates were centrifuged at 500 rpm for 2 min and then incubated for 4 h at 37°C. After incubation 0.1 ml supernatants were removed and counted in a gamma counter (Wallace LKB 80000 gamma sample counter). Each target was assayed at a series of Effector:Target (E:T) ratios and each ratio was carried out in triplicate. Results are expressed as mean per cent specific ^{51}Cr release calculated to the following formula :

$$\% \text{ specific } ^{51}\text{Cr release} = \frac{\text{Experimental release - spontaneous release}}{\text{Maximal release - spontaneous release}}$$

Spontaneous and maximal ^{51}Cr release were determined by incubating target cells alone or with detergent respectively. Standard errors of the mean of replicate samples were < 5% and spontaneous ^{51}Cr release was < 25%.

Metabolic labelling of cellular proteins

10^6 cells were washed twice with DMEM and resuspended in 1 ml methionine-free DMEM containing 25 µCi ^{35}S-methionine (Amersham).

Proliferation Assay (^{3}H-thymidine (^{3}H-TdR) incorporation)

5×10^3 -5×10^5 cells in 0.2ml volumes in 96-well flat bottom plates were pulsed with 10 µl methyl ^{3}H thymidine (100 µCi/ml) and incubated for 6 h at 37 $^\circ$C. The plates were harvested on a Dynatech Microtiter Automash cell harvester. The filter paper was dried and each piece emersed in 3 ml OptiScint scintillation fluid (LKB) and counted on a liquid scintillation counter (LKB Rackbeta 1214). Each assay point was carried out 10 times and the mean cpm calculated.

Fluorescent staining for flow cytometry

To 10^6 cells in 50 µl MEM-H +5% HS +0.1% sodium azide in round-bottomed 96-well plates was added 50 µl antibody, diluted in MEM-H +5% HS +0.1% azide, to achieve a final concentration of approximately 2 µg/well (individual antibodies or culture supernatants were generally titrated before use). This was incubated for 45 min at 4°C and washed 3x in MEM-H +5% HS +0.1% azide, by centrifugation of the plate (500g, 5 min) and flipping off the supernatant. In the case of directly conjugated antibodies, cells were resuspended in filtered PBS and analyzed on a flow cytometer (FACScan, Becton and Dickinson). Where a second antibody was required, this was added to the pellet of cells as

before, and the cells were again incubated at 4°C for 45 min, prior to washing and analyzing.

Cell cycle enrichment

In order to enrich for a population of cells at a particular point in the cell cycle, cells were separated according to size on a discontinuous percoll gradient. Osmolarity adjusted percoll was prepared by the addition of nine parts to one part (v/v) 1.5M NaCl. Percoll of densities ranging from 1.04g/ml to 1.08g/ml were prepared by mixture of this osmolarity adjusted percoll with 0.15 M saline in the following ratios:

Density	% osmolarity adjusted percoll
1.04	29
1.05	38
1.06	47
1.07	55
1.08	64

A discontinuous gradient was prepared by layering 2 ml of the different densities of percoll on top of each other in 15 ml Falcon tubes. The cells to be separated were layered on top in 1 ml MEM-H. The tube was centrifuged at 10000g for 20 min without brake. Bands of cells were sucked off with a glass pipette and washed 3x in MEM-H before analysis to assess their cell cycle status.

Cell cycle analysis

Assessment of cell cycle status was carried out by staining cells with Propidium Iodide (PI) and with an antibody to Brd-U, after pulsing with Brd-U. Brd-U is an analogue of thymidine and can be incorporated specifically into the DNA. Hence the antibody identifies those cells in S phase at the time of pulsing. PI is a stain which binds to DNA, and the intensity of staining can be used to distinguish between cells in G_1/G_0 and in G_2/M .

10^7 cells were suspended in appropriate tissue culture medium at 10^6 per ml and Brd-U was added to achieve a final concentration of 10mM. The cells were incubated for 20 min at 37°C, washed 2x in PBS and resuspended in 100 μl of normal saline. This suspension was chilled on ice and to it was added 2 ml of cold 70% ethanol with vigorous mixing, and it was fixed at 0°C for 30 min. In order to denature the DNA, 2 ml 4N HCl was added and the cells were incubated at room temperature for 30 min. The cells were centrifuged at 500g for 5 min and resuspended in 1 ml 0.1M sodium borate, pH8.5, to neutralize the acid. Cells were aliquoted onto 96 well plates at 10^6 per well, centrifuged again (500g, 5 min) and the pellet resuspended in 50 μl 0.5% Tween 20 in PBS. 5 μl directly conjugated antiBrd-U-FITC were added and the suspension was underlayered with 50 μl 10% (w/v) BSA in PBS and incubated for 45 min at 4°C. The cells were washed 2x in PBS +10% BSA and finally resuspended in 1 ml PBS containing 20 μg PI, for Facs analysis.

DNA fragmentation Assay (Duke et al 1983)

Target cells (2×10^6) were labeled in 10 ml of medium in a 25cm^2 flask containing 200 μCi ^{3}H-thymidine (or 25 μCi ^{125}I-uridine) for 24 h. The labelled target cells were washed once, incubated for 2 h at at 37°C to chase out unincorporated nucleotides, and then washed twice more before use in the assay. Cells were then used either as targets in a CTL-killing assay, as described previously, or in measurement of the kinetics of death of growth factor dependent cell lines. After appropriate incubation, plates were spun gently (150 x g, 3min), and radiolabel was counted in the culture supernatant. To the residual pellet was added PBS containing 0.2% triton-X 100, this was transferred to eppendorf tubes, the volume made up to 1 ml with PBS + 0.2% triton-X 100, and centrifuged for 10 min at 1500g in the cold. This supernatant was then

3 4

counted. Total uptakes were determined by adding 1 ml PBS containing 0.2% triton-X 100 and 3 µg/ml of bovine pancreatic DNAse 1 and incubating at 37° C for 1 h before processing as before. The percent ^{3}H-TdR (or ^{125}I-UdR) release was calculated in the same way as percent ^{51}Cr release.

Assessment of cell viability

Trypan blue exclusion

A 20 µl aliquot of cells was mixed with an equal volume of 0.2% (w/v) trypan blue in PBS and counted in a haemocytometer. Cells which failed to exclude trypan blue were scored as dead. In kinetic studies, where time allowed, 3-5 samples were taken at a given time-point and cell death was expressed as a mean percentage. Any time-points for which the standard deviation of the percentage of cells that were dead exceeded 10% of the mean percentage of dead cells were excluded from the study. Trypan blue was dissolved in PBS, filtered through a 0.4 µ filter and stored at room temperature with 0.1% sodium azide.

Propidium Iodide (PI) exclusion

The ability of cells to exclude the fluorescent DNA binding dye PI was used to score percentages of dead cells on the Facs. 5×10^5 cells were pelleted and resuspended in 1ml PBS containing 20 µg PI and analysed on the Facs. Stock PI was made up in PBS at 2 mg/ml and stored in the dark at -20°C.

MTT assay

This is a colourimetric assay which is dependent on the ability of mitochondria to utilize a tetrazolium salt (Tada et al 1986). Only live cells are capable of taking up the MTT (3-(4,5-dimethylthiazol-2-yl)-

growth medium in Terasaki plates at concentrations ranging from less than 1 to >100 cells per well. One plate (60 wells) was set up at each concentration and left for 5 days before scoring for the number of wells containing at least one viable colony per well. Clonogenic potential was calculated by assuming it conforms to a Poisson distribution. The proportion of cells estimated to be capable of proliferating is estimated by plotting the negative natural log of the proportion of negative wells out of total wells plated, against cells plated per well. If the only limiting factor is the number of cells plated per well, then this plot will give a straight line. The clonogenic potential is estimated from the line, by finding the proportion of cells that would be plated when the ln(negative/total) = -1 (Langhorne and Lindahl 1981).

Transmission Electron Microscopy

Fixation and Embedding

Cells were fixed in 2.5% glutaraldehyde, 0.12M cacodylate buffer, pH7.4, for 1 hour on ice, pelleted by centrifugation and the pellet embedded in low melting point agarose. This agar-embedded pellet was then fixed in 1% OsO_4, 0.12M cacodylate buffer for 90 min on ice, washed 5 times in distiled water and incubated overnight in 0.5% aqueous uranyl acetate at 4^oC in the dark. Dehydration through an alcohol (EM grade ethanol 99.97% BDH chemicals Ltd) series (25%, 50%, 75%, 90%, 100%(w/v)) proceeded 10 min incubation in propylene oxide (PO), and 2 hour incubation in PO:resin, 1:1, while being mixed on a rotary mixer. The pellet was then placed in 100% resin on the rotary mixer overnight, and the resin was changed and mixed for a further 2 hours prior to pouring out into a plastic flat embedding tray and baking at 60 ^oC for 2 days.

Resin was Polarbed 812 low viscosity embedding media (Polaron Equipment Ltd).

For blocks of medium hardness   Polarbed 812 (resin) 21 ml
DoDecenyl succinic anhydride 11 ml
Nadic methyl anhydride 13 ml

These components were mixed and aliquoted into 6.4 ml portions which were stored at -20 $^{\circ}$C and to which 0.1 ml of the reaction accelerator. Tri[dimethylaminomethyl]phenol was added directly before use.

Sectioning and staining

Resin blocks were allowed to age for a minimum of one week before being sectioned with a diamond knife (Du Pont [UK] Ltd) on an LKB ultra-microtome (LKB Ultratome III). Gold/silver sections (70-110nm) were collected on copper grids (3.05mm diameter, 300 mesh). Grids were stained for 35 min in saturated uranyl acetate (8%w/v in 50% v/v ethanol) and for 5 min in lead citrate stain (Reynolds 1963). Staining was achieved by floating grids, sections face down, on a small volume of the stain on a hydrophobic surface. The grids were washed for 5 min in 50% ethanol and for 5 min in distilled water between stains, and for 5 min in distilled water after lead citrate staining.

Lead citrate stain   lead nitrate   1.33g
sodium citrate   1.76g
dH_2O 30 ml
1M NaOH 8 ml

The lead nitrate, sodium citrate, and water were shaken vigorously for 1 min and then allowed to stand for 30 min with intermittent shaking, to ensure complete conversion from lead nitrate to lead citrate. The NaOH was added and the suspension diluted with distiled

water to a final volume of 50 ml. The solution was mixed by inversion
and stored at room temperature for up to 6 months; old solutions were
centrifuged before use.

Viewing

Sections were viewed with a Jeol JEM-100CX II electron microscope.

Biochemistry

O^6-methylguanine-DNA methyltransferase assay (Bogden 1981)
This assay measures the removal of a radioactive methyl group from O^6-
MeG by the enzyme in cellular extracts.

substrate: heated ^{3}H-MNU treated M.luteus DNA
                (Amersham)

Assay mix: 70mM Hepes KOH, pH7.8
              10mM DTT
              1mM EDTA
              + substrate to give counts between 1-2 x 10^3
                  cpm/100μl

Extraction Buffer:
              50mM Tris HCl, pH7.5
              1mM EDTA
              10mM DTT
              0.2 % Triton X-100

Cell extracts (eg. 0, 5, 10, 20 μl up to about 150 μl protein) were added to
100 μl assay mix and incubated at 37° C for 20 min. 10 μl heat denatured
carrier DNA at 2 mg/ml and 120 μl 0.8M ice cold TCA were added and the
solution was mixed and incubated for 5 min on ice. Samples were
centrifuged for 10 min at 500 g. Supernatants were removed and
counted on a β-counter. To each pellet was added 100 μl 0.1 M HCl and
this was incubated at 70° C for 30 min and on ice for 5 min. Samples
were spun at 500 g for 10 min and 80 μl of the supernatant was counted.
Activity of the enzyme was expressed as cpm removed by the enzyme,
which was determined by subtraction of cpm in the presence of cell

extract from cpm with no extract present. This was plotted against μg protein in the extract. The protein concentration of each cell extract was determined in a Bradford protein assay. In each experiment extracts from cells known to have high activity of the enzyme, or no activity, were run as controls. A Raji tk$^+$ cell line was used as positive control and a mutant of this cell line which has lost activity of O^6 MeG DNA methyltransferase and has lost thymidine kinase expression (P. Karran, personal communication) was used as negative control, and is designated Raji tk$^-$.

Bradford Protein Assay

Preparation of protein reagent: 100 mg of Coomassie Brilliant Blue G-250 (Eastman) was dissolved in 95% ethanol, to which was added 100 ml 85% (w/v) Orthophosphoric acid. The solution was made up to 1 litre with distilled water and was stored at 4°C in the dark. To 10 μl of cell extract was added 90 μl distilled water and 1 ml of protein reagent. This was vortexed and the absorbance was read at 595 nm on tungsten between 2 min and 1 h after addition of the protein reagent. A standard curve for the reagent to calibrate the assay was made on each day of use by reading absorbances when the assay was carried out with 0, 1, 2, 4, 8 and 10 μg BSA. The assay is only linear up to 10 μg protein.

<u>Molecular Biology</u>

Methods in this section were taken from Maniatis et al (1982) unless otherwise referenced.

Phenol extraction

UNC phenol	500g phenol ("Analar" grade)
	111ml 2M tris pH7.5
	114 ml dH$_2$O
	28 ml m-cresol
	1.1 ml 2-ME

4 0

555 mg 8-hydroxyquinoline

The phenol was heated to 65 °C until melted and the other ingredients added. Phenol was them stored in a darkened bottle at room temperature. Solutions were phenol extracted by addition of an equal volume of UNC phenol, shaking in a poly-propylene tube, and centrifugation at 10000g, 20 °C, 10 min. The upper phase was taken and extracted with an equal volume of chloroform/isoamylalcohol [IAA] (24:1), centrifuged as before, and the upper phase taken.

Ethanol precipitation of nucleic acid

0.1 volumes of 3M NaOAC and 2 volumes of 99% ethanol were added to the sample, which was incubated on dry-ice for 20 min and centrifuged at 10000g, 4°C, 15 min. The pellet was washed once with 70% ethanol and allowed to air dry, and the nucleic acid was taken up into the appropriate buffer. Where small quantities of DNA/RNA were present 5 µg tRNA was added prior to the addition of the ethanol, to act as a carrier.

Plasmid preparation

Solution 1	100 mM tris pH7.0
	10 mM EDTA
	15% sucrose

| Solution 2 | 0.1M NaOH |
| | 1% SDS |

| Solution 3 | 3M sodium acetate |
| | 2M acetic acid |

| TE-8 | 10mM tris (pH8) |
| | 1mM EDTA |

3ml bacteria cultures were grown overnight, 1.5 ml taken and the cells pelleted in eppendorf tubes. The cells were resuspended in 100 µl solution 1 and incubated at room temperature for 5 min. 200 µl

solution 2 was added, the tubes incubated on ice for 5 min, followed by addition of 150 µl solution 3 and incubation on ice for a further 10 min. The solution was centrifuged for 10 min at 10000g, 4 °C, the supernatant taken and extracted once with phenol/chloroform and once with chloroform. The nucleic acid was ethanol precipitated and the pellet resuspended in TE-8 and stored at -20 °C.

Restriction digest of plasmid DNA

Restriction enzymes were used according to supplier's recommendations.

10X DNA digestion buffer	100mMtris-HCl pH7.5
	100mM MgCl$_2$
	100 mM EDTA
	10mM DTT

NaCl was added to this basic digestion buffer to produce a range of buffers which contained 0mM, 50mM, 100mM, and 150mM NaCl at 1x dilution.

Preparation of RNA

Glassware was chromic acid washed and autoclaved before use. Plastics were untouched by human hands and autoclaved.

Solution A	4M Guanidine thiocyanate
	0.5%(w/v) Nlauroylsarcosine (Na salt)
	25mM sodium citrate
	100mM 2-ME
Adjusted to pH7, filtered through 0.45 µ filter	

Solution B	5.7M Caesium chloride
	25mM sodium acetate, pH5
	0,2% diethyl pyrocarbonate
Autoclaved and filtered through 0.45 µ filter	

3×10^7 cells were pelleted in Falcon tubes and frozen by pouring liquid nitrogen on to the pellet. 8ml Solution A was added to the frozen pellet which was homogenized immediately using a Polytron

42

probe. The solution was centrifuged for 10 min at 2500g to reduce foaming and loaded onto a cushion of 4 ml Solution B and centrifuged for 18 h at 32000g at 20 °C in a Europa 65 ultra-centrifuge. The supernatant was aspirated and the RNA pellet was air-dryed before resuspension in DCP-treated water and ethanol precipitation. Final concentrations of RNA were estimated by reading optical densities at 260 and 280. RNA in aqueous solution was stored at -70 °C.

Poly(A) Selection of RNA (Chromatography on Oligo(dT)-cellulose)

TS buffer	10mM Tris-HCL, pH7.4	
	0.1% (w/v) SDS	
NTS buffer	0.5M NaCl	
	10mM Tris-HCl, pH7.4	
	0.1% SDS	

The column was prepared by plugging a disposable plastic syringe with siliconized glass wool and filling it with oligo(dT)-cellulose in TS buffer. TS buffer was washed through several times and then 0.1M NaOH was run through the matrix. The column was left to stand at room temperature for 30 min and the NaOH was washed away with NTS buffer. The column, now ready for use, was stored at 4 °C. For fractionation, total RNA was suspended in 5 ml NTS buffer and this solution was applied to the column and the flow-through buffer collected and reapplied to the column twice more. The bound RNA (PolyA$^+$) was eluted with 5 ml TS buffer and was ethanol precipitated as described previously.

Electrophoresis of Nucleic acids

Running buffer	10x TAE	400mM tris
		200mM NaOAC
		100mM EDTA
		pH adjusted to 8.3

Loading buffer   (10x)   0.1% solid orange G
                                       20% ficoll
                                       10mM EDTA
                                       9.5x TAE

DNA was electrophoresed through 1% agarose gels in horizontal

submarine gel systems supplied by Pharmacia.   Agarose was melted

in 1X TAE.   Gels were stained with ethidium bromide (5 μg/ml in 1X

TAE) for 5 min and the nucleic acid visualized on a u.v.

transilluminator (u.v. Products Ltd).

RNA gels (For Northern Blotting)

Running buffer   10X MOPS   0.2 M morpholinopropane-
                                    sulfonic acid
                                    50mM sodium acetate
                                    5mM EDTA
                                    pH adjusted to 7.5

                  Sample buffer   50%   formamide (de-ionized)
                                    18% formaldehyde   (12.3M)
                                    10% 10x MOPS
                                    22% H_2O

                  Loading buffer   50% glycerol
                                    1mM EDTA
                                    0.4% bromophenol blue
                                    0.4% xylene cyanol

Gel preparation (per 100 ml)   1g agarose
                                   10 ml 10X MOPS
                                   72 ml dH_2O
                                   18 ml formaldehyde (2.2M)

RNA gels were electrophoresed through 1% agarose gels in

horizontal submarine gel systems.   Agarose was melted in water and

10x MOPS and this was cooled to 50 ^{o}C before addition of

formaldehyde and pouring of the gel.   Aqueous RNA solutions were

incubated at 65 ^{o}C in 5x volume of sample buffer for 3 min prior to

loading in 1x loading buffer.   Gels were run at 21mV and running

buffer (1x MOPS) was recirculated with a peristaltic pump.

Transfer of RNA

Gels were blotted on to Nylon membranes (Genescreen-Plus). The membrane was soaked in 10x SSC for 30 min and then placed on the gel and any air bubbles trapped between the gel and the membrane were removed. A stack of Whatman 3MM paper soaked in 10x SSC was placed above the gel and a stack of dry 3MM paper was placed below the gel. This was sandwiched between two glass plates, wrapped in cling-film and left for 12 h with a weight on top of it. After transfer the membrane was baked for 2 hours at 80 $^\circ$C.

20x SSC per litre	175.3g NaCl
	88.2g sodium citrate
	Adjusted to pH7 with Na OH

Hybridization of Northern blot

Hybridization buffer	50% deionized formamide
	5% 100x Denhardt's
	30% 20x SSC
	1mM EDTA
	1% SDS
	0.01% salmon sperm DNA
	2% H_2O
	10% Dextran sulphate

100x Denhardt's	2% (w/v) ficoll
	2% (w/v) polyvinylpyrrolidone
	2% (w/v) BSA

Blots were pre-hybridized in a plastic bag at 45 $^\circ$C for 2 h before addition of the radio-labelled probe. Hybridization was carried out at 45 $^\circ$C for 12 h in a shaking water bath. The blot was washed at an appropriate stringency (ie. salt concentration and temperature) and exposed to pre-flashed X-OMAT AR diagnostic film (Kodak) at -70 $^\circ$C.

Preparation of probes

Plasmid DNA was digested with appropriate restriction enzymes and the desired fragment was gel isolated and the DNA extracted from the

labelled with an oligo-labelling kit supplied by Pharmacia, using [α32-P]dCTP (Amersham). Unincorporated nucleotides were separated on a Sephadex G-50 NICK column supplied by Pharmacia. Probes were boiled for 5 min and held on ice for 5 min before addition to separate the strands of DNA.

Bacteriological Methods (Hanahan 1985)

Bacteriological Strains

For general cloning and sub-cloning of recombinant plasmids E. coli strains LE392 and DH5 were used.

Growth Media and Agar Plates

All specialized media components were obtained from Difco Laboratories Ltd, Michigan.

LB medium per litre:	10g bacto-tryptone 5g yeast extract 10g NaCl
2X agar per litre:	30g bacto-agar
2x LB per litre:	20g bacto-tryptone 10g yeast extract 20g NaCl

These solutions were sterilized by autoclaving and stored at room temperature.

1000x ampicillin	40 mg/ml ampicillin

This solution was sterilized by filtration through a 0.22 μ filter (Millipore Corp.) and stored in aliquots at -20 °C. All strains of E. coli used were grown in LB medium at 37 °C with constant shaking of the culture. Where appropriate the medium was supplemented with ampicillin at a concentration of 40 μg/ml. Agar plates were prepared

46

by combining equal volumes of 2x LB solution with 2x agar solution, while heating until the agar had melted. The solution was autoclaved and under sterile conditions was poured onto 10cm diameter petri dishes (Falcon) and allowed to cool until solid. When plates were supplemented with 40 µg/ml ampicillin, the LB/agar solution was cooled to below 55 °C before the ampicillin was added. Plates were stored at 4 °C and were air dried at 37 °C for 1 h prior to use, to remove surface liquid.

Preparation of Competent Bacteria

Tfb I   30mM   potassium acetate
                         100mM rubidium chloride
                         10mM calcium chloride
                         50 mM manganese chloride
                         15% glycerol (v/v)
Adjusted to pH5.8 with 0.2M acetic acid

Tfb II   10mM MOPS
                         75mM calcium chloride
                         10mM rubidium chloride
                         15% glycerol (v/v)
Adjusted to pH6.5 with KOH

Both solutions were filter sterilized and stored at -20 °C. 200ml of LB medium was innoculated with 50 µl of an overnight culture of DH5 cells and the culture was shaken at 37°C until the O.D.$_{550nm}$ was between 0.2-0.4. The cells were chilled on ice for 5 min and pelleted at 6000 rpm at 4 °C for 5 min. The bacteria were resuspended in 40 ml of ice-cold Tfb I by gentle vortexing and placed on ice for 5 min. The bacteria were then repelleted, resuspended in 5 ml Tfb II and placed on ice for 15 min. Aliquots (200 µl) were placed into round bottom screw-cap cryotubes (NUNC) on ice and then snap-frozen in liquid nitrogen and stored at -70 °C.

Transformation of Bacteria with Plasmid DNA

DNA was added to competent cells at less than 100ng DNA per 100 μl cell suspension and incubated on ice for 10 min, followed by a 5 min heat-shock at 37 °C. The cells were transferred to 3 ml LB (no antibiotics), incubated at 37 °C for 15 min, pelleted and resuspended in 100 μl LB. This was then spread onto LB-agar plates (supplemented with antibiotics if appropriate) and incubated at 37 °C overnight. Colonies were picked into 3 ml liquid cultures with sterile gilson tips.

Long-term Storage of Recombinant Bacteria

Long-term stocks of bacterial strains and bacteria hosting recombinant plasmids were produced by adding 15% glycerol to overnight bacterial cultures and storing these at -20°C. Bacteria were recovered by inoculating 3 ml cultures with 10 μl of glycerol stock and growing the culture overnight at 37°C with constant agitation.

CHAPTER THREE:

DEATH OF GROWTH FACTOR-DEPENDENT CELL LINES

The aim of this work was to establish an *in vitro* model system in which cell death was readily inducible in order to study the mechanism of apoptosis. Previous studies had frequently used glucocorticoid-mediated lysis of thymocytes or thymomas (Wyllie 1980, Wyllie et al 1984, Cohen and Duke 1984), but these systems seem inappropriate to assess the question of whether or not apoptosis requires protein synthesis, as glucocorticoids act by turning on new gene expression (Yamamoto 1985). IL2- and IL3-dependent cell lines are used in bioassays to measure lymphokine production because they rapidly cease to proliferate and die in the absence of their growth factor (Gillis et al 1978). I have used these cells to try to answer the following questions: (1) Do these cells die by apoptosis (as defined morphologically) on withdrawal of their growth factors? (2) Is cell death an active process? (3) What is the mechanism of death?

The cell lines used in this study were the IL2-dependent T cell line CTLL (Gillis and Smith 1977) and the IL3-dependent cell lines FDCP-2 and A4 (Dexter et al 1980). CTLL is a murine $CD8^+$ T cell line which dies rapidly when deprived of IL2. FDCP-2 and A4 are murine cell lines, derived from long term bone-marrow cultures. Early isolates of the latter cell lines had many properties of haemopoietic progenitor cells, and were able to form spleen colonies in irradiated mice. Although these properties were lost in long-term culture, these cell lines have retained their ability to differentiate in response to haemopoietic growth factors and to self-renew in the presence of IL3 (Spooncer et al 1986). Thus these cells provide a model for the study of the mechanism of action of IL3, and the control of self-renewal. It has been shown that FDCP-2 cells die rapidly on removal of IL3 and that their death can be

delayed by addition of ATP to the tissue culture medium (Whetton and Dexter 1983). Here, I show that this cell death falls into the category of apoptosis and seems to be an active process that depends on DNA fragmentation.

RESULTS

All experiments were carried out on the three growth factor dependent cell lines. Except for discrepancies in the results between the different cell lines, however, I shall show data for FDCP-2 cells only.

Kinetics of Death

All three growth factor dependent cell lines examined died rapidly and asynchronously on removal of their respective growth factors. All FDCP-2 cells died within 35 h of IL3 removal, all CTLL cells died within 35 h after IL2 removal, and A4 cells died more rapidly, within 24 h of IL3 deprivation. The disintegration of the plasma membrane is a late event in death by apoptosis (Duvall and Wyllie 1986) and in some instances even apoptotic bodies have been shown to exclude dye (Sheridan et al 1981, Wyllie 1981). For this reason, I measured the ability of IL-3 dependent cells to form clones on re-exposure to IL3. In the case of FDCP-2 cells loss of clonogenic potential on re-exposure to IL3, as assessed by limiting dilution cloning, preceeded loss of ability to exclude dye (Fig. 1a). For CTLL cells the kinetics of loss of ability to exclude dye and of clonogenic potential did not differ appreciably (Fig. 1b).
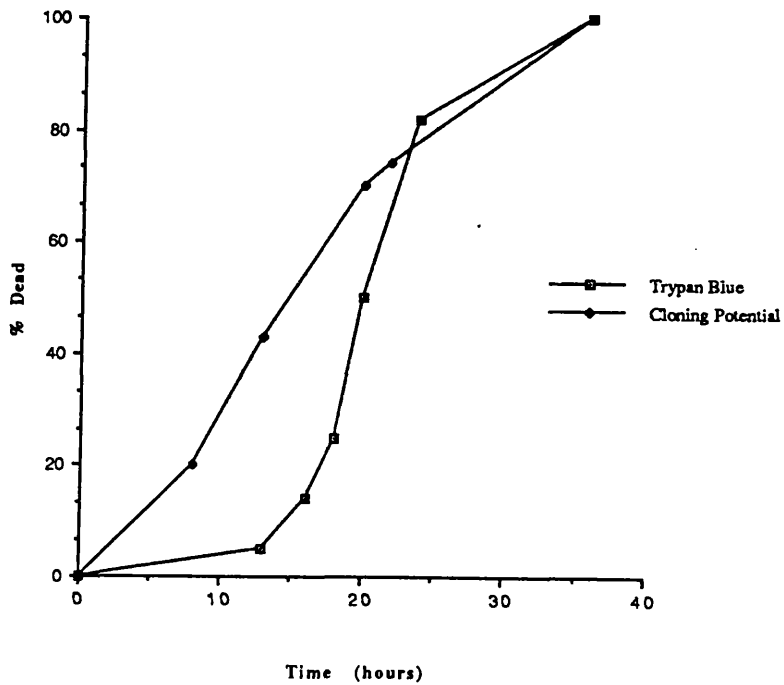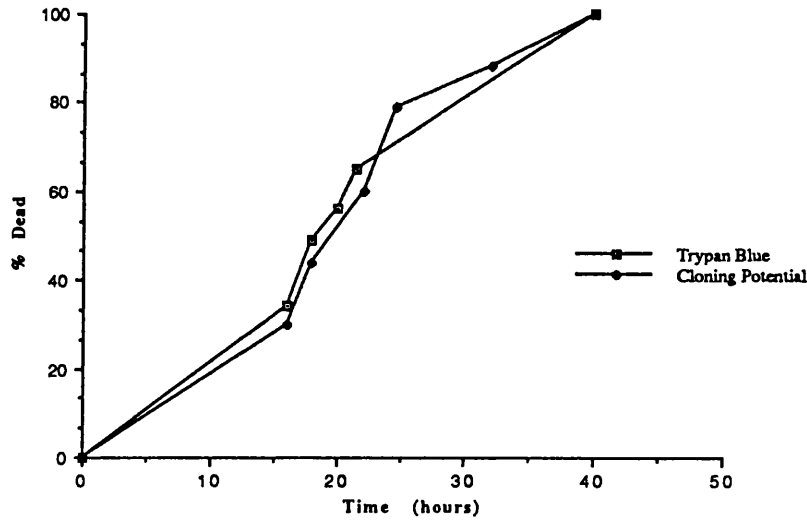
51

Fig. 1a

Time course of death of FDCP-2 cells on IL3 removal, measured by trypan blue exclusion and clonogenic potential. Clonogenic potential was determined by limiting dilution cloning in Terasaki plates, on readdition of IL3, and was estimated from a best fit line of a plot of cells per well against ln(negative/total wells plated). Each point of trypan blue exclusion curve is a mean of 5, with $\sigma_n < 10\%$ of mean.

52

Fig. 1b

Time course of death of CTLL cells on IL2 removal, measured by trypan
blue exclusion and clonogenic potential. Clonogenic potential was
determined by limiting dilution cloning in Terasaki plates, on
readdition of IL3, and was estimated from a best fit line of a plot of cells
per well against ln(negative/total wells plated). Each point of trypan
blue exclusion curve is a mean of 5, with $\sigma_n < 10\%$ of mean.

Electron Microscopy

Only electron micrographs of experiments performed with FDCP-2 cells are shown.
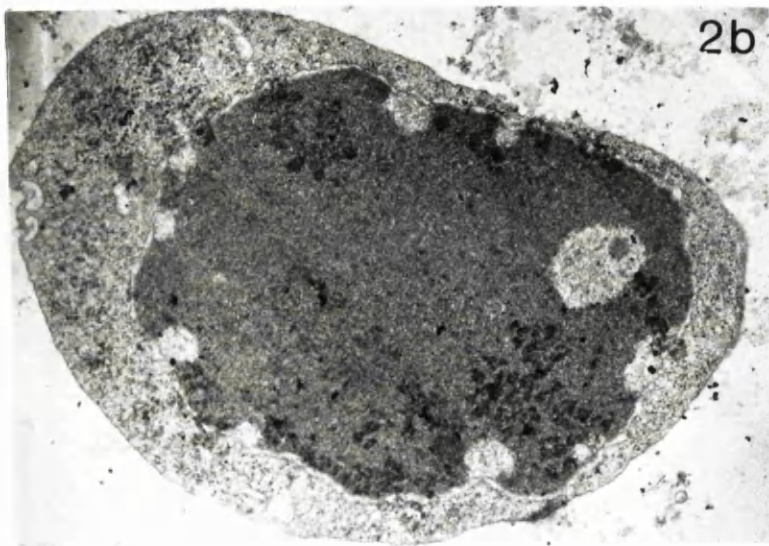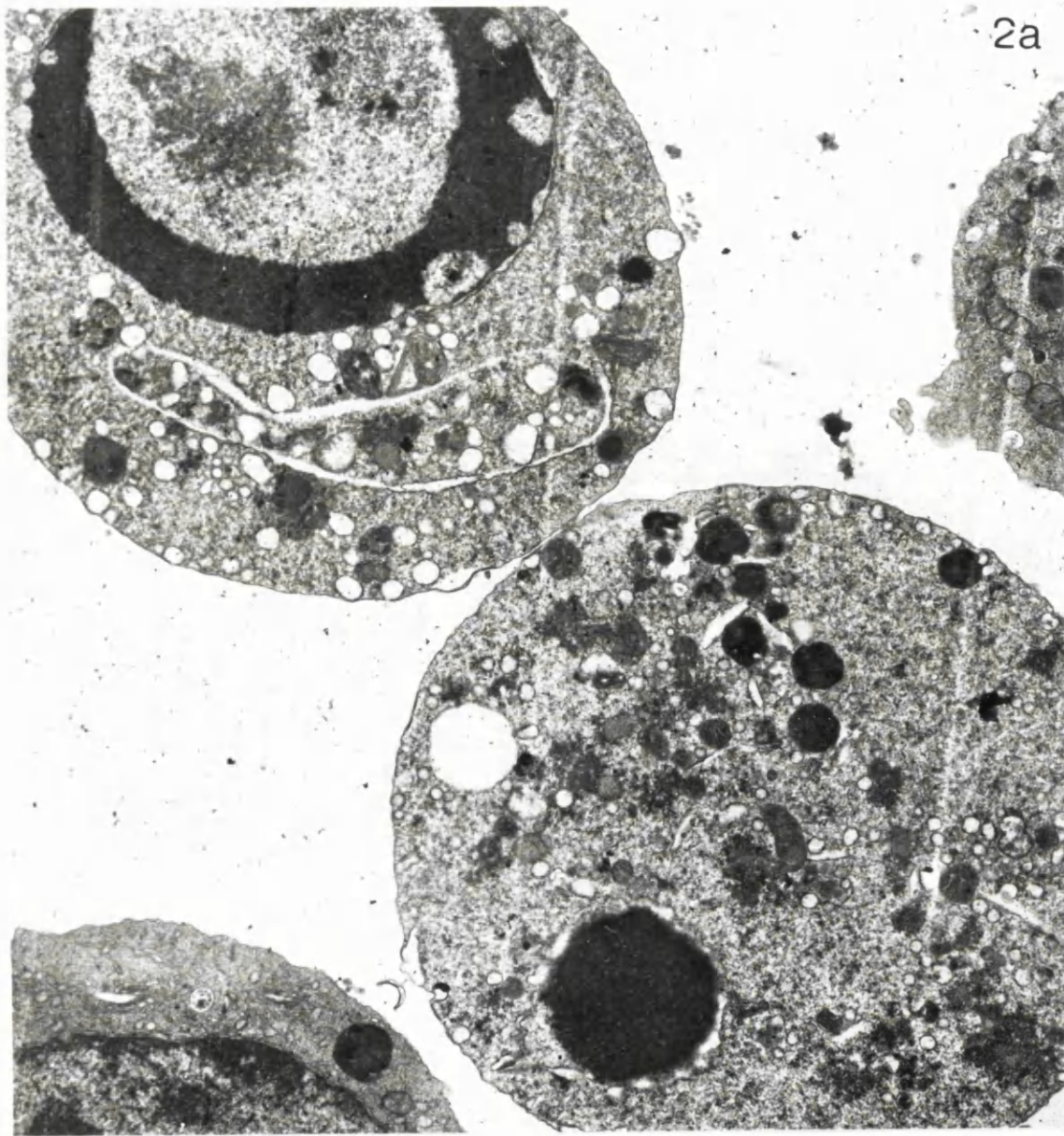
Electron micrographs of FDCP-2 cells 20 h after removal of IL3 indicate that the cells died by apoptosis. The morphology of apoptosis (reviewed by Wyllie et al 1986) is characterized by nuclear condensation and margination of the chromatin to form dense masses that abut on the nuclear membrane, such as is seen in Fig. 2a, in which the chromatin of one FDCP-2 cell has formed a crescent shaped aggregate lining the nuclear membrane. The integrity of organelles is initially maintained. A later event is the break up of the cell into membrane-bound "apoptotic bodies", often containing nuclear fragments, as can be seen in Fig. 2b. In some planes of sections the nucleus may appear uniformly dense (Kerr et al 1987), as in Fig. 2c. In Fig. 2d blebbing can be seen in the nuclear membrane. Only a proportion of cells at a given time-point after IL3 withdrawal displayed apoptotic morphology, but this is explicable by the fact that the cells died asynchronously (Fig 1a). That the nuclear condensation was not an artifact of fixation is shown in Fig. 2e, which shows an FDCP-2 cell which had been maintained in IL3, and in Fig. 2f, which shows an FDCP-2 cell which had been incubated with complement and an antibody to CD45 (Dennert et al 1980), and hence is dying by necrosis. The nucleus of this cell was unchanged but the plasma membrane was disintegrating.

<u>Fig.2</u>
Fig. 2a-d Electron micrographs of FDCP-2 cells 20 h after removal of IL3.

<u>Scale</u>

Fig. 2a: —— 1μ

Fig. 2b   ———————— 1μ

Fig. 2c   ———— 1μ

Fig. 2d   ——— 1μ
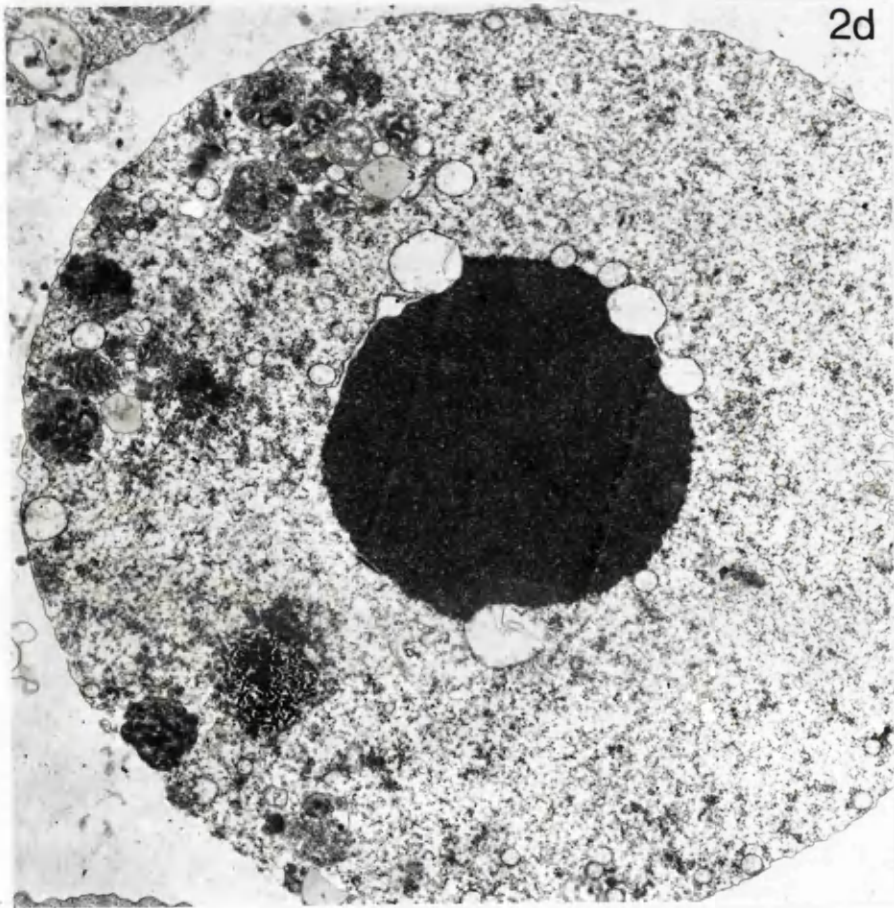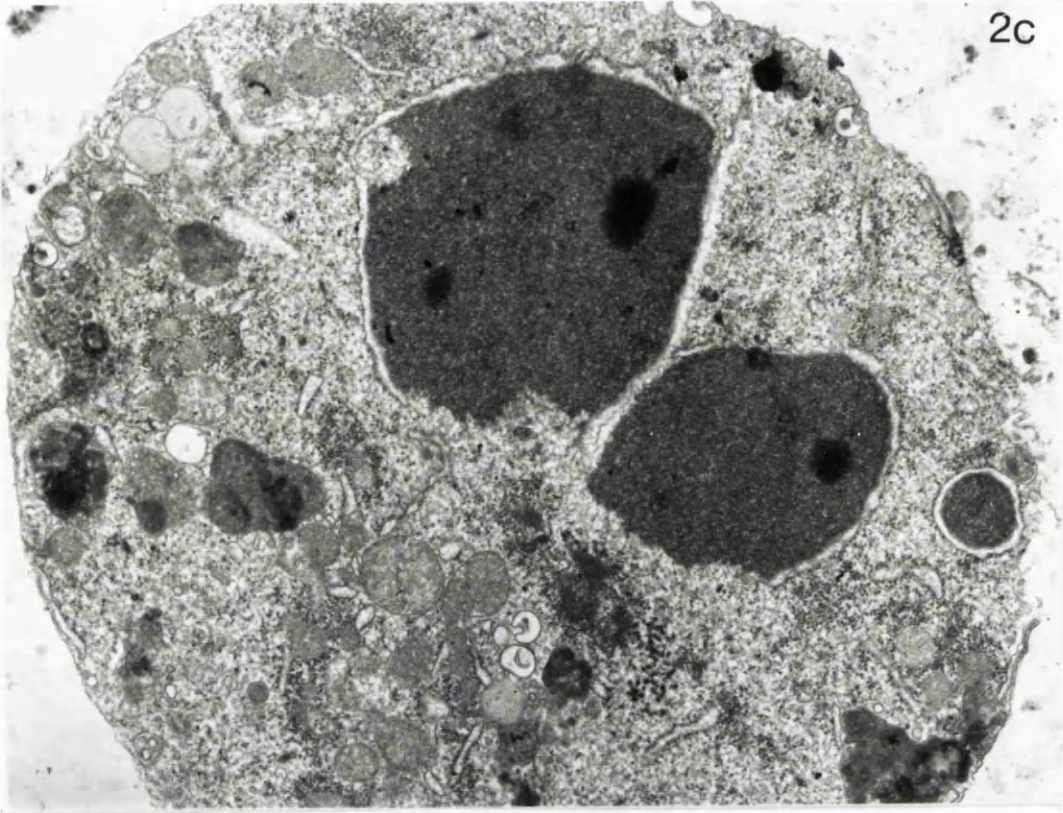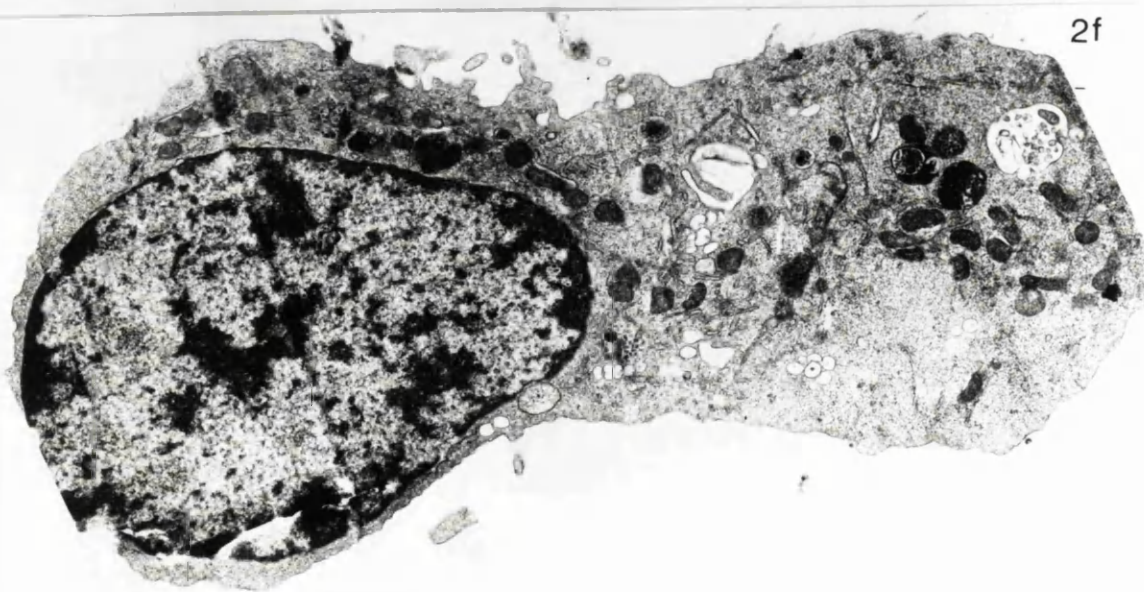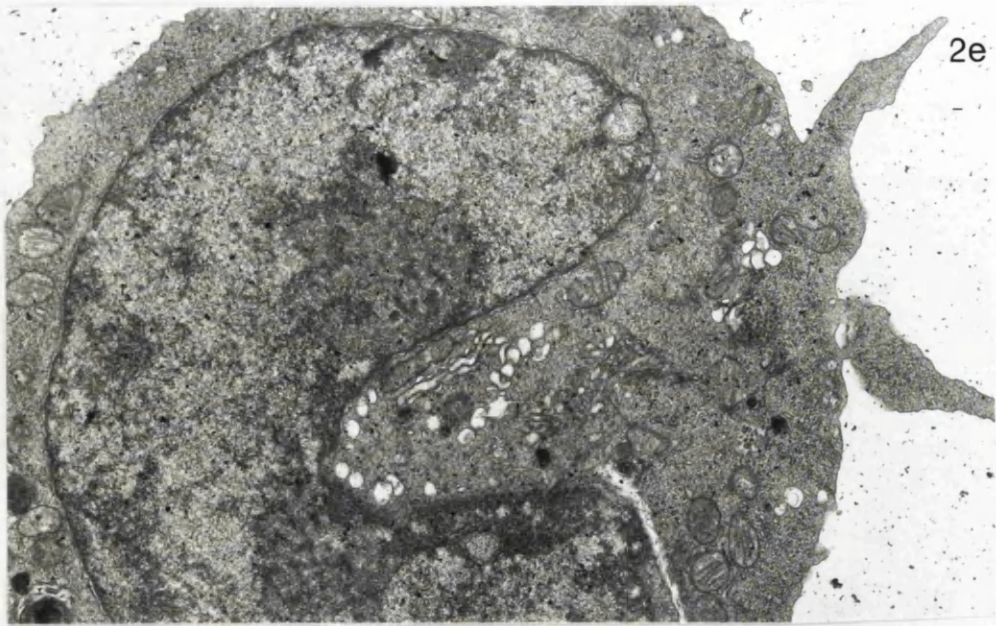
2a

2b

<u>Fig.2</u>
Fig. 2e Electron micrographs of a viable FDCP-2 cell cultured in the presence of IL3.

Fig. 2f Electron micrograph of an FDCP-2 cell that is dying by necrosis due to antibody and complement treatment. The cell was incubated on ice with mab M1-89 (anti-CD45) for 1 h, followed by 20 min at $37^{o}C$ with complement, prior to fixation.

<u>Scale</u>

Fig. 2e: ————— 1µ

Fig. 2f   —————— 1µ

2e

2f

DNA Fragmentation

After incubation of FDCP-2 cells for 18 h without IL3, the DNA had fragmented, so that a typical "ladder" was seen when the DNA was run on an agarose gel (Fig. 3a, tracks c and d). The DNA was cleaved into fragments of multiples of about 200 base pairs, presumably as a result of preferential digestion of internucleosomal DNA by an endonuclease. DNA prepared from cells maintained in IL3 was not fragmented (Fig. 3b, tracks a and b). A ladder was also observed when DNA was prepared from CTLL cells deprived of IL2 for 18 h, and A4 cells deprived of IL3 for 18 h.

When isolated nuclei were prepared from FDCP-2 cells maintained in IL3 and exposed to high concentrations of Ca^{2+} and Mg^{2+} for 2 h internucleosomal DNA cleavage was activated (Fig. 3b, tracks c and d). The DNA of isolated nuclei incubated for 2 h in 2mM EGTA (to chelate Mg^{2+} and Ca^{2+}) was not fragmented (Fig. 3b, tracks a and b).

The kinetics of DNA fragmentation, as quantified by release of ^{3}H-thymidine from pre-labelled cells, adapted from the method of Duke et al (1983) (see Materials and Methods), corresponded closely to the kinetics of death. This is shown for A4 cells in Fig. 3c. The shape of the curves suggests that both the loss of ability to exclude dye and the release of radiolabel were normally distributed, and this suggests that in the case of an individual cell DNA cleavage is a fairly fast event after initiation of death. The background ^{3}H-thymidine release when A4 cells are kept in IL3 is quite low, but nevertheless prohibits the inference that DNA cleavage is occurring before loss of plasma membrane integrity.
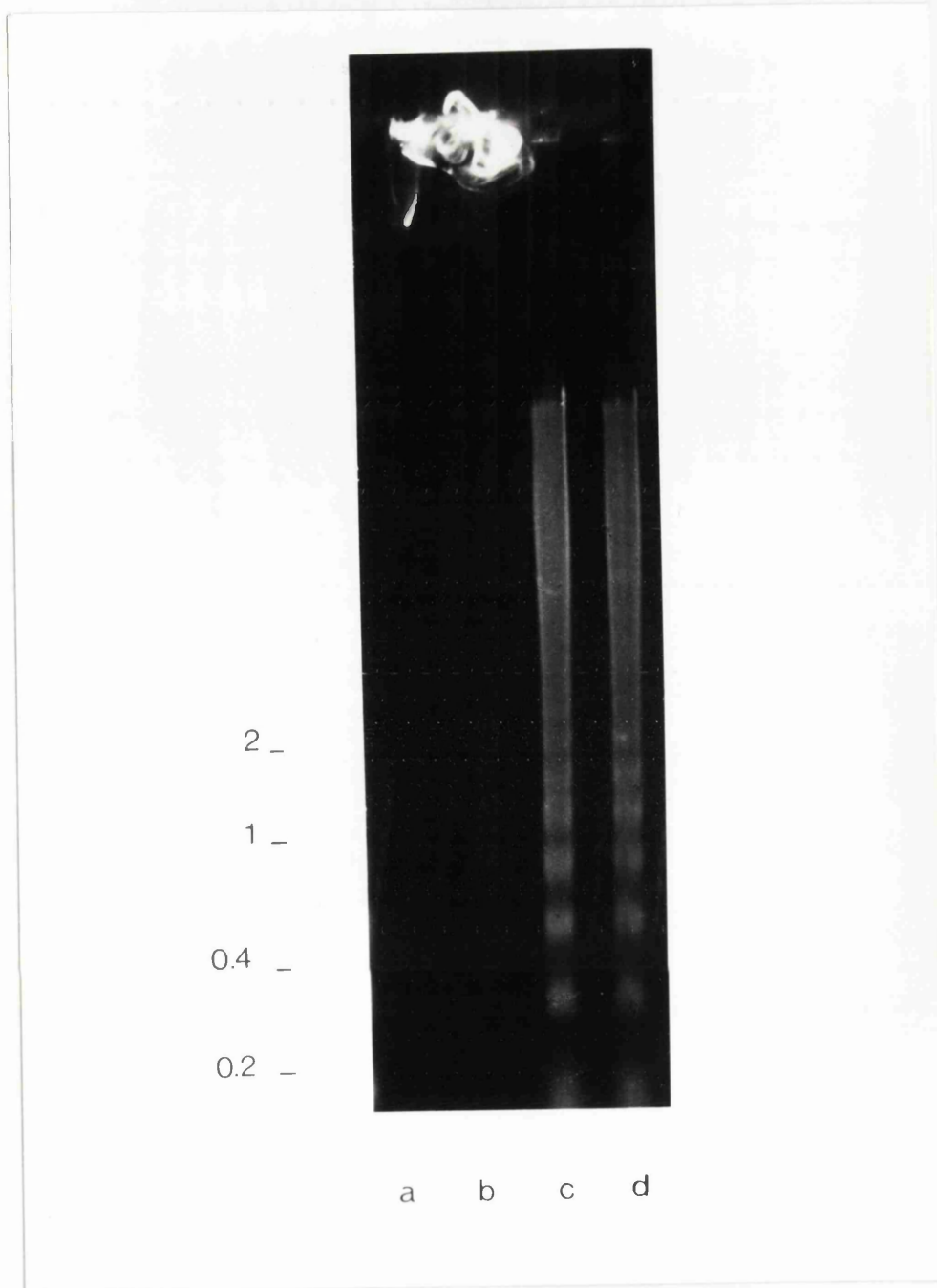
Fig. 3
Fig. 3a Ethidium bromide stained 1% agarose gel showing DNA prepared from FDCP-2 cells grown with IL3 (tracks a and b) and from FDCP-2 deprived of IL3 for 18 h (tracks c and d). Size is given in kilobases.
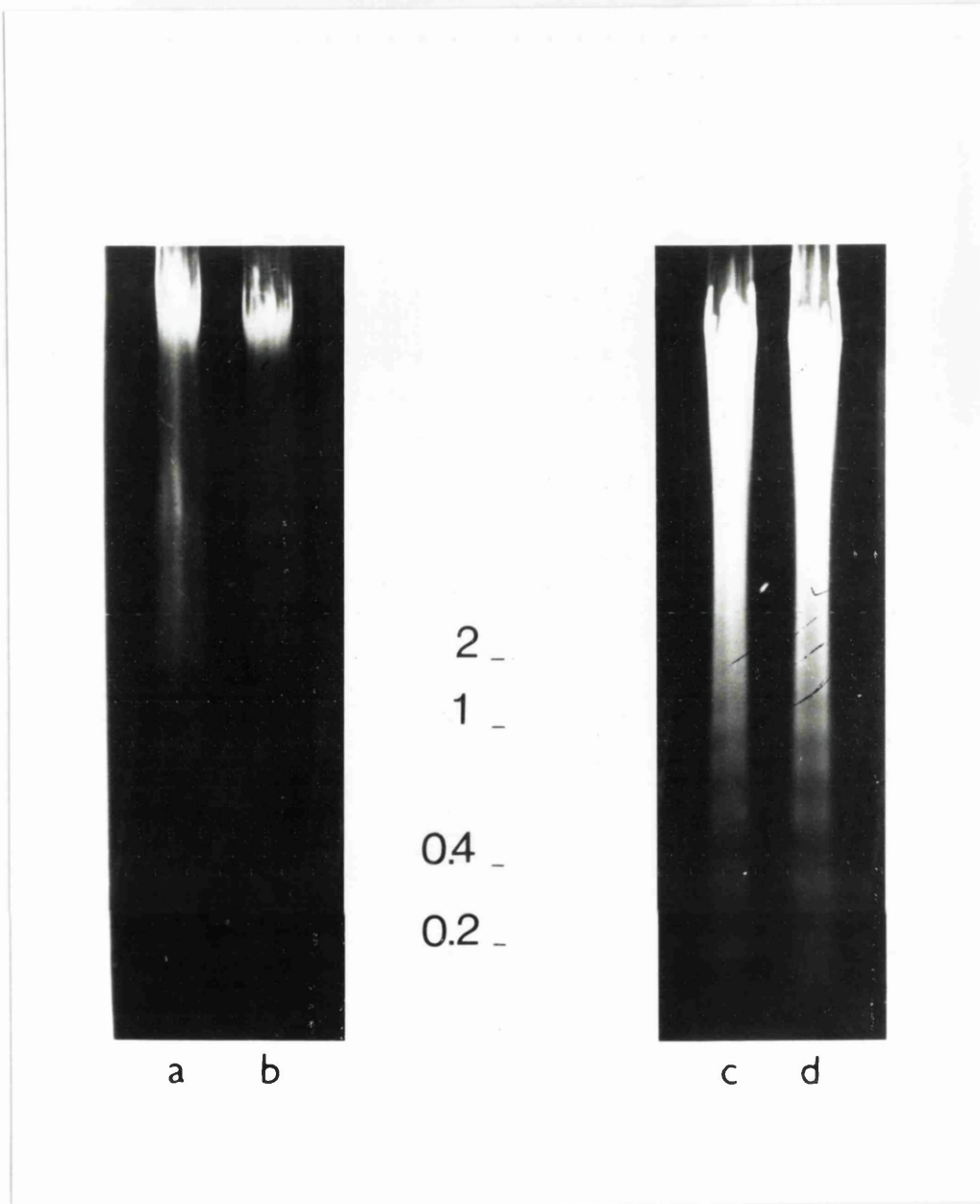
Fig. 3

Fig. 3b Ethidium bromide stained 1% agarose gel showing DNA prepared from isolated nuclei of FDCP-2 cells which were incubated in 1 mM Ca^{2+} and 1 mM Mg^{2+} for 2 h (tracks c and d), and isolated nuclei of FDCP-2 cells incubated in 2 mM EGTA for 2 h (tracks a and b). Size is given in kilobases.
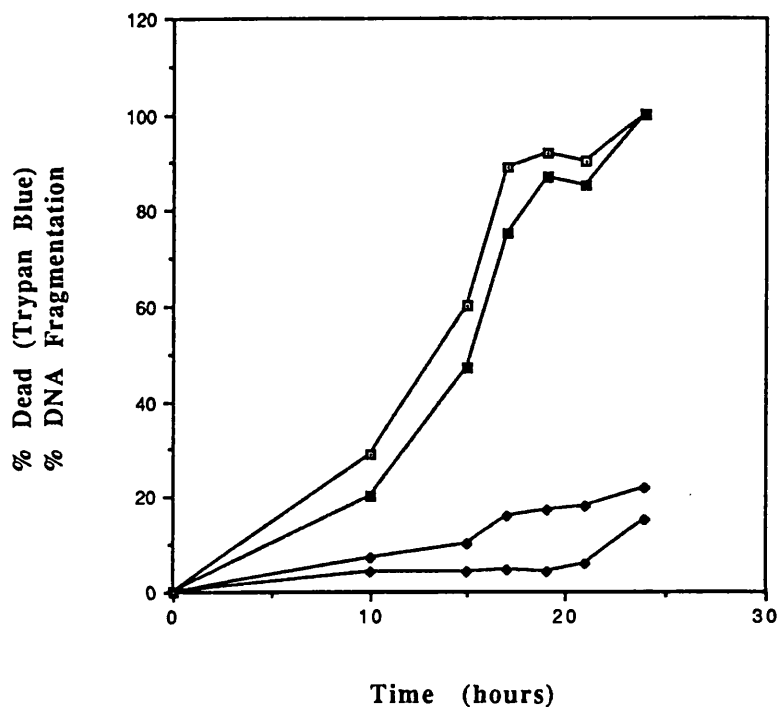
Fig. 3c

Fig. 3
Fig. 3c shows the kinetics of DNA fragmentation and cell death of A4 cells, on IL3 removal. DNA fragmentation was determined by measuring the release of ^{3}H-thymidine fron pre-labelled cells, and is expressed as a percentage of total radiolabel present. Each point is a mean of 6, with $\sigma_n < 10\%$ of the mean. Death was measured by loss of ability to exclude trypan blue. Each point is a mean of 5, with $\sigma_n < 7\%$ of mean. .

—□— % DNA fragmentation - IL3

—♦— % DNA fragmentation + IL3

—■— % Dead (trypan blue) - IL3

—♦— % Dead (trypan blue) + IL3

63

Effect of aurintricarboxylic acid

In order to ask if DNA fragmentation is causal in the death of FDCP-2 cells deprived of IL3, I tested the ability of the nuclease inhibitor aurintricarboxylic acid (ATC) (Hallick et al 1977) to increase viability. As loss of clonogenic potential of FDCP-2 cells preceeded loss of ability to exclude dye (Fig. 1a), and loss of plasma membrane integrity is generally a late event in apoptosis (Duvall and Wyllie 1986), alternative measures of viability to dye exclusion were employed. ATC caused a clear dose-dependent increase in viability, as measured by MTT uptake and breakdown (see Materials and Methods) (Fig. 4a-c). In addition, as an alternative assay, I measured the ability of IL3-dependent cells to be stimulated to incorporate ^{3}H-thymidine on re-exposure to IL3. ATC greatly increased cell survival when this ^{3}H-thymidine assay was used (Fig. 5). Fig. 4d shows that ATC did not inhibit protein synthesis, as assessed by ^{35}S-methionine incorporation.

Effect of cycloheximide

Apoptosis has been shown in several instances to depend on protein synthesis (Duke and Cohen 1986, Shi et al 1989, Pratt and Greene 1976). As shown in Fig. 6a, cycloheximide prolonged survival of FDCP-2 cells on removal of IL3 over a 32 h period, when cell viability was determined by dye exclusion. That cycloheximide at these concentrations was inhibiting protein synthesis was shown by its inhibition of ^{35}S-methionine incorporation (Fig. 6b). As an alternative measure of viability, the ^{3}H-thymidine incorporation assay was used. As shown in Fig. 7, cycloheximide increased the proportion of cells able to incorporate ^{3}H-thymidine on being returned to IL3 after 24 h in its absence. When added at the start of the experiment, cycloheximide (at 0.5 μg/ml and 0.25 μg/ml) enhanced the response (Fig. 7a), although the

6 4

response was much less than that of cells which were exposed to IL3 throughout the experiment (Fig. 7b). Another protein synthesis inhibitor, emitine, had the same effect, and enhanced survival of a small proportion of cells. Both cycloheximide and emitine also increased survival of CTLL and A4 on growth factor deprivation.

Cell Cycle Analysis

To ask if the time it takes a cell to die depends on which point it was at in the cell cycle when its growth factor was removed, cells were synchronized and the kinetics of death in synchronized vs unsynchronized populations of cells was compared. As DNA degradation may be causal in cell death, it seemed inappropriate to use agents that inhibit DNA handling enzymes, such as hydroxyurea (Sinclair 1967), to synchronize cells, and so cells were synchronized by separating the cells by size, and culturing cells of uniform size. That size is an appropriate parameter by which to synchronize FDCP-2 cells is shown in Fig. 8. FDCP-2 cells were incubated with BrdU and then permeabilized and stained with αBrdU (Fluorescence (FL) 1) and with PI (FL 2). Using "paint a gate" soft-ware (Becton Dickinson), cells which stain brightly with PI, and so have more DNA and are in G_2, can be seen to be larger (painted blue) and comprise 36% of total (Fig. 8a). *See arrow* When this staining was carried out after 4 h incubation with 1.25 mM hydroxy-urea, a drug which blocks progression from G_1 to G_2 by preventing DNA synthesis through inhibition of ribonucleotide reductase, the volume profile, as assessed by fluorescence side scatter (FSC) becomes clearly *(See arrow)* bimodal, with only 17.6% of cells in G_2 (Fig. 8b).

FDCP-2 cells were separated according to density (volume) on a discontinuous percoll gradient, and FSC, as an estimate of size, was measured by flow cytometry (Fig. 9a). The proportion of cells in each

fraction in G_1 or G_2 was determined by PI staining (Fig. 9b). Each fraction was washed to remove IL3, cultured in the absence of IL3 and cell death was measured by PI exclusion after various times. Fig. 9c shows that there was no difference in the kinetics of death between the different fractions. This experiment was carried out in 5% HS, but as serum contains many factors and hormones, the experiment was repeated in serum-free medium (Iscove and Melchers 1985) and again no difference in kinetics was observed, except that all cells died slightly more rapidly and after 24 h viability was reduced by approximately 10 % in the controls that were kept with IL3.

It therefore seems unlikely that death is initiated at the putative "restriction point" (R) in G_1 (Pardee 1974). However, it has been suggested that some cells, such as activated B cells, have more than one restriction point (Melchers et al 1985). If FDCP-2 cells had more than one restriction point that required IL3 (or another factor contained in Wehi-3b supernatant) to pass through, then death might be initiated at two restriction points, and hence be cell cycle-dependent, without these experiments being able to detect it. Agents which arrest cells in cycle would theoretically increase the proportion of cells able to recover on re-exposure to growth factor by preventing a proportion of cells from reaching the restriction point(s). Neither hydroxyurea (Fig. 10a) nor aphidicolin, a competitive inhibitor of DNA-polymerase-α (Spadari et al 1982), (Fig. 10c) increased the ability of FDCP-2 cells to be stimulated by re-exposure to IL3. That hydroxyurea and aphidicolin were non-toxic at the concentrations employed is shown in Fig. 10b and Fig. 10d respectively. That hydroxyurea was an effective cell-cycle block for FDCP-2 cells is seen in Fig. 8b. That aphidicolin arrested FDCP-2 cells at 0.015 mM is shown in Fig. 10e.

A final objection to the conclusion that apoptosis is not initiated at the cell cycle restriction point is that commitment to death might take place at R, but that thereafter the cells might take a varying amount of time to die, as defined by dye exclusion. To exclude this objection one would need to perform limiting dilution cloning of synchronized and unsynchronized populations over a time-course after IL3 removal. This experiment seemed impracticable, but as the kinetics of ^{3}H-thymidine release from pre-labelled cells suggested that the release approximates to a binomial distribution for individual cells (Fig. 3c) and as DNA fragmentation seemed to cause death (Fig. 6), the kinetics of ^{3}H-thymidine release from pre-labelled synchronized cells was compared. A4 cells were used in this experiment because they died more quickly and so background release was reduced. Fig. 11 shows that there was no difference in the kinetics of DNA fragmentation between the synchronized populations. The possibility that cells take the same interval of time to die once the mechanism of death is initiated but that there is an interval between commitment to death at R and initiation of the mechanism of death, dependent on a stochastic event, is not excluded. A precedent for this suggestion is the control of proliferation, in which after commitment at R there is variability in the time before DNA synthesis is initiated (Zetterberg and Larsson 1985), consistent with a transition probability event (Brooks 1985).

DNA Repair

The activity of the DNA repair enzyme, O^6methylguanine-DNA methyltransferase was measured in cells deprived of growth factor, using a specific assay for this enzyme (Bodgen et al 1981). Fig. 12 shows that there was no increase in the activity of this enzyme relative to total

cellular protein content in FDCP-2 cells and in CTLL cells on interleukin withdrawal.
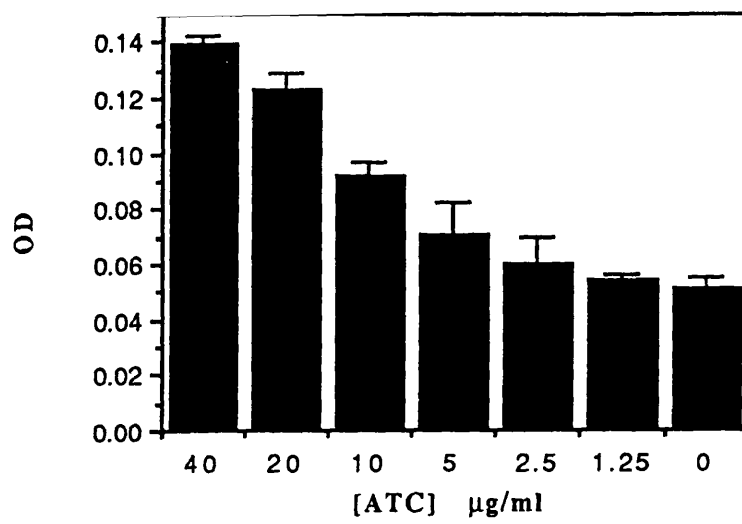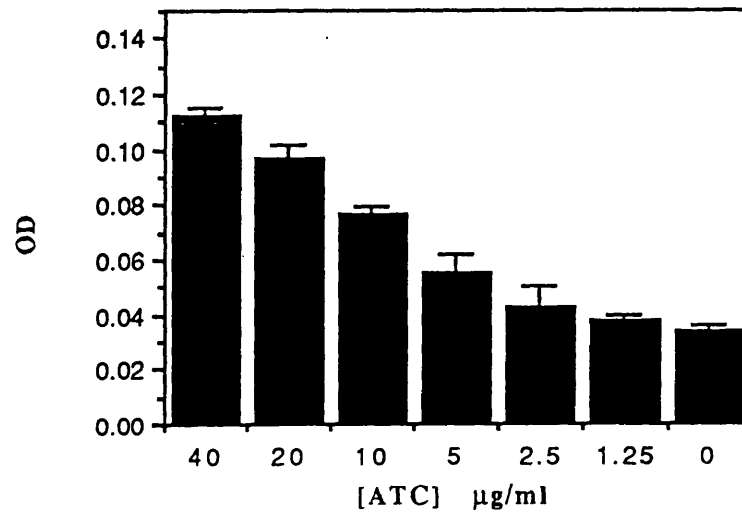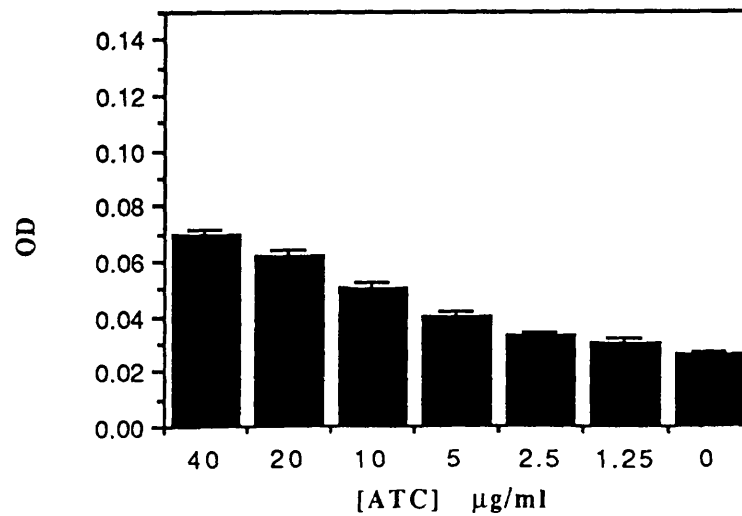
Fig. 4a

Fig. 4b

Fig. 4c

Fig. 4
Viability of FDCP-2 cells, measured by their ability to breakdown MTT, when incubated with ATC, after 16(Fig. 4a), 22(Fig. 4b) and 41(Fig. 4c) h of IL3 deprivation. In this and all further figures, bars represent standard deviations. [O.D. of FDCP-2 + IL3 = 0·17]

69
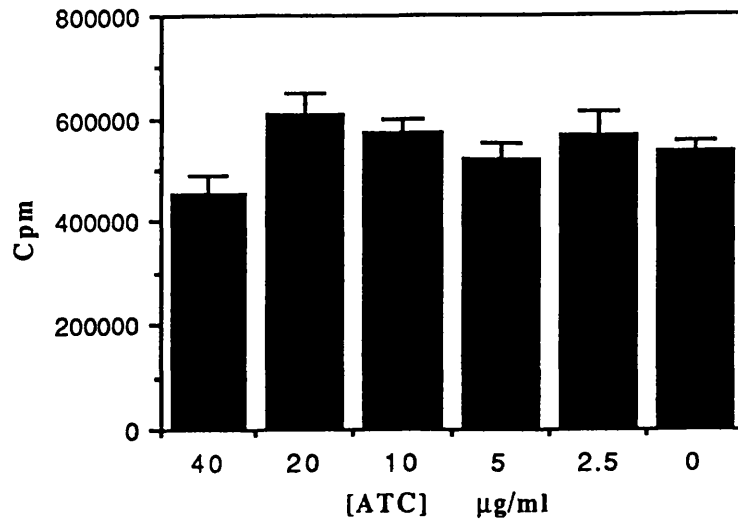
Fig. 4d

Fig. 4d

Incorporation of ^{35}S-methionine by FDCP-2 cells in presence of ATC.
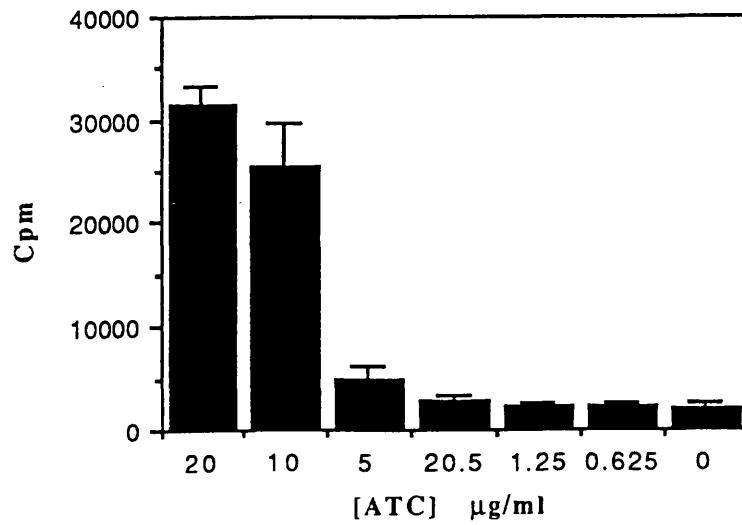
Fig. 5

Fig. 5

Incorporation of ^{3}H-thymidine by FDCP-2 cells on re-exposure to IL3, after 24 h deprivation of IL3, in the presence or absence of ATC.
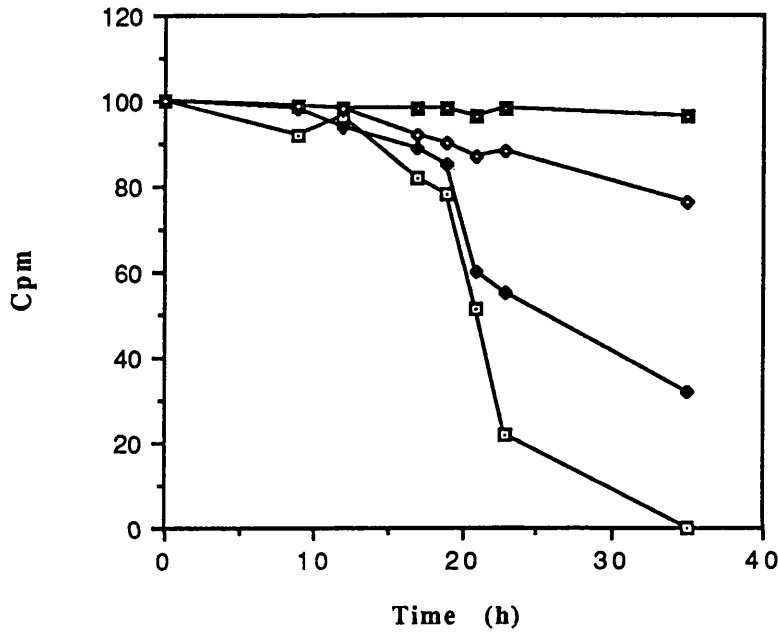
Fig. 6a

Fig. 6a

The kinetics of FDCP-2 cell death, measured by PI exclusion, on IL3 removal in the presence of 0.5μg/ml cycloheximide. Each point is a mean of 5, with σ_n < 3% of the mean. Differences are statistically significant from t = 16.

▪	+ IL3
◇	+ IL3 + cycloheximide
▫	- IL3
◆	- IL3 + cycloheximide
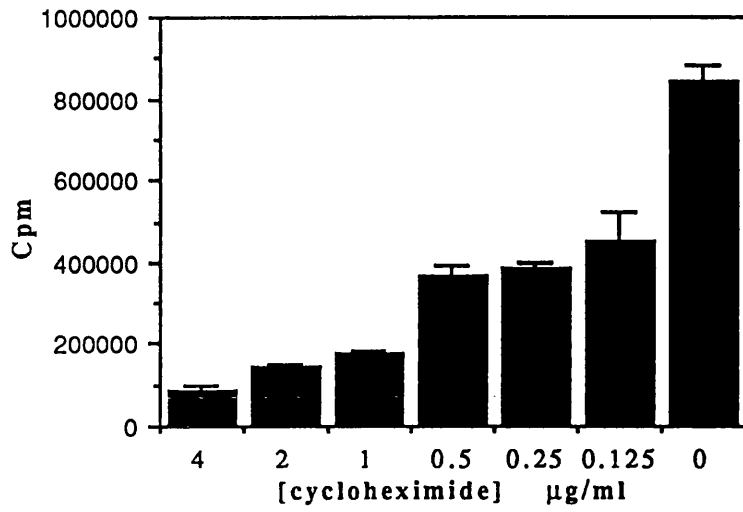
Incorporation of ^{35}S-methionine by FDCP-2 cells in presence of cycloheximide.
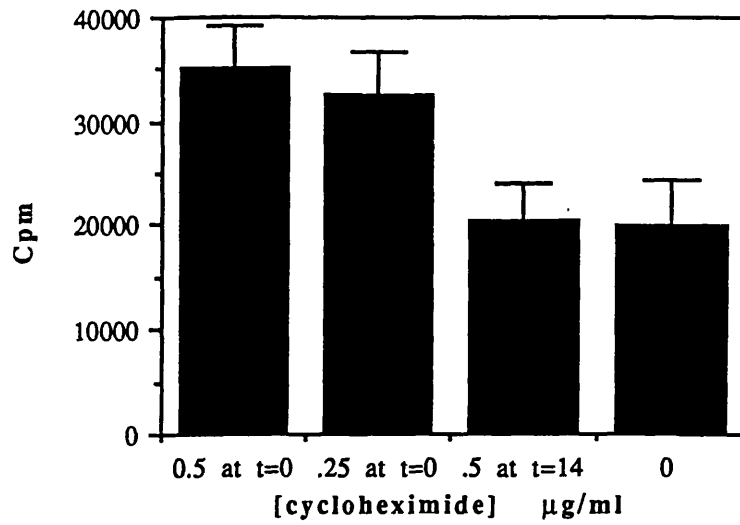
Fig. 7a

^{3}H-thymidine incorporation by FDCP-2 cells on re-exposure to IL3, after 24 h incubation in absence of IL3, but presence of cycloheximide.
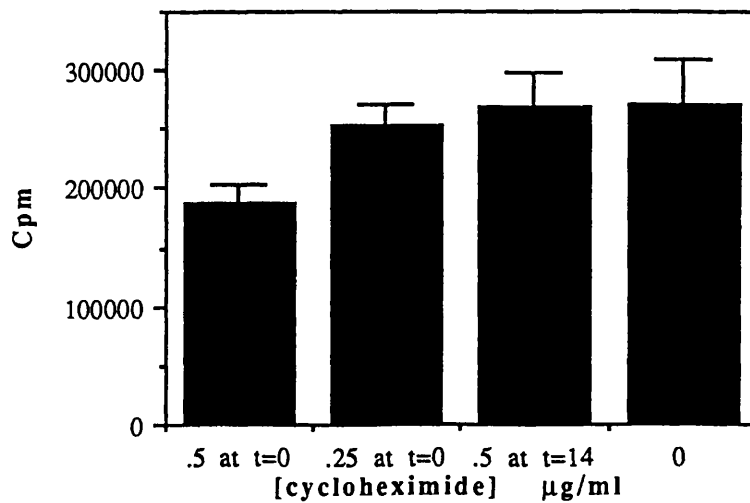
Fig. 7b

Fig. 7b
^{3}H-thymidine incorporation by FDCP-2 cells after 24 h exposure to cycloheximide.

73

Fig. 8a

Flow cytometry of FDCP-2 cells stained with anti-BrdU (FL1) and PI (FL2), analysed using "paint a gate" soft-ware. Cells painted blue are in G_1/M.

Fig. 8b

As above, but prior to staining cells were incubated with 1.25 mM hydroxyurea for 4 h.

Key

FSC   Volume

FL1   fluorosonce 1

FL2   fluoresence 2

SSC   side scatter

%= 43.0 1.1 1.0 36.1 0.0 3.6 0.0
RGBO gate; +- mode; <> size
0-5 plot; FZH$s

Fig. 8a
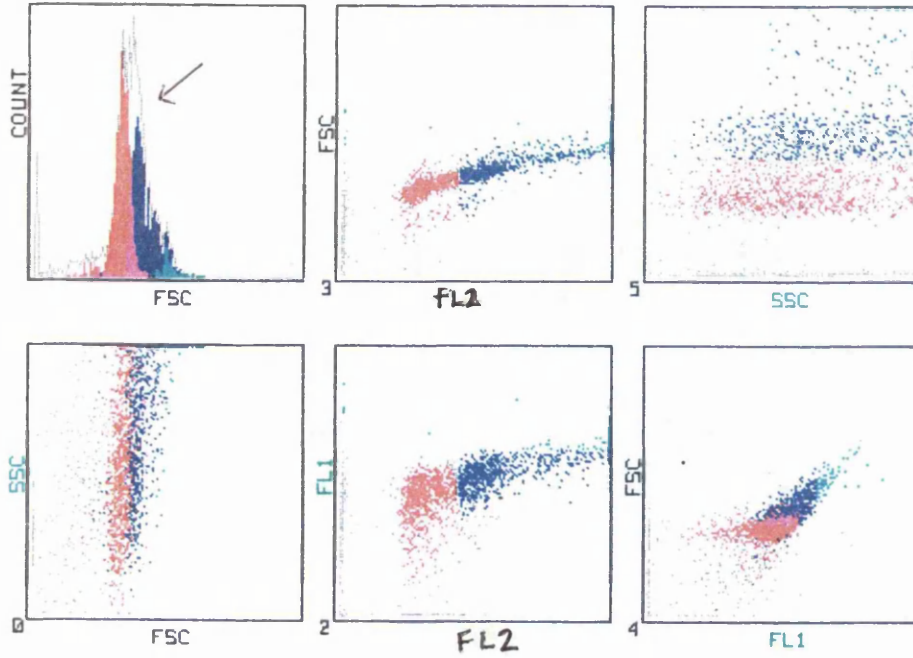
%= 43.2 3.3 17.6 0.0 6.8 0.0
RGBO gate; +- mode; <> size
0-5 plot; FZH$s

Fig. 8b

Fig. 9a

Fig. 9b
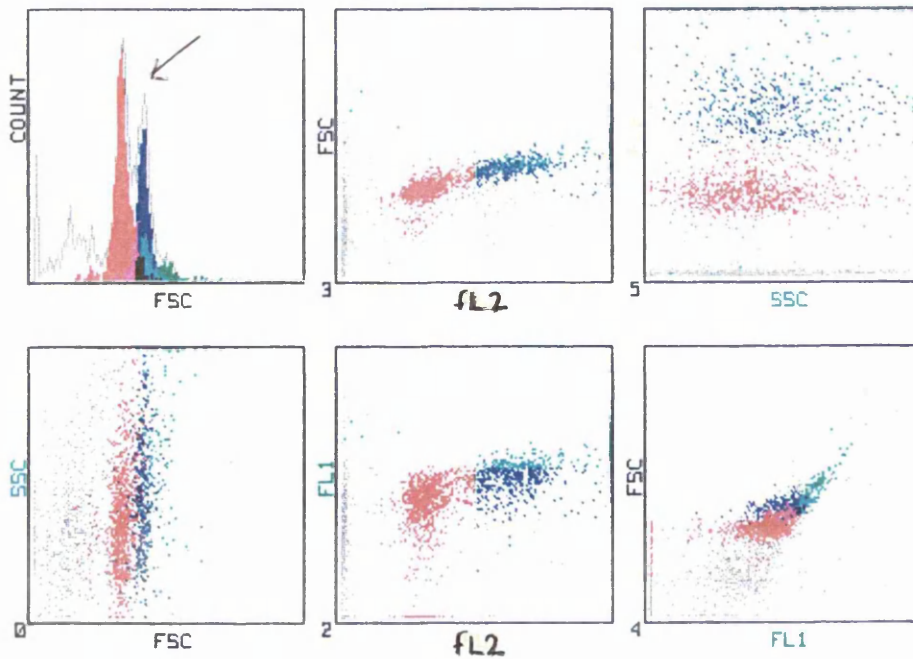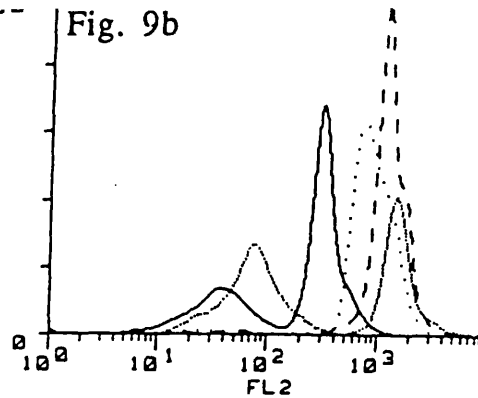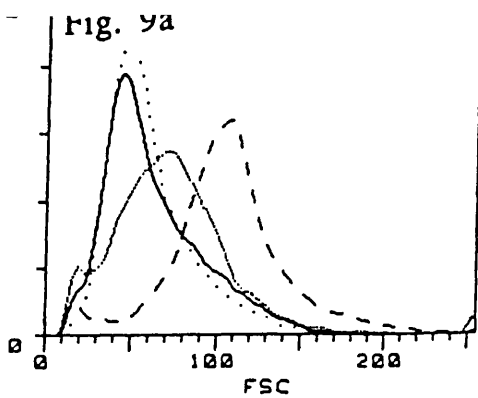
1.) DATA002   A

2.) DATA004   ....

3.) DATA005   FDCP-2

4.) DATA006   B

Fraction	% G_0/G_1	% G_2/M
FDCP-2	54	46
A	78	22
B	13	87

Fig. 9c

FDCP-2 + IL3
A + IL3
B + IL3
FDCP-2 - IL3
A - IL3
B - IL3
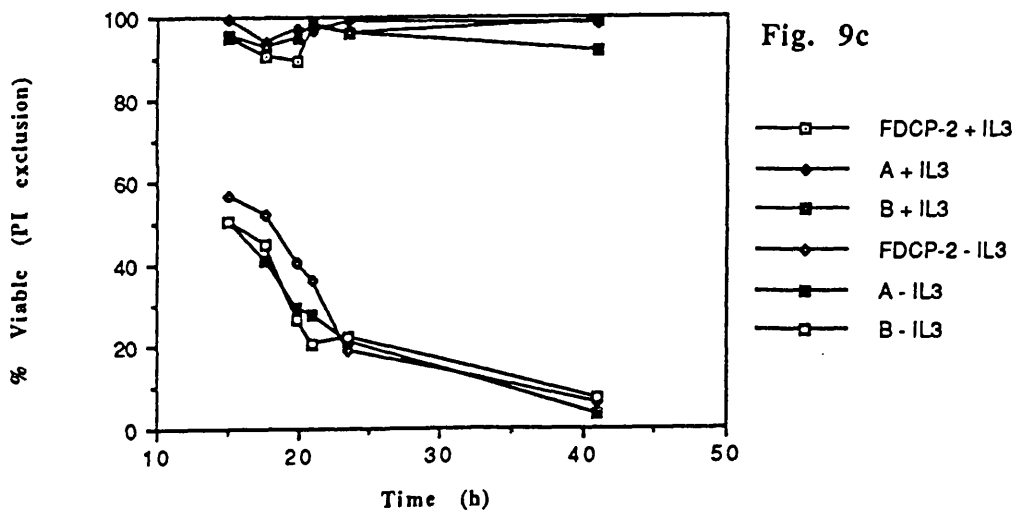
Fig. 9

Fig. 9a Volume (FSC) profiles for the populations of FDCP-2 cells
separated on a discontinuous percoll gradient and for unfractionated
FDCP-2 cells. Volumes may be slightly distorted because cells have been
fixed with 70% ethanol. 'A' is enriched for cells in G1 and 'B' is enriched for cells in G2.
Fig. 9b Same fixed cells stained with PI, and an estimate of their cell
cycle ststus. 'A' is enriched for cells in G_1 and 'B' is enriched for cells in
G_2.
Fig. 9c Kinetics of cell death in the three groups, as determined by PI
exclusion by flow cytometry. Each point is the mean of five readings
and in all cases the standard deviation was less than 3% of the mean.

76

<u>Fig. 10a</u>
^{3}H-thymidine incorporation by FDCP-2 cells on restimulation with Il3, after 24 h incubation in the absence of IL3, but presence of 0-1.25 mM hydroxyurea.

<u>Fig. 10b</u>
^{3}H-thymidine incorporation by FDCP-2 cells after 24 h incubation with 0-1.25 mM hydroxyurea.

<u>Fig. 10c</u>
^{3}H-thymidine incorporation by FDCP-2 cells on restimulation with IL3, after 24 h incubation in the absence of IL3, and presence of 0-0.06 mM aphidicolin.

<u>Fig. 10d</u>
^{3}H-thymidine incorporation by FDCP-2 cells after 24 h incubation with 0-0.06 mM aphidicolin.

<u>Fig. 10e</u>
Flow cytometry of FDCP-2 cells stained with anti-BrdU (FL1) and PI (FL2), analysed using "paint a gate" soft-ware. Cells painted blue are in G2/M. Cells were treated with 0-0.15mM aphidicolin for 4 h prior to staining.

(For Key see fig. 8 legend)

Fig. 10a

Fig. 10b
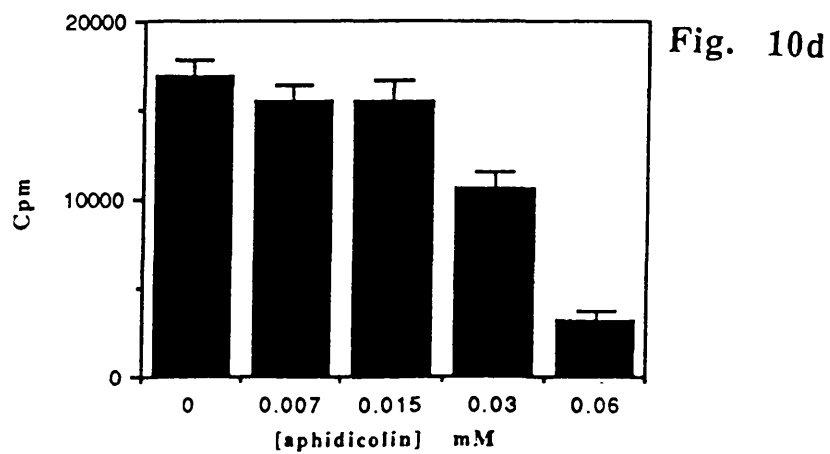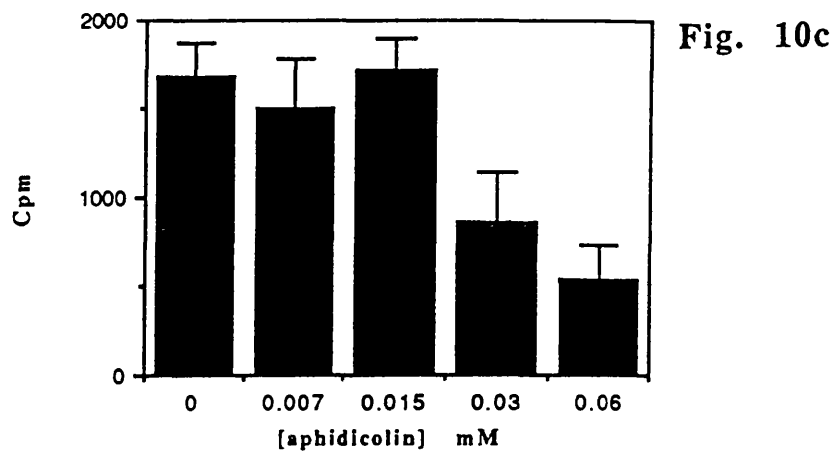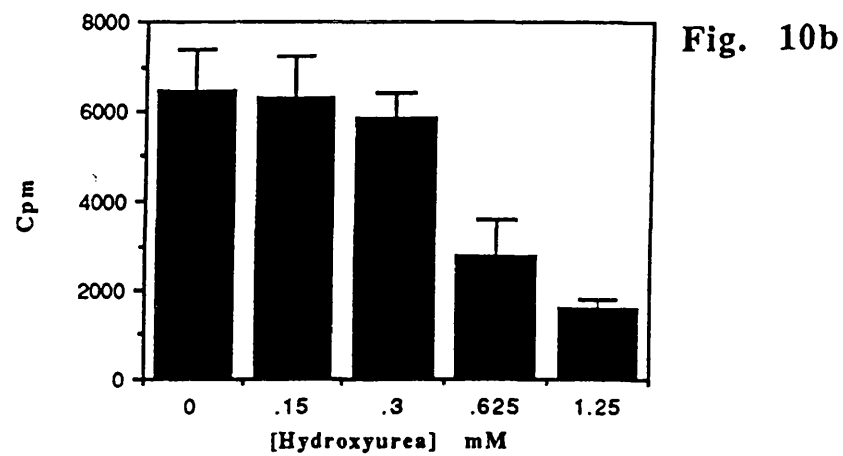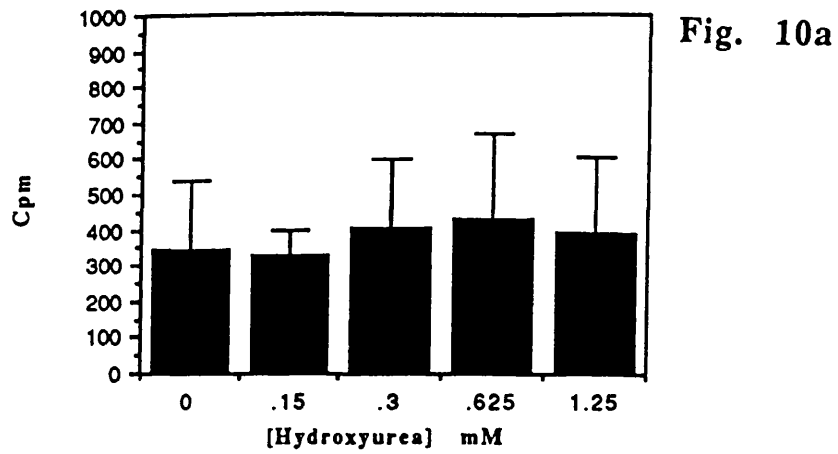
Fig. 10c

Fig. 10d

%= 51.0 0.9   21.4 0.4 4.6 0.0
RGBO gate; +- mode; <> size
0-5 plot; FZH$s

Fig. 10e
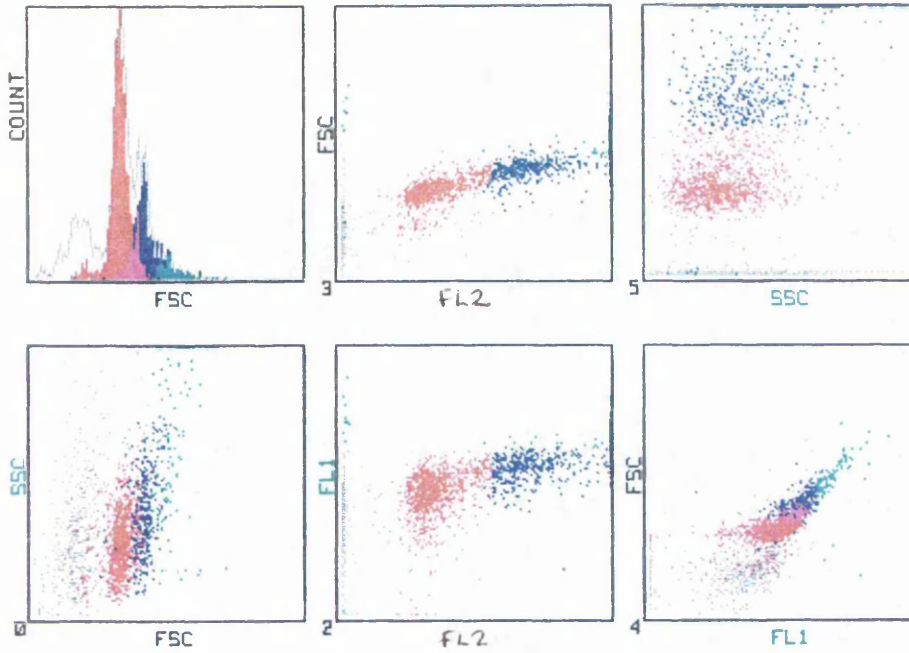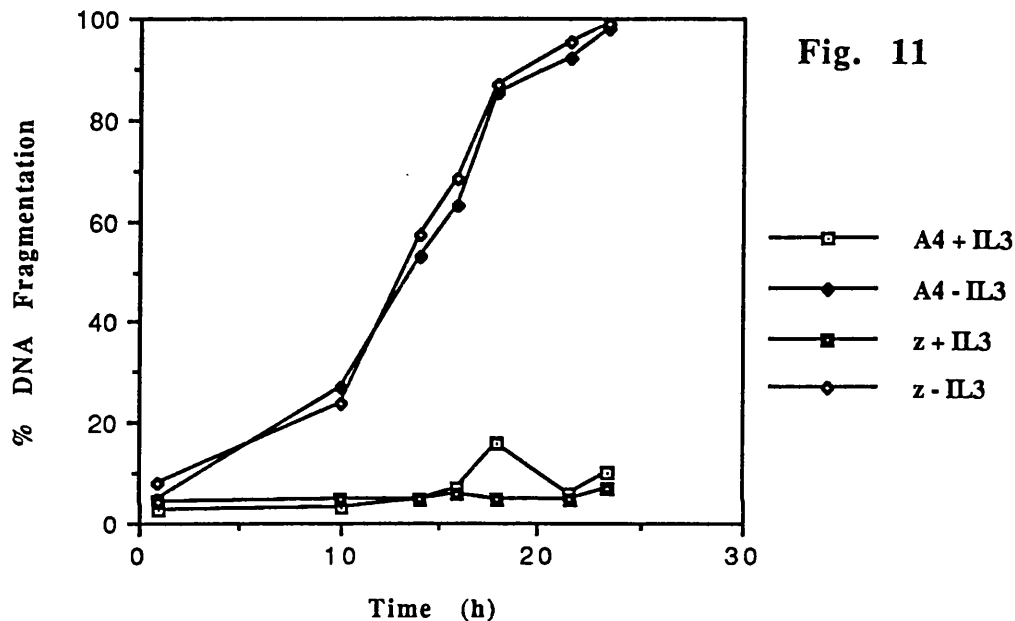
Fig. 11

Fig. 11

Kinetics of DNA fragmentation by A4 cells, and by a population of A4 cells enriched for cells in G_2/M on a discontinous percoll gradient (designated 'z'). The unfractionated population of A4 cells contained 54% cells in G_0/G_1 and 46% in G_2/M, whereas 'z' contained 85% cells in G_2/M. DNA fragmentaion was determined by measuring the release of ^{3}H-thymidine from pre-labelles cells, and is expressed as a percentage of total radiolabel present. Each point is a mean of 6, and σ_n was < 10% of the mean in all cases.
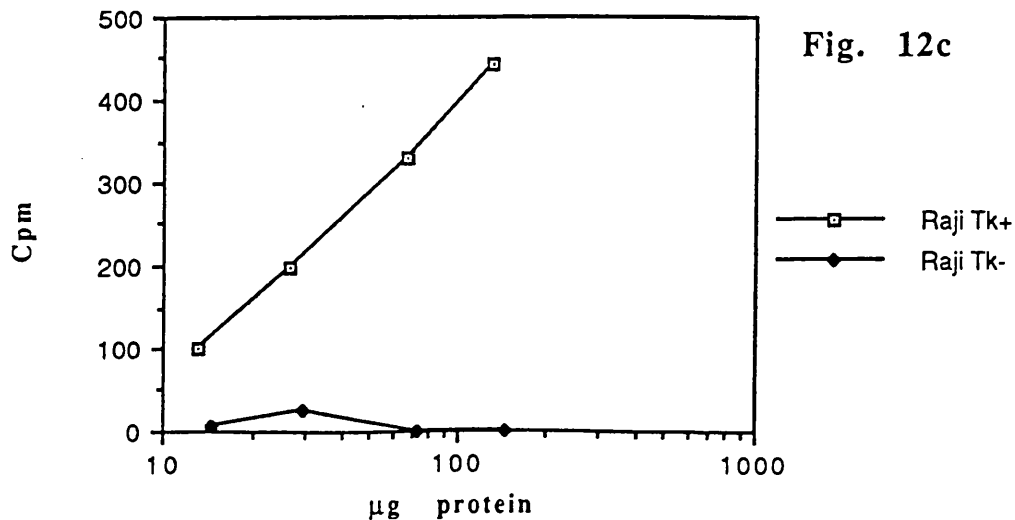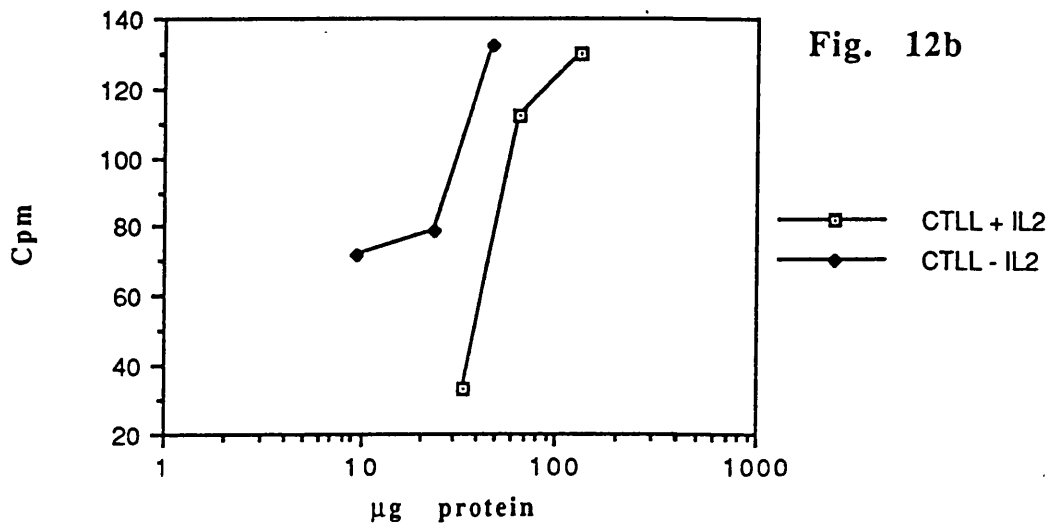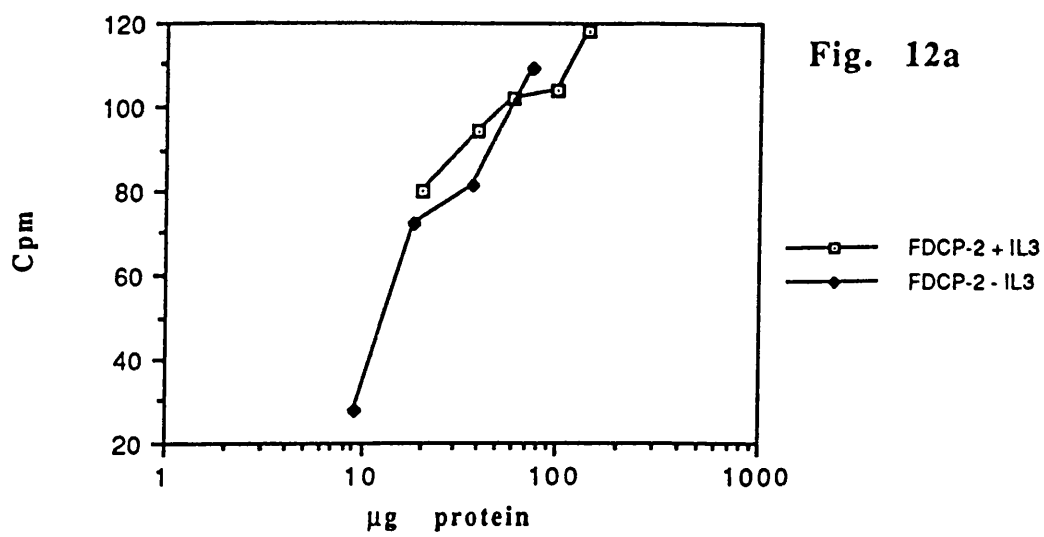
Fig. 12

Activity of O^6-methylguanine-DNA methyltransferase in cellular extracts from FDCP-2 cells, cultured with or without IL3 for 15 h (**Fig. 12a**), CTLL cells, cultured with or without IL2 for 15h (**Fig. 12b**), and Raji Tk^+ and Raji Tk^- cells, as positive control (**Fig. 12c**). Cpm shown are counts that the enzyme removed from a pre-labelled sustrate.

DISCUSSION

There are several reported examples of programmed cell death being induced by withdrawal of a growth factor : IL2-dependent T cells die on removal of IL2 (Duke and Cohen 1986); cultured rat sympathetic neurons die on withdrawal of nerve growth factor (NGF) (Martin et al 1988); epithelial cells of the rat prostate die when androgen is withdrawn by castration (Stanistic et al 1978); and IL3-dependent FDCP-2 cells, A4 cells (this report), FDCP-1 cells and FDCP-mix cells (Williams et al 1990) die on removal of IL3. In all cases death is inhibited by the protein synthesis inhibitor cycloheximide, and this has been interpreted to mean that death is an active process that requires the synthesis of specific proteins essential for cell death. The finding that the removal of a signal causes the cell to commit suicide is surprising. Intuitively, one might think that growth factors stimulate cell proliferation and that their removal would lead to the cell withdrawing from the cell cycle; if death followed it might be due to a gradual decline in metabolism, rather than to active suicide. Is it possible, then, that there is an alternative explanation for the results obtained with cycloheximide?

In the case of proliferating cells, such as FDCP-2 cells, cycloheximide might, in principle, enhance survival by slowing the rate at which cells progress through the cell cycle. FDCP-2 cells die asynchronously on IL3 withdrawal (Fig. 1a), suggesting that death might be initiated at a specific point in the cell cycle, such as the "restriction point" (R). Cycloheximide might inhibit cell death by causing a proportion of the cells to arrest early in G_1 (Zetterberg and Larsson 1985), so that they never reach R during the timespan of the experiment. This does not seem to be true for FDCP-2 cells, however, as

synchronization of the cells does not alter the kinetics of cell death, suggesting that the cells are dying at different points in the cycle. It is still possible that FDCP-2 cells are incapable of going into G_0 so that R is the commitment point for either proliferation or death, but that after commitment to death, FDCP-2 cells take a varying amount of time to die. The fact that agents that cause cell cycle arrest such as hydroxyurea and aphidicolin do not seem to enhance survival on re-exposure to IL3 in the ^{3}H-thymidine incorporation assay suggests that this is not the case, as does the fact that cell cycle synchrony does not alter the kinetics of DNA fragmentation in the ^{3}H-thymidine release assay, even though for an individual cell this assay seems to be measuring an all-or-none phenomenon, rather than a gradual release of radio-label. In addition, transfection of the related IL3-dependent cell line FDCP-1, with the proto-oncogene *bcl-2* suppresses cell death on IL3 removal without stimulating proliferation (Vaux et al 1988), suggesting that these cells can become quiescent, and that the action of IL3 to promote survival is distinct from its ability to stimulate proliferation. Thus, cell cycle arrest does not seem to explain the inhibition of FDCP-2 cell death by cycloheximide.

The above arguments are not relevant to the action of cycloheximide in prolonging neuron survival when NGF is withdrawn. Cycloheximide increases survival of neurons in the absence of NGF *in vitro* (Martin et al 1988) and *in vivo* (Oppenheim and Prevette 1988) even though these cells do not proliferate. Perhaps, rather than preventing the *de novo* synthesis of proteins that kill the cell, cycloheximide acts to prolong the life of an essential mRNA, normally made when the cell is stimulated by growth factor. Stimulation of quiescent cells with growth factors activates a number of genes, such as *c-fos* and *c-myc*, and cycloheximide has been shown to superinduce the

mRNA levels of these and other genes, both by inhibiting the shut off of their transcription and by prolonging the life-times of the mRNAs, perhaps by inhibiting a labile RNAase (Lau and Nathans 1986, Greenberg et al 1985). Unfortunately, examination of c-fos and c-myc mRNA levels would not definitively distinguish between these two mechanisms for cycloheximide's effect in inhibiting cell death, as it has been demonstrated that c-fos and c-myc are activated when rat prostatic cells die in response to androgen withdrawal (Buttyan et al 1988).

The internucleosomal DNA cleavage observed in apoptosis has also been reported in forms of cell death that are not thought to be active, such as complement-mediated lysis of mouse B and T cells (Bachvaroff et al 1977). Exposure of isolated nuclei from T and B cells (Bachvaroff et al 1977), thymocytes (Cohen and Duke 1984), and FDCP-2 cells (this report) to high concentrations of Ca^{2+} and Mg^{2+} activates internucleosomal DNA cleavage, suggesting that the enzymes that catalyze the cleavage are always present in these nuclei and are not synthesized only on induction of apoptosis. Is DNA fragmentation, then, a secondary, non-causal phenomenon in apoptosis? The inhibition of death by the nuclease inhibitor ATC suggests that DNA fragmentation might mediate cell death in apoptosis. ATC blocks nuclease activity by inhibiting nucleic acid binding proteins (Blumenthal and Landers 1973). Therefore, it can also act as a general protein synthesis inhibitor by preventing the initiation of polypeptide synthesis (Stewart et al 1971). At the concentrations used in my experiments, however, the effect of ATC on overall protein synthesis was negligible (see Fig. 6d), suggesting that its effect on apoptosis resulted from nuclease inhibition. This interpretation is supported by the finding that cycloheximide, at concentrations that effectively prevented protein synthesis, had a much weaker effect than ATC in promoting cell

84

survival. ATC also inhibits death of thymocytes, induced by glucocorticoids or calcium ionophores (McConkey et al 1989).

The data presented here show that FDCP-2 cells die by apoptosis on IL3 removal and that DNA fragmentation probably causes death. Is this consistent with an earlier report that viability of FDCP-2 cells can be increased on IL3 withdrawal by addition of ATP to the culture medium (Whetton and Dexter 1983)? A theory of cell death which links ATP depletion to breaks in DNA, and so would explain the effects of both ATC and ATP to prolong survival of FDCP-2 cells, is the suicide hypothesis first proposed by Berger (1985). This postulates that it would be advantageous to an organism for there to be a mechanism by which cells with extensive DNA damage kill themselves, as the extra pressure placed on the DNA repair apparatus in cases of extreme DNA damage might increase the risk of error in repair, and associated mutagenesis. The theory suggests that the enhanced poly(ADP-ribosyl)ation of DNA that occurs under conditions of extreme DNA damage (Farzeneh et al 1982) actually functions to deplete cellular NAD^+ (Sims et al 1983). One of the consequences of this depletion of NAD^+ is a decrease in glycolysis (Berger et al 1986), and decline in the production of ATP. However, the function of poly(ADP-ribose) synthetase remains controversial (Gaal et al 1987) and some authors have suggested that poly(ADP-ribosyl)ation instead functions to stabilize the chromatin structure and that its action is directly antagonistic to programmed cell death (Wielckens et al 1987). In a study of glucocorticoid induced death of thymocytes Wielckens argues that death only occurs when this antagonistic potential is exhausted and that death can be regarded as a struggle between DNA repair enzymes and the endonucleases that cleave the DNA. Given that it is not clear if poly(ADP-ribose) synthetase is involved in DNA repair, I decided to look for evidence of increased activity of another well

8 5

defined DNA repair enzyme, O^6methylguanine-DNA methyltransferase. No increase in activity of this enzyme was observed in the dying cells.

What is the physiological significance of the induction of apoptosis by growth factor withdrawal? The NGF-dependence of sympathetic neurons has been frequently described *in vivo*, and can be explained functionally, in that it facilitates the quantitative matching of targets with their sources of innervation.

The physiological role of interleukin-dependence is less well documented. IL2-dependence of activated T cells would in theory help dampen down an immune response when antigen is no longer present. When antigen is cleared, IL-2 production by T helper cells subsides and most effector cells disappear from the animal (MacDonald et al 1973). If all these effector cells became quiescent, they could potentially interfere with subsequent immune responses to other unrelated antigens (Moller and Sjoberg 1970) by competing for limiting concentrations of growth factors, and in addition they would use up energy with no advantage to the animal. It seems that the majority of antigen specific T cells do not merely cease dividing, but actually die on interleukin removal (Gillis et al 1978, Bishop et al 1985). Duke and Cohen (1986) have demonstrated IL2-dependence and active cell death *in vitro* in CD8$^+$ T cell lines, MLR blasts and Con A blasts. They also demonstrate DNA fragments *in vivo* in lymph node cells draining a site of antigen administration. Apoptosis has been observed in lymph node (Searle et al 1982) and spleen (Swartzendruber and Congdon 1963). Taken together this evidence suggests that apoptosis induced by IL2 withdrawal does have a role in regulating the immune response.

Does apoptosis due to IL3 withdrawal have a function in the control of normal haemopoiesis? Normal somatic cells have a finite lifespan and in culture tend to undergo a limited number of cell

divisions before they reach a crisis point and die (Hayflick 1965).
However an apparent exception to this inevitable cell death is the case
of stem cells. Haemopoietic stem cells from aged mice can be repeatedly
grafted into young mice, so that the cells proliferate and survive for at
least five times the lifespan of a normal mouse, and the decline in
transplantability that is finally observed may only be due to technical
difficulties (Ross et al 1982). It is tempting to speculate that growth
factor dependence might be the physiological control of this system of
self-renewal. Normal haemopoietic cells rapidly die in culture in the
absence of their appropriate colony stimulating factor (CSF) (Metcalf
1970). Haemopoiesis can be supported in culture by stromal cells in the
absence of added IL3 or GM-CSF and, under these circumstances, the
growth factors are not detectable in the culture medium (Roberts et al
1987), but it is likely that they are produced by the stromal cells and
retained on their plasma membranes in association with the
extracellular matrix (Roberts et al 1988). Thus it seems probable that
the self-renewal and differentiation of haemopoietic stem cells *in vivo*
is controlled by membrane bound CSFs produced by the bone-marrow
stroma. It has not been possible to abrogate production of CSFs and
demonstrate haemopoietic cell death in vivo, but elevation of serum
levels of these factors for several days leads to dramatic increases in the
numbers of precursor cells and mature cells in the bone marrow
(Bronchud et al 1988, Gabrilove et al 1988). This response is likely to be
due to stimulation of proliferation of bone marrow cells, but may also at
least in part be due to enhanced survival of the precursor population.

CHAPTER FOUR:

DO CTL KILLING AND GLUCOCORTICOID -INDUCED LYSIS SHARE A

COMMON PATHWAY?

INTRODUCTION

Cytotoxic T cells (CTL) control viral infections by killing infected cells (reviewed by Nabholz and MacDonald 1983). The killing is specific and occurs after recognition by the T cell receptor (TCR) on the CTL of a foreign peptide in association with self Class I MHC molecules on the target cell-surface (Townsend 1987). The molecular interactions of the recognition process are well characterized, but the mechanisms of killing remain controversial.

CTL-mediated lysis involves plasma membrane and nuclear damage (Russell 1983), following obligatory CTL-target cell contact up to delivery of the lethal hit, after which death proceeds in the absence of the killer cell (Golstein and Smith 1976 and 1977). CTL contain secretory granules (Podack and Konisberg 1984; Henkart et al, 1984), which in isolated form have strong calcium-dependent cytotoxicity. On CTL-target cell conjugation the CTL reorientates its Golgi apparatus (Kupfer et al 1985) and granules (Yanelli et al 1986) and secretes individual granules into the intracellular space between the two cells. Granules contain cytolytic proteins, the perforins (Podack and Dennert 1982; Dennert and Podack 1983; Masson and Tschopp 1985), serine proteases (Masson and Tschopp 1987; Hameed et al 1988) proteoglycans (Schmidt et al 1985), and cytotoxins homologous to TNF (Liu et al 1987).

Perforin is homologous to the C9 component of complement (Shinkai et al 1988) and is cytolytic by virtue of its calcium-dependent binding to phospholipids (Tschopp et al 1989, Yue et al 1987), membrane insertion and intramembraneous polymerization (Podack 1986). Thus it has been suggested that CTL-lysis is mediated by perforins, which cause plasma membrane lesions, in a manner analogous to the complement system (Young and Cohn 1986).

There are, however, several problems with this theory : (1) Some CTL, and particularly CTL *in vivo*, do not appear to have cytotoxic granules (Berke and Rosen 1987) and do not have detectable perforin activity (Allbritton et al 1988); (2) Even though serine esterases are presumed to be found in the same granules as perforin, in the absence of extra-cellular Ca^{2+} and after depletion of the killer cell's intracellular Ca^{2+}, serine esterase secretion is depleted, but lysis is not inhibited (Ostergaard and Clark 1987; Trenn et al 1987), and ; (3) Inhibitors of Ca^{2+}-dependent granule secretion, such as cyclosporin A, block both the constitutive and induced pathways of cellular secretion, yet only slightly affect CTL-mediated lysis (Clark et al 1988; Lanki et al 1989).

Current opinion remains divided between those who hold that CTL kill by a mechanism very similar to that of complement killing (reviewed by Podack 1989) and those who emphasize the differences between the two processes and who place importance on the nuclear lesion induced by CTL (reviewed by Martz 1989). There seems to be reasonable evidence that at least in some instances CTL killing does not involve perforin (Ostergaard and Clark 1989) and one alternative mechanism that has been suggested is that CTL kill by inducing a suicide mechanism in their target cells (Golstein 1987).

The evidence in favour of target-cell suicide came largely from the experiments of Ucker (1987), who reported a correlation between resistance to dexamethasone (dex)-induced lysis and resistance to cytotoxic T cell (CTL)-mediated killing in mutants of the thymoma, S49,4RD (Sibley and Tomkins 1974). Furthermore, he showed that a single step reversion could restore sensitivity to both lethal stimuli. These data strongly implied a common pathway of cell death and suggested that the genetic locus which restored sensitivity might

encode an element of an endogenous suicide pathway. The suggestion of a common pathway seemed attractive because CTL killing had been shown to induce the morphology of apoptosis in its targets (Kerr et al 1987), as had glucocorticoid-induced lysis of thymomas (Wyllie et al 1984). Both stimuli also lead to regular DNA fragmentation (Russell et al 1979, Wyllie et al 1984).

Unfortunately, Ucker's findings proved controversial because other groups were unable to repeat his work with the cells he provided, or with cells from the original source, supplied by Yamamoto's laboratory (E. Martz, personal communication) and Ucker's original cell line was subsequently lost. In an attempt to confirm the evidence for a common pathway of cell death, I decided to select independently for dex-resistant mutants from a sensitive parent cell line and test them for susceptibility to CTL-induced lysis. DNA fragmentation does not always occur in CTL-induced lysis (Gromkowski et al 1988) and it has been suggested that whether or not DNA fragmentation takes place might be dependent on a property of the target cells and not of the CTLs (Howell and Martz 1987a). It was therefore important to use a target cell line which does exhibit DNA fragmentation on CTL-induced lysis.

The murine mastocytoma P815 seemed an appropriate choice because it is well documented that this cell line does undergo DNA fragmentation when it is killed by CTL (Duke et al 1983, Russell et al 1979), and because the CTL clone CTL-P35:10 (Maryanski et al 1982), which is specific for P815, was available in our laboratory. P815 is of DBA/2 origin and is H-2^d. The subline used in this study was P815-X.

RESULTS

Cloning

A clone of P815-X, designated P815α was isolated by a limiting dilution procedure. This clone was used in all subsequent experiments and was the parent from which dex-resistant clones were selected. Cloning efficiency was between 0.7-0.9.

Sensitivity of P815α to dexamethasone

P815α cells were dexamethasone sensitive and died after 3 days in culture in dexamethasone. Fig. 13a shows the death of P815α cells in dexamethasone for 3 days, as measured by trypan blue exclusion. ^{3}H-thymidine incorporation after 3 days of culture in different concentrations of dexamethasone was used as an alternative measure of glucocorticoid sensitivity and is shown in Fig. 13b. This dose response to dexamethasone was then used to distinguish resistant from sensitive variants. An electrophoretic ladder was obtained when DNA prepared from P815α cells incubated in 10^{-6} M dex for 2 days was run on an agarose gel (Fig. 14), confirming that the glucocorticoid induced cell death of these cells was apoptosis.

Sensitivity of P815α to CTL killing

As was expected, P815α was sensitive to killing by the CTL clone P35:10 and by CTL raised in a mixed lymphocyte reaction (MLR) of CBA spleen cells and irradiated Balb/c spleen cells (Fig. 15a and b respectively). DNA was fragmented and the release of ^{125}I-uridine on Triton X-100 permeabalisation (Duke et al 1983) preceded ^{51}-Cr release at an Effector:Target (E:T) ratio of 10:1, suggesting that DNA fragmentation was an early event (Fig. 15c). The experiment shown in Fig. 15c was carried out using CTL from an MLR.
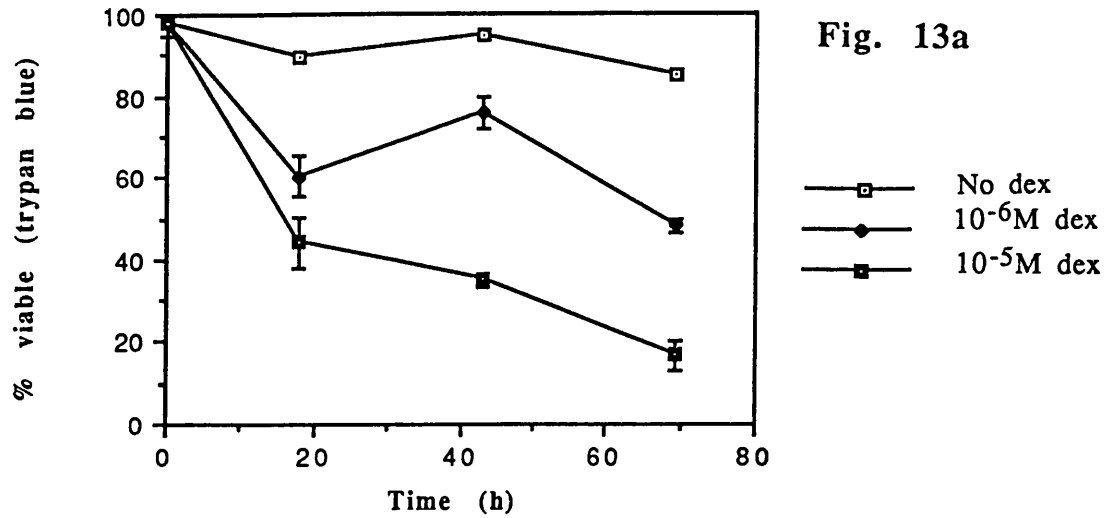
Fig. 13a

Fig. 13a

Death of P815α on culture in 10^{-5} M and 10^{-6} M dex. Viability is determined by trypan blue exclusion and each point is a mean of 5 readings.
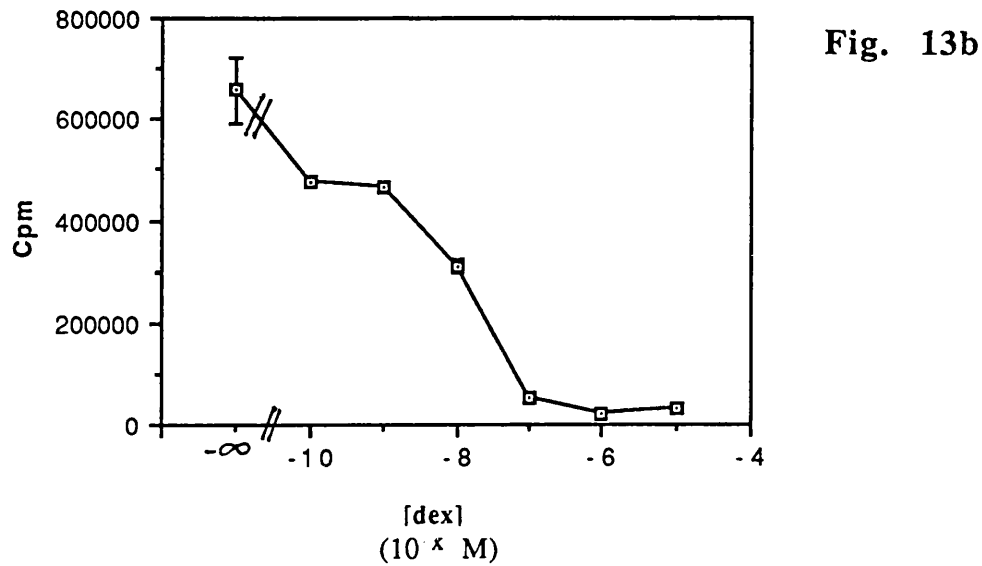
Fig. 13b

Fig 13b

Proliferation of P815α after 3 days culture in dex at concentrations from 10^{-5} M to 10^{-10} M. Proliferation is expressed as counts per minute (Cpm) and was measured by ^{3}H-thymidine incorporation. Each point is a mean of 6.

Fig. 14
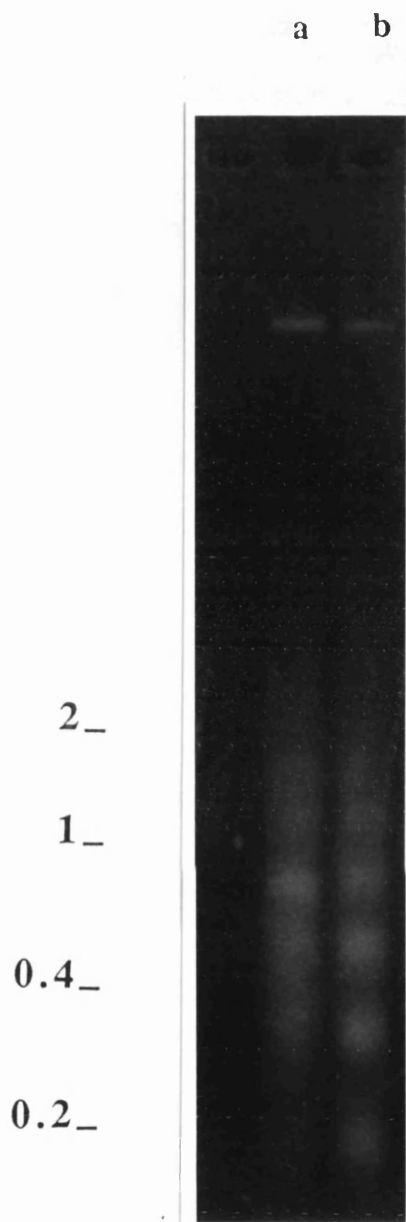
Fig. 14
Ethidium bromide stained 1% agarose gel showing DNA prepared from
P815α cells grown for 3 days in 10^{-7}M dex (track a) and 10^{-6}M dex (track
b). Size is given in kilobases.
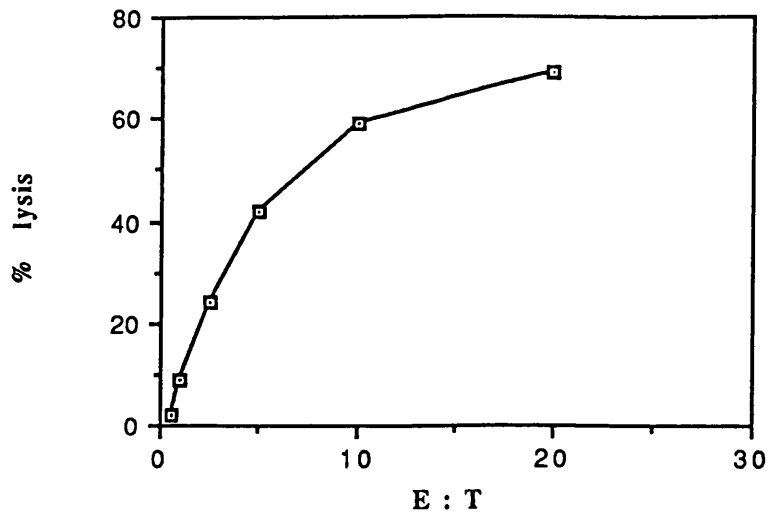
Fig. 15a

<u>Fig 15a</u>

Lysis of P815α by CTL:P35:10. Lysis was determined by ^{51}Cr release. Each point is a mean of 3 with $\sigma_n < 5\%$ of the mean. Background release was less than 20% of total release.
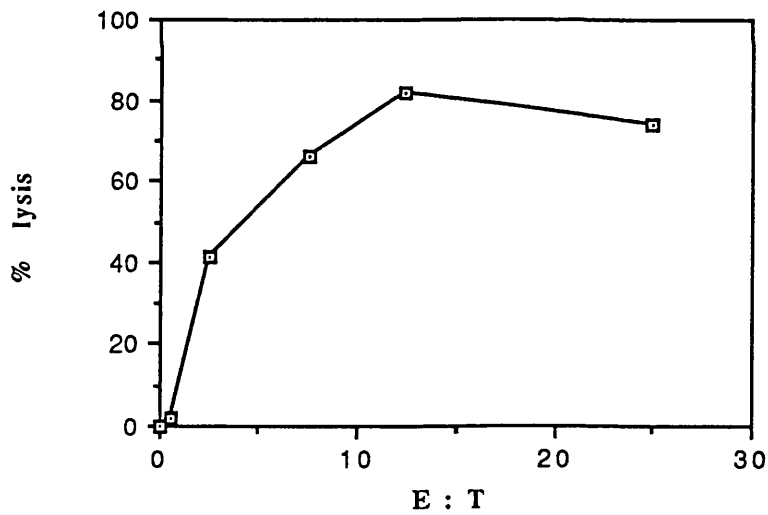
Fig. 15b

<u>Fig 15b</u>

Lysis of P815α by CTL raised in a MLR, determined as in Fig 15a.
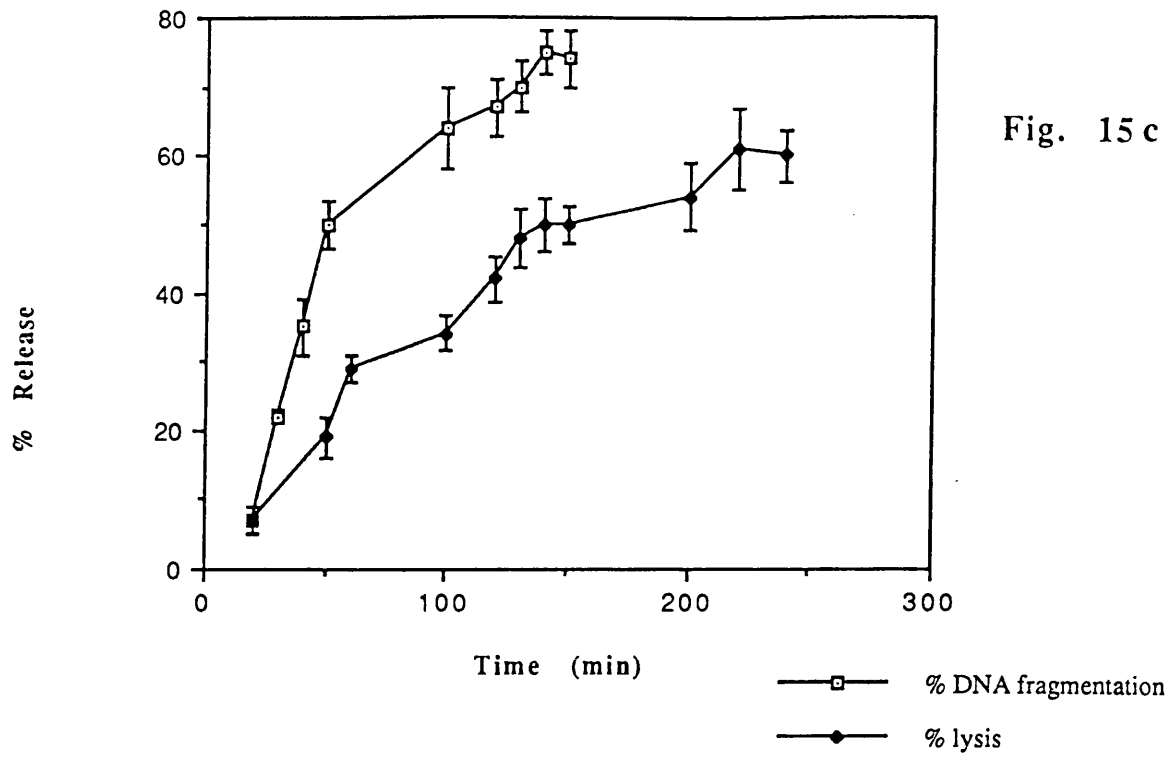
Fig. 15 c

Fig 15 c

The kinetics of lysis, as determined by ^{51}Cr release, and DNA fragmentation, as determined by ^{125}I-uridine release, in the killing of P815α by CTL raised in an MLR.

Selection of dexamethasone-resistant clones

Dex-resistant clones were selected by two protocols. Either they were isolated by two rounds of limiting dilution cloning in 10^{-6} M dex, and were designated R1-R22, or they were cloned in dex from a bulk culture which had been first treated with the mutagen ethylmethane sulfonate (EMS), and were designated RA-RF. EMS treatment was carried out according to the protocol of Glimcher et al (1983). Cells were treated with a dose that caused about 40% toxicity for 16 h before washing and limiting dilution cloning in dex. Twenty-two dex-resistant clones were selected by the first method and six clones were selected by the second method. Resistant clones fell into two categories. Some showed no response to dexamethasone and six clones proliferated more in dexamethasone in a dose-dependent manner. The dose-response in the ^{3}H-thymidine assay of a representative clone from each category is shown in Fig. 16a. In selecting for dex-resistance it would seem very likely that one would obtain clones that have lost functional glucocorticoid receptors (Wyllie et al 1984), but this cannot be the case for those clones which proliferate more in dex, and resistant clones with functional receptors have been isolated from sensitive parents in other studies (Zawdyiwski et al 1983; Gasson and Bourgeois 1983). Approximately 20% of the resistant clones isolated showed a dose dependent response to dex, and this is the same proportion of resistant isolates with functional glucocorticoid receptors as has been reported in other studies (Sibley and Tomkins 1974). Increased proliferation is not surprising, as murine monocyte cell lines initially proliferate more when they are treated with dex (Norton 1989), and dex is known to induce the expression of multiple genes (Voris and Young 1981). Figs. 16b and 16c show the response to dex in the ^{3}H-thymidine incorporation assay of all resistant clones isolated without EMS treatment, and Fig. 16d

shows the response of clones isolated after EMS treatment. No increase in proliferation with dex was seen in any of the latter clones.

Sensitivity of dex-resistant clones to killing by CTL-P35:10

All dex-resistant clones were sensitive to killing by the cytotoxic T cell clone CTL-P35:10, and no differences in sensitivity between the resistant clones and the dex-sensitive parent were observed. Fig. 17a shows a representative experiment.

Sensitivity of dex-resistant clones to killing by CTL raised in a MLR

It has been suggested that there is more than one killing mechanism used by cytotoxic T cells, and that it is a perforin independent mechanism which causes DNA fragmentation in target cells (Ostergaard and Clark 1989). Culturing CTL clones in IL2-containing medium seems to favour the generation of perforin-containing cytoplasmic granules (Allbritton et al 1988) and perforin, being homologous to the C9 component of complement, is thought to act by permeabalizing the plasma membrane of the targets (Young et al 1986; Lowrey et al 1989). Granules derived from CTL clones can lyse targets without causing DNA degradation (Gromkowski et al 1988). Therefore, it seemed appropriate to test the sensitivity of the dex-resistant clones to CTL from another source. CTL were prepared in a 5-day MLR of CBA spleen cells and irradiated Balb/c spleen cells. Killing was specific against H-2^d and so was not mediated by NK or LAK cells, as inappropriate targets, such as Dab L cells transfected with H-2^k (a gift from H. Stauss) were not killed. All dex-resistant clones were killed and were as sensitive to killing as the dex-sensitive parent. A representative experiment is shown in Fig. 17b. These experiments

provide no evidence for the hypothesis that CTL and dex kill P815 cells by a common pathway, though clearly the limited number of dex-resistant clones tested does not rule out a common pathway.

Effect of E:T ratio on kinetics of DNA degradation

One of the arguments that is made in favour of the position that there is more than one way in which CTL can kill their targets, is that DNA degradation does not always occur, and that the cell whose DNA is fragmented dies in a fundamentally different way from the one whose DNA is not (Golstein 1987). DNA fragmentation is associated with the putative perforin-independent mechanism (Ucker 1987; Ostergaard and Clark, 1989).

This seems illogical. Just because DNA fragmentation has been shown to be causal in apoptosis induced by growth factor withdrawal (see Chapter 3) and in apoptosis in developing thymocytes (McConkey et al 1989, and Chapter 5), one cannot conclude that whenever it occurs it is necessary, and not just sufficient, for cell death. DNA fragmentation has occasionally been observed in killing by antibody and complement (Bachvaroff et al, 1977; Shipley et al 1971) and has recently been shown to occur in P815 cells killed by purified perforin (Hameed et al 1989). It seems possible that when death is mediated both by perforin and complement, whether DNA fragmentation takes place could be a function of the concentrations of perforin or complement + antibody and the speed of death. This suggestion is supported by the observation that at low concentrations a Ca^{2+} ionophore induced apoptosis and DNA fragmentation in immature thymocytes (Kizaki et al 1989), which other authors have shown to be inhibitible by ATC (McConkey et al 1989), whereas a higher dose of the ionophore caused cell lysis, but not DNA fragmentation or apoptosis. Thus, DNA fragmentation could be the

result of Ca^{2+} signalling which only takes place if the membrane lesion creates the appropriate Ca^{2+} flux to trigger apoptosis. If the Ca^{2+} flux is too great because the membrane lesion is greater, then, as in the situation with the higher dose of Ca^{2+} ionophore the cell would lyse without apoptosis being triggered. Kinetics of death would also be critical, as if the break up of the plasma membrane were very fast, soluble components, possibly including the relevant nucleases, would leak out of the cell before DNA degradation could occur.

In search of evidence in favour of this hypothesis, I looked at the kinetics of DNA fragmentation in P815 cells at different E:T ratios. There was no decrease in DNA fragmentation as E:T ratios were increased (Fig. 18a). I then measured release by target cells of ^{125}I-uridine and ^{51}Cr into the culture medium in parallel assays, after 1 h incubation with CTLs. Release of ^{125}I-uridine increased with increasing E:T ratio, up to an E:T ratio of 10, and thereafter declined (Fig. 18b), but ^{51}Cr release continued to increase with increasing E:T ratio (Fig. 18c). At high E:T ratios, where after 1 h the plasma membrane of about 35% of targets had disintegrated, the nuclear membranes had remained intact. Incubation of isolated nuclei in appropriate ion concentrations leads to DNA fragmentation (see Chapter 3) and so this could account for some of the DNA fragmentation observed in the experiment in Fig. 18a.
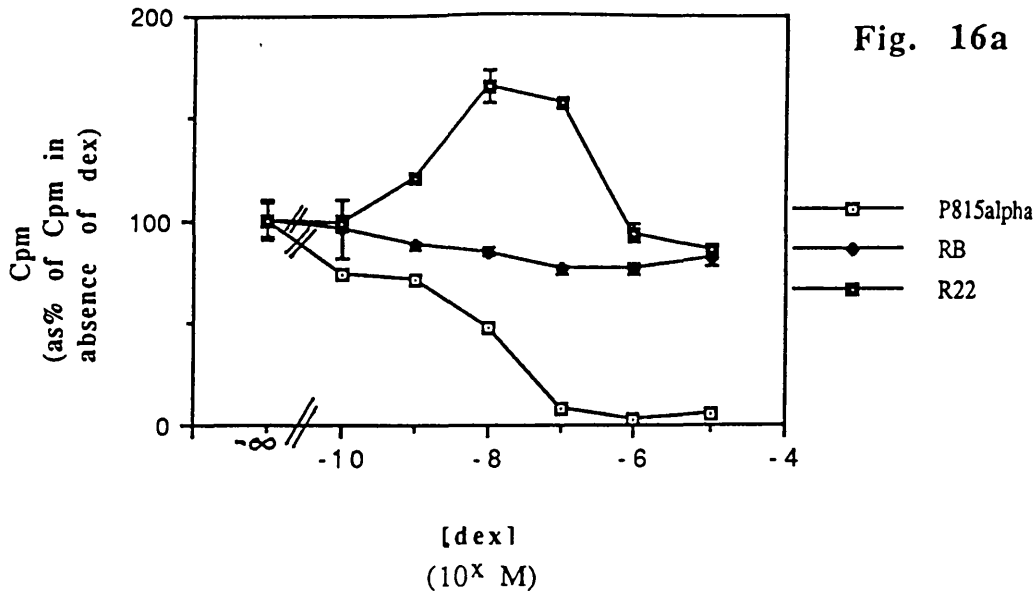
Fig. 16a

Fig. 16a

Proliferation of clones of P815 after 3 days culture in dex at concentrations from 10^{-5} M to 10^{-10} M. Each point is a mean of 6. Proliferation was measured by ^{3}H-thymidine incorporation and is expressed as a percentage of proliferation in the absence of dex. Resistant clones were isolated by limiting dilution cloning.
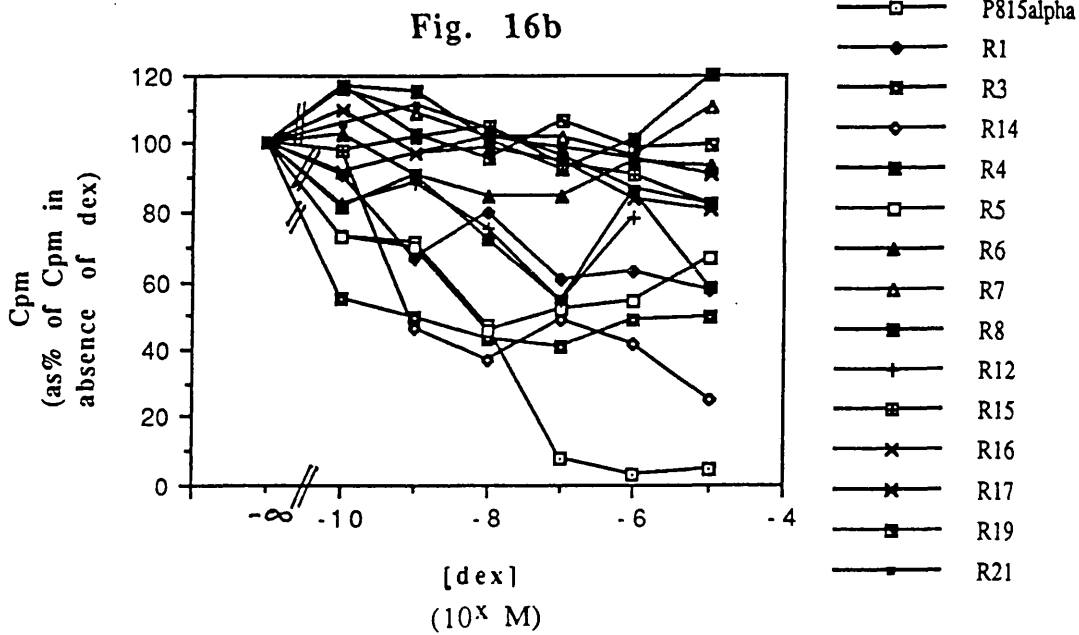
Fig. 16b

Fig 16b

As above. Standard deviations are not shown, but in all cases they were less than 5 % of the mean.

101

Fig. 16c

Legend:
- PS15alpha
- R18
- R22
- R13
- R11
- R2

X-axis: [dex] 10^x M

Y-axis: Cpm (as % of Cpm in absence of dex)
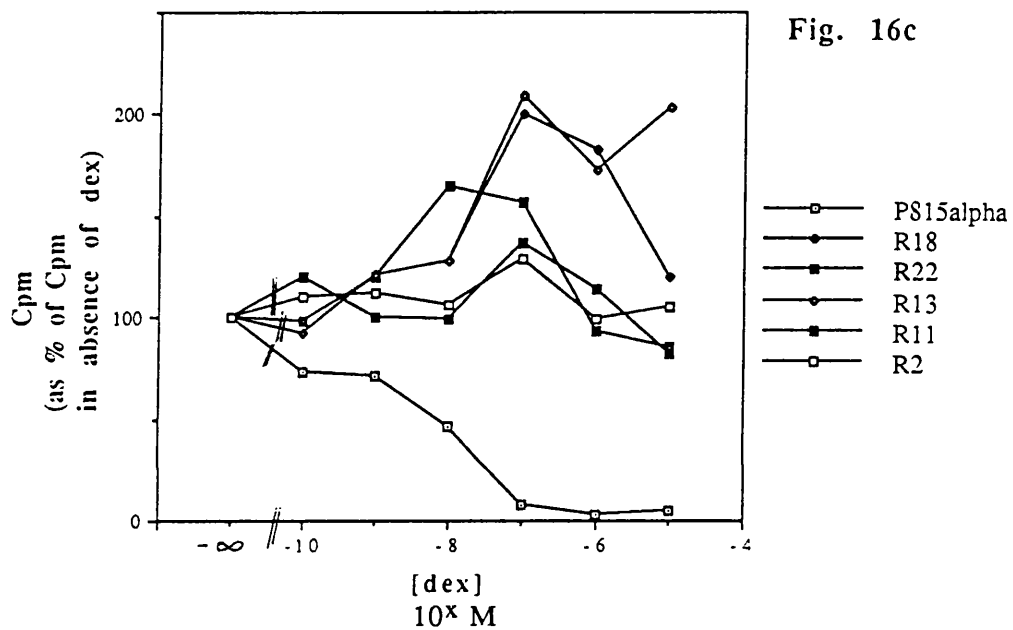
Fig. 16c

Proliferation of clones of PS15 after 3 days of culture in dex at concentrations from 10^{-5}M to 10^{-10}M. Each point is a mean of 6, with σ_n < 5% of the mean. Proliferation was measured by ^{3}H-thymidine incorporation and is expressed as a percentage of proliferation in the absence of dex. Resistant clones were isolated by limiting dilution cloning.

102
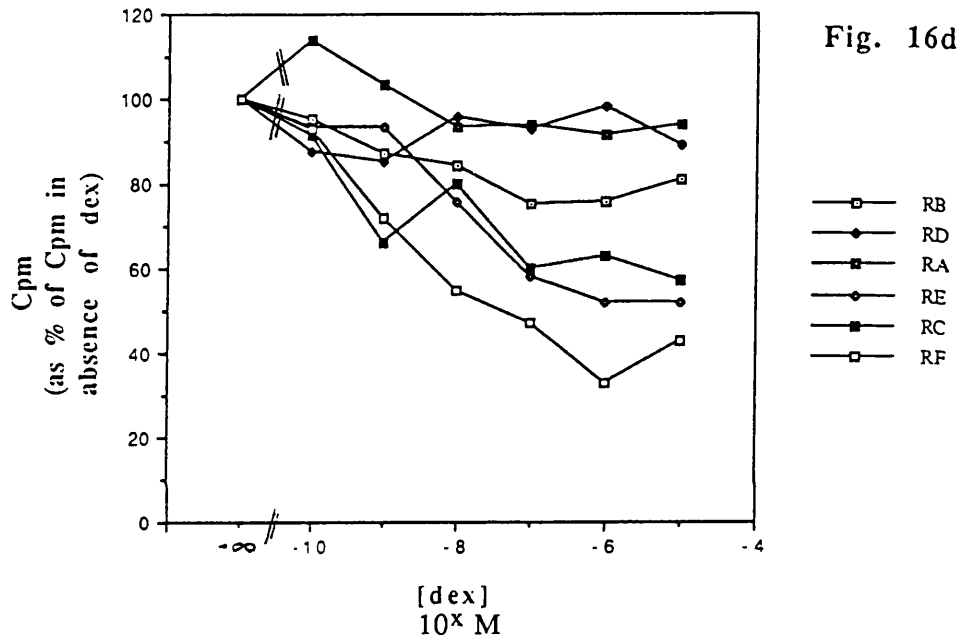
Fig. 16d

Fig. 16d

Proliferation of clones of PS15 after 3 days of culture in dex at concentrations from $10^{-5}M$ to $10^{-10}M$. Each point is a mean of 6, with σ_n < 5% of the mean. Proliferation was measured by ^{3}H-thymidine incorporation and is expressed as a percentage of proliferation in the absence of dex. Resistant clones were isolated following EMS treatment.
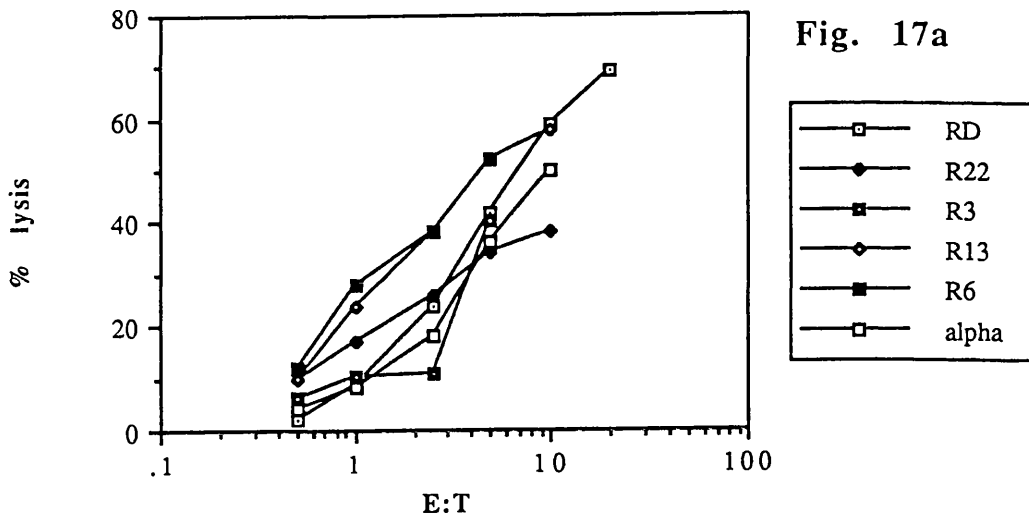
Fig. 17a

Fig 17a

Lysis of clones of P815 by CTL:P35:10. Lysis was determined by ^{51}Cr release. Each point is a mean of 3, with $\sigma_n < 5 \%$ of the mean. Spontaneous release was less than 20 % of total.
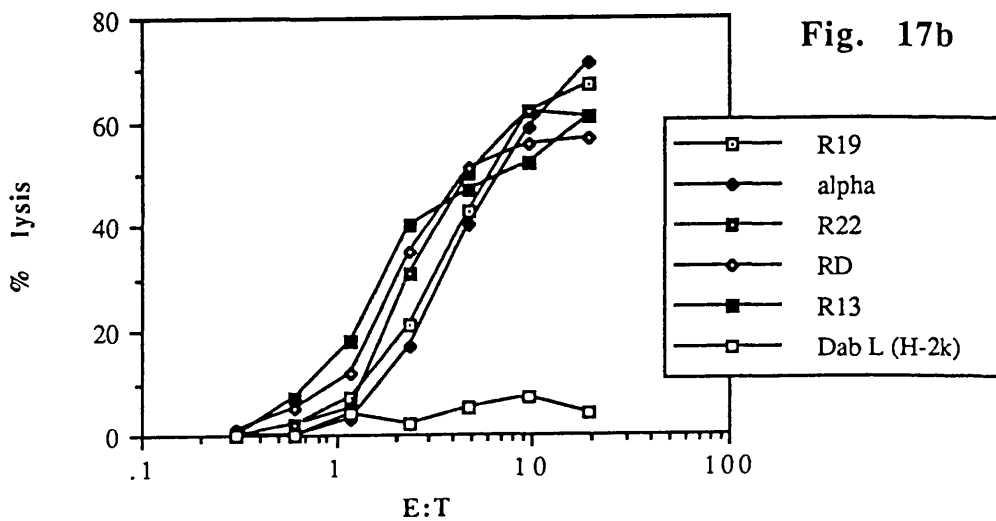
Fig. 17b

Fig. 17b

As above, but CTL were derived from an MLR.

Fig. 18a

Legend:
- t=60
- t=120
- t=240
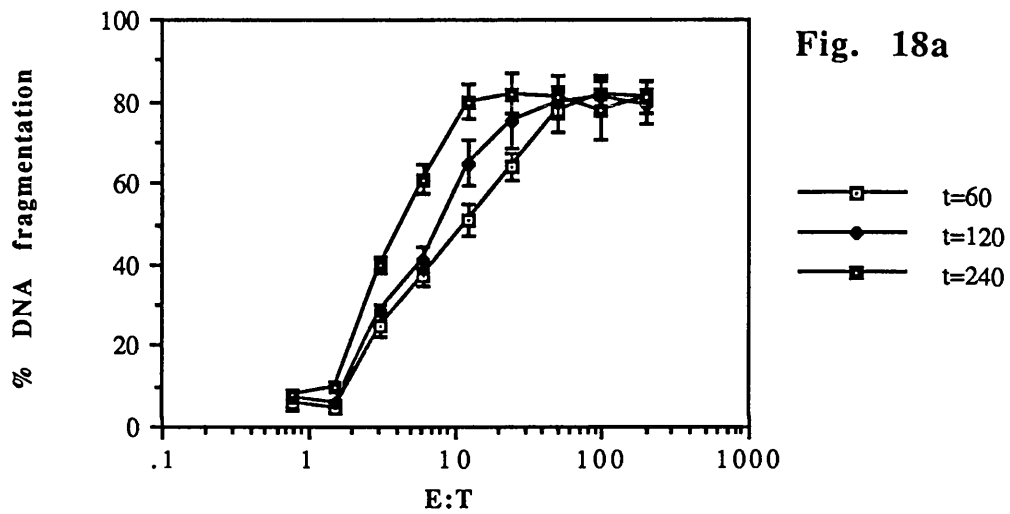
Fig. 18a

Kinetics of DNA fragmentation when P815α is killed by CTL derived from
an MLR. DNA fragmentation was determined by the Triton-X100
solubilization assay and is expressed as a percentage of total
incorporated radiolabel. Each point is a mean of 6. Time is given in
minutes.
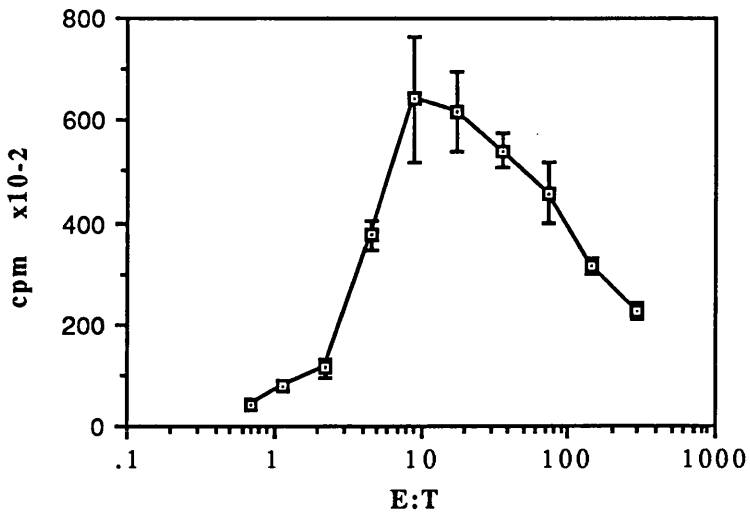
Fig. 18b

Fig 18b

Release of ^{125}I-uridine into the culture medium after 1 h incubation of P815α with CTL derived from an MLR. Each point is a mean of 6.
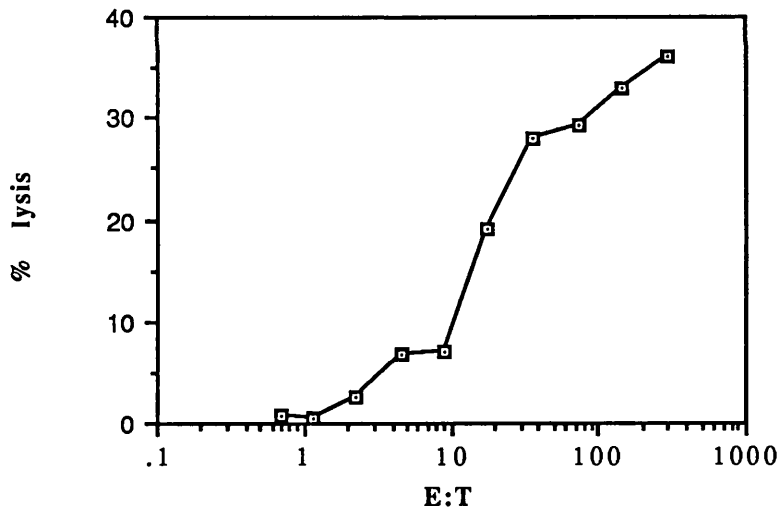
Fig. 18c

Fig 18c

Lysis of P815α , as determined by ^{51}Cr release, after 1 h incubation with CTL derived from an MLR, carried out in parallel to the experiment shown in Fig. 18b. Each point is a mean of 3.

Effect of 5-azacytidine on P815α clones

Treatment of cells in culture with the drug 5-azacytidine (5-azaC), an inhibitor of DNA methylation, has been shown to cause new gene expression (Groudine et al 1981), and in general, in eukaryotes, the DNA of inactive genes is more heavily methylated than that of active genes (reviewed by Cedar 1988). Treatment of glucocorticoid resistant lymphoid cell lines which have functional glucucorticoid receptors with 5-azaC has been shown to restore dexamethasone-sensitivity (Gasson and Bourgeois 1983) and spontaneous *de novo* methylation of these dex-sensitive cells is accompanied by the acquisition of the glucucorticoid resistant phenotype (Gasson et al 1983).

As dex-resistant clones were isolated from P815α at a high frequency (cloning efficiency of P815α in 10^{-6} M dex was reduced from 70-90% to approximately 1 in 2.3 x 10^3) it seemed possible that I had selected cells which had undergone a functional change due to an epigenetic or differentiatative event rather than a mutation in DNA sequence. To ask if dex-sensitivity might be restored to resistant clones by a decrease in DNA methylation, the dex-resistant clones were cultured in dexamethasone and 5-azaC for 5 days, according to the method of Gasson and Bourgeois (1983), and then bulk cultures were tested for glucucorticoid sensitivity. No lytic response was observed during the 5 day culture and Fig. 19a shows that after removal of 5azaC from the cultures there was no difference in their responses to dex in the ^{3}H-thymidine incorporation assay from the untreated cultures. No evidence in favour of the inactivation by methylation of a gene involved in lysis was found for any of the resistant clones.

Surprisingly, preliminary experiments showed that 5azaC treatment resulted in the acquisition of resistance by the sensitive parent P815α. Fig 19b shows the dose response of P815α in dex after 5

107

days 5azaC treatment and without treatment. In this experiment 5azaC treatment was carried out in the absence of dex, to exclude the criticism that resistance was acquired simply by selection in dex. Such bulk acquisition of dex-resistance was observed on several occasions. Cloning efficiency of P815α in dex was increased 100-fold by 5azaC treatment, although treatment itself caused a reduction in cloning frequency to about 30%. The most straight forward interpretation of these data would suggest that in contrast to the lymphoid cell lines used in the work of Gasson et al, for P815 cells glucocorticoid resistance is an active function and not just acquired by the loss of a lysis capability. This would be in line with studies on the acquisition of resistance to apoptosis induced by tumour necrosis factor (TNF) (Laster et al 1988), in which TNF resistance is found to be dominant in cell fusion experiments (Nophar et al 1988). Clearly, to conclude this one would have to show that 5azaC was actually causing a decrease in DNA methylation; it is possible that, as 5azaC is itself toxic, it is selecting for cells that are more resistant to lysis, and not causing a bulk reversion to resistance by allowing new gene expression.

Northern blot analysis of glucocorticoid receptor mRNA levels

The cDNAs for the glucocorticoid receptor (GR) have been cloned in human, rat and mouse (Hollenberg et al 1985; Miesfeld et al 1986; Danielson et al 1986). The inactive glucucorticoid receptor is thought to be associated with the heat shock protein Hsp90 (Sanchez et al 1985), and in the presence of glucucorticoid hormones the Hsp90 dissociates and the receptor/steroid complex translocates to the nucleus and binds to the DNA at defined sequences, called the glucocorticoid response elements (GREs), thereby activating or inactivating specific gene

108

expression (reviewed by Gustafsson et al 1987; Yamamoto 1985). The hormone-binding and GRE-binding regions of the receptor have been defined by analysis of mutants (Okret et al 1986; Giguere et al 1986; Godowski et al 1987) and are at the C terminal of the receptor, separated by a short hinge region (Carlstedt-Duke and Gustafsson 1987).

In an effort to further elucidate the nature of the resistance to dex-lysis of the clones selected from P815α, I carried out Northern blot analysis of the glucucorticoid receptor mRNA levels in the different clones, using a probe isolated from the plasmid pBS-0.7PR, which was a gift from Mike Jacobson. This probe is a 0.7kb GR cDNA fragment encoding the C-terminal region of the murine GR. Fig. 20a shows that this probe hybridized to two bands of molecular weights 7.2 kb and 5.2 kb in polyA$^+$ RNA prepared from P815α cultured in the absence of dexamethasone. These two different mRNA species have been reported previously and may be due to alternative polyadenylation sites (Rosewicz et al 1988; Kalinyak et al 1987).

Glucucorticoids are known to down-regulate GR mRNA levels by influencing gene transcription (Okret et al 1986; Rosewicz et al 1988). A possible explanation for the difference in sensitivity to dex of P815α and the resistant receptor-positive sub-clones might lie either in their receptor levels or in their relative abilities to down-regulate GR mRNA expression on exposure to dex. Perhaps the sensitive cells fail to down-regulate their receptor levels and so over express genes that are induced by the hormone, which are then lethal to them over a period of days. Quantative comparisons of GR mRNA levels on exposure to dex was carried out by dot-blot analysis, using the 0.7kb GR cDNA probe. Poly A$^+$ mRNA was prepared from P815α, R19 (a resistant clone which has no response to dex) and R22 (a resistant clone which proliferates more in dex). Autioradiographs were scanned using a densitometer (Hoefer

109

Scanning densitometer GS-300) and integration of density curves was carried out using GS-370 electrophoresis data reduction system (Hoefer). A chicken β-actin probe (a gift from N. Pringle) was used as positive control and to make sure that equivalent amounts of RNA were compared.

Fig. 20b shows densitometer readings from a dot-blot, indicating the relative levels of GR receptor in R19 and P815α and R22 at time points after addition of Dex. Units are an arbitrary measure of area and all figures are adjusted by comparison with actin mRNA levels from re-screening the same blot with an actin probe, to normalise mRNA concentrations. Both cell lines down-regulate their receptors at a similar rate, with R19 reaching one half its original level in 19 h and P815α in 17 h. P815α had about 2.6 times more GR mRNA than R19. R22, like P815α, also had high levels of GR, which was down-regulated by one half in 17.9 h. When grown continously in 10^{-6} M dex both R19 and R22 had GR mRNA concentrations similar to those after culture in the absence of dex, and after 48 h in dex [GR mRNA] in R19 is rising again.

Thus, these three cell lines manifest no difference in their abilities to down-regulate the GR on exposure to dex, but R19, a clone which does not respond to dex has strikingly less GR mRNA than R22 and P815α.
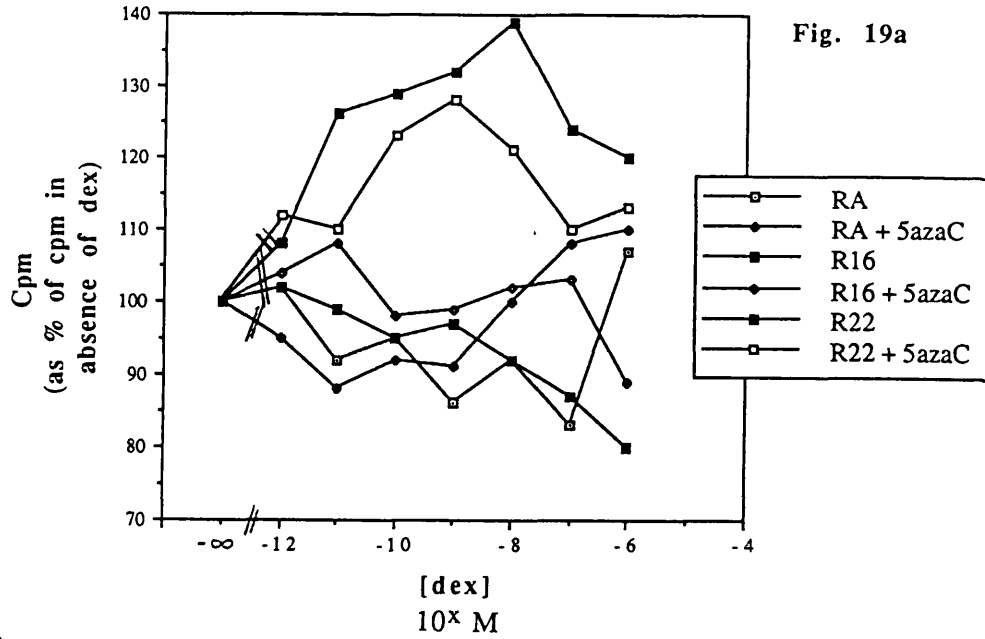
Fig. 19a

Fig 19a

Proliferation of dex-resistant P815 clones after 3 days culture in dex. Clones have been treated with 5azaC. Proliferation was measured by thymidine incorporation and is expressed as a percentage of proliferation in the absence of dex. Each point is a mean of 6 with $\sigma_n < 5\%$ of the mean.
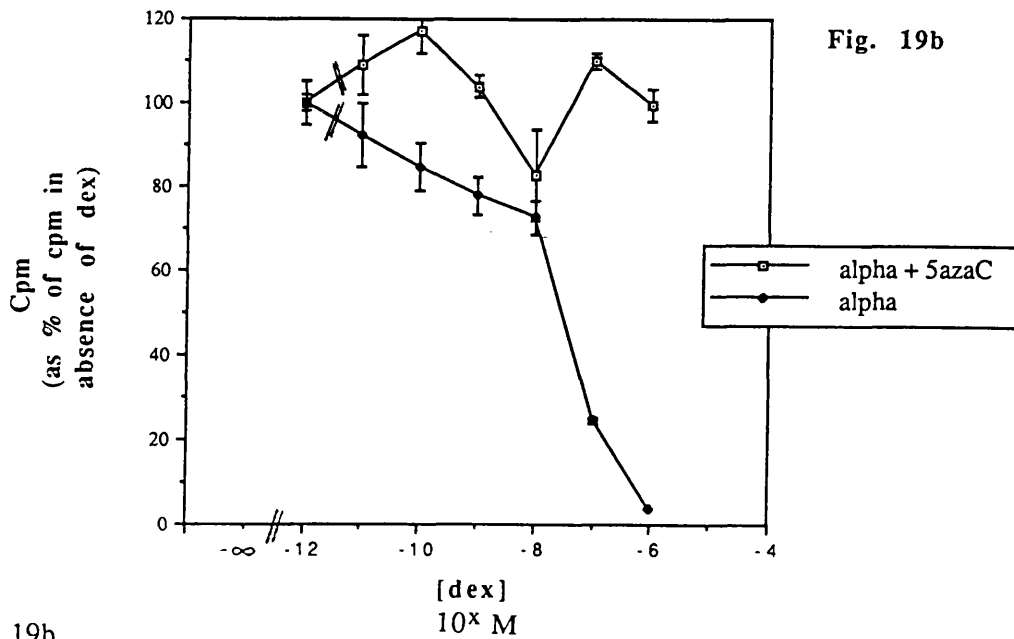
Fig. 19b

Fig 19b

As above, but showing the response of the parent P815α after 5azaC treatment.

111
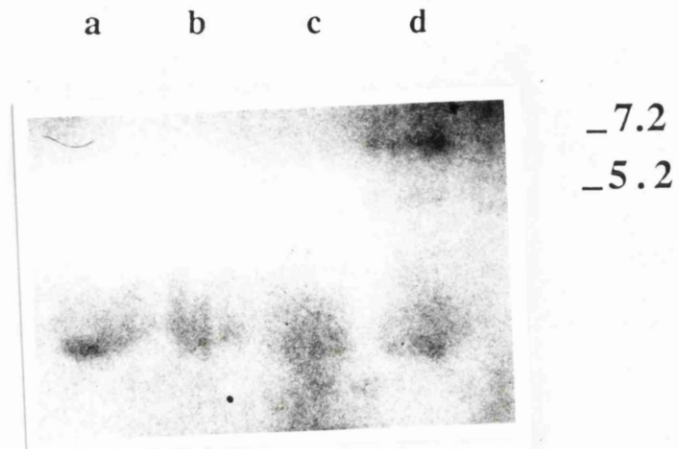
Northern blot of PolyA$^+$ RNA prepared from RA (track a), RB (track b), RD (track c) and P815α (track d), probed with a 0.7 kb GR cDNA probe. The probe hybridized to two bands of molecular weights 7.2 kb and 5.2 kb in track d (P815α).
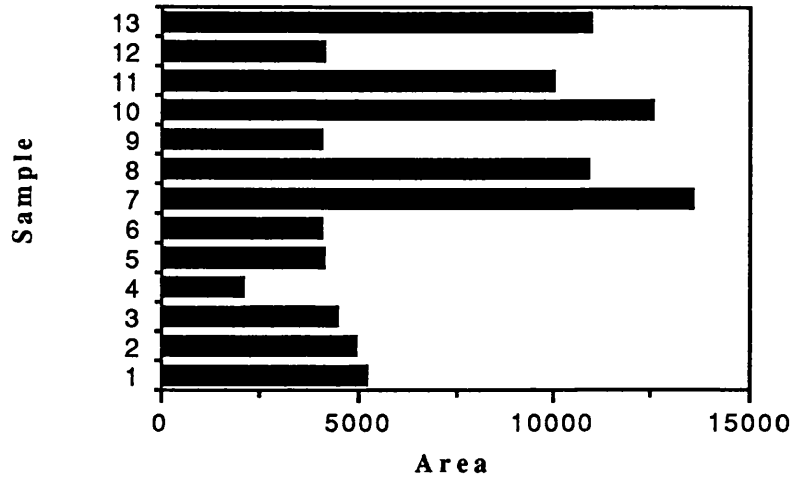
Number	Sample
1	R19, No dex
2	R19, 10^{-6}M dex, 2h
3	R19, 10^{-6}M dex, 4h
4	R19, 10^{-6}M dex, 24h
5	R19, 10^{-6}M dex, 48h
6	R19, 10^{-6}M dex, 2 weeks
7	P815α, No dex
8	P815α, 10^{-6}M dex, 4h
9	P815α, 10^{-6}M dex, 24h
10	R22, No dex
11	R22, 10^{-6}M dex, 4h
12	R22, 10^{-6}M dex, 24 h
13	R22, 10^{-6}M dex, 2 weeks

Fig. 20b

The relative concentrations of glucocorticoid receptor mRNA in P815α, R19 and R22 on exposure to dexamethasone. Units are an arbitrary measure of area, derived from integration of densitometer readings of an autoradiograph. Integration was carried out using the software GS-370 electrophoresis data reduction system (Hoefer).

DISCUSSION

This study failed to provide evidence in favour of a common pathway of cell death induced by glucocorticoids and by CTL. None of the dex-resistant clones isolated showed a reduction in susceptibility to CTL lysis, indicating that the defects selected for were not part of a common suicide pathway, although it is possible that common elements in the death pathways exist downstream from these lesions. Other investigators have also been unable to demonstrate a common killing mechanism by analysis of mutants (Dennert et al 1988), which is a pity because a mutant cell line that is resistant to multiple forms of killing would be invaluable for investigating the molecular mechanisms of apoptosis. It always seems that a high percentage of dex-resistant mutants isolated in any study are defective in their glucocorticoid receptors (Sibley and Tomkins 1974; Wyllie et al 1984), which are unlikely to be involved in CTL killing. It might be possible to increase the probability of isolating a cell line with a mutation at a common point in a lysis pathway by first transfecting the dex-sensitive parent clone with multiple copies of the glucocorticoid receptor gene, thus reducing the chance of selecting a cell with defective receptor function.

The question remains, however, whether any cell is truly resistant to CTL lysis per se, if the need for target cell recognition by CTL is by-passed. In their search for a correlation between resistance to dex and resistance to CTL, Dennert et al examined several cell lines with reduced sensitivity to CTL-lysis and found in all but one case that these cells had reduced expression of MHC Class I; the sensitivity to CTL-lysis could be restored by increasing Class I expression. The only cell line where the failure to be killed could not be explained by lack of Class I expression was a subclone of the line S49-4RD, used in Ucker's study.

S49-4RD had normal levels of cell surface Class I but failed to induce the reorientation of the Golgi apparatus and microtubule-organising centre (MTOC) in the CTL, which is known to be a prerequisite for lysis (Kupfer and Dennert 1984; Kupfer et al 1985). S49-4RD's sensitivity to CTL was normal when the lectin ConA was added to by-pass the need for CTL recognition. This was interpreted to imply that the CTL must interact with another undefined molecule on the target cell surface in addition to Class I in order to reorientate its Golgi apparatus and MTOC. Other cells which have been reported to be resistant to CTL killing are neuroblastomas and gliomas (Main et al 1985). These cells have low levels of surface Class I expression, which can be increased with γIFN treatment, but in some cases they remain resistant to CTL. However, when the need for adequate recognition is by-passed by the addition of lectins, these cells are killed (Main et al 1988), again implicating an undefined target cell-surface molecule in the recognition phase. Perhaps, previously reported CTL-resistant cells (Gasson and Bourgeois 1983) were deficient in such a cell surface molecule rather than in an element of a lysis pathway.

The final category of cells resistant to CTL-killing are CTL themselves (Kranz and Eisen 1987; Blakely et al 1987). The necessity for these cells to be resistant to their own lysis apparatus is obvious and it has been suggested that they have cell surface molecules which protect them from the membrane lesions induced by perforin (Zalman et al 1987; Muller-Eberhard 1988), in the same way that a homologous restriction factor (HRF) protects erythrocytes of a given species from injury by complement of that species (Zalman et al 1986). The role of HRF in protecting CTL remains controversial (Jiang et al 1989). Other authors have suggested that perforin must be activated by a cascade involving the granule-contained serine proteases, analogous to the

complement cascade, and that in the cell, the components of this cascade are seqestered within the granules (Masson and Tschopp 1987). The cytoplasmic granules themselves have T cell receptors on their surfaces, providing specificity in their lysis action (Peters et al 1989). An HRF-like protection mechanism would only protect CTL against a perforin-dependent mechanism of lysis. Authors who favour a perforin-independent killing process suggest that this type of killing occurs where the perforin-dependent mechanism fails (Ostergaard and Clark 1989). As CTL have been reported to be resistant to lysis not only by perforins (Shinkai et al 1988), but also by cytoplasmic granules (Verret et al 1987), and by CTL (Skinner et al 1987; Kranz and Eisen 1987; Blakely et al 1987), those in favour of an alternative mechanism of killing would have to postulate that CTLs are also resistant to this perforin-independent mechanism. If the alternative mechanism involves the triggering of an auto-destruct process of apoptosis (Golstein 1987), then CTL should be unable to undergo this process. This seems not to be the case: many CTL clones, although resistant to glucocorticoid-induced lysis (Nguyen and Nabholz 1985), are IL2-dependent (Gillis and Smith 1977) and die by apoptosis on IL2-withdrawal (Duke and Cohen 1986).

What is the mechanism of the resistance to glucocorticoids of the selected dex-resistant clones? Resistance has frequently been described in cell lines which have lost expression of functional glucocorticoid receptors (GR) (Sibley and Tomkins 1974), but this cannot be the explanation in all cases because some of the clones still show a response to dex, which increased their proliferation. Previous studies have indicated that there is little difference in the ligand affinity of the GR between different resistant and sensitive cell types (Schleche et al 1982;

116

Shipman et al 1983; Zawydiwski et al 1983) but degree of sensitivity may, correlate with the concentration of GR. As steroid hormones are used in the treatment of leukaemia, numerous studies looking for a correlation between GR levels and sensitivity to the hormone (and hence between GR levels and prognosis) have been carried out in humans. Some such studies report a good correlation (Lipman et al 1978; Costlow et al 1982), whereas others find no correlation (Mastrangelo et al 1980). A similar discrepancy exists between studies looking for a correlation between GR levels and the differential sensitivity of normal murine lymphoid cells (Bradley and Mishell 1982; Lippman and Barr 1976). This work measured GR concentration using ligand binding assays, an approach which has been criticised because of the difficulty of assuring a total exchange of receptor-bound unlabelled steroids for labelled hormone and of preventing the binding of labelled glucocorticoid ligand to heterologous receptors (Okret et al 1986). To avoid these criticisms I looked directly at GR mRNA levels in P815α and two resistant sub-clones. R19, which shows no response to dex in proliferation assays, had about three times less GR mRNA than the sensitive parent or than R22, a resistant clone which undergoes increased proliferation in dex. This result suggests that in the case of R19 the lack of sensitivity to dex may simply be due to reduced expression of GR.

Ligand binding studies have suggested that GR concentration is regulated by the hormone (Cidlowski and Cidlowski 1981; Svec and Rudic, 1984), which also regulates GR mRNA concentrations (Okret et al 1986; Rosewicz et al 1988). Rosewicz's study of rat and human cell lines shows that corticosteroid-induced down-regulation of GR is caused by a decrease in the rate of gene-transcription, whereas other authors have also suggested that the hormone shortens GR half-life (McIntyre and Samuels 1985). Okret uses immunoprecipitation to analyse GR-DNA

117

interactions and shows that there are GR-specific binding regions within a GR cDNA clone, suggesting that GR down regulation might be due to interaction of the GR protein with its own gene. He also shows that down regulation is decreased by the protein synthesis inhibitor cycloheximide, implicating a trans-acting repressor protein. As 5azaC seems to convert dex-sensitive cells to dex-resistance, hinting that resistance might be the dominant state, it seemed possible that the difference between P815α and the resistant clones might be the expression of such a trans-acting repressive factor. Therefore, I examined down-regulation of GR by R19, R22 and P815α in 10^{-6} M dex. P815α down-regulated its GR at the same rate as the resistant cells. The time for GR mRNA to decrease by one half was about 18 h. This is similar to the findings of Okret who showed that in rat hepatoma cells in culture [GR mRNA] was decreased by 50-95% in 24 h, whereas in Rosewicz's study the decrease was much faster, with a one-half time of 3 h for a human lymphocyte cell line and 6 h for a rat pancreatic acinar cell line. P815 cells in prolonged culture in dex re-express GR mRNA at their original levels. This concurs with Okret's study, which showed that levels return to normal after 72 h in dex, but Rosewicz found no return to the original level of expression.

The observation that 5azaC confers dex-resistance to the dex-sensitive parent cells needs further investigation, and suggests that resistance results from the acquisition of something which protects the cell from lysis or changes its response to glucocorticoids, rather than as a result of the loss of part of a lysis pathway. Unfortunately, this means that these cells were probably not appropriate to use in the search for a correlation between resistance to CTL and to dex (unless one believes that resistance to CTL is also active). Resistance to tumour necrosis factor (TNF) has been shown to be a positive function in some cells

118

(Nophar et al 1988) and heat shock proteins have been shown to protect WEHI-164 cells from apoptosis induced by TNF (Jaattela et al 1989). It would be interesting to examine levels of heat shock proteins in P815 cells in response to dex.

CHAPTER FIVE:

STUDIES ON APOPTOSIS IN THE THYMUS

.

.

INTRODUCTION

Possible roles for cell death in T cell development in the thymus.

Since the 1960s it has been known that there is massive cell death in the mammalian thymus (Matsuyama et al 1966; Metcalf 1966), but, until recently, the assumption had been that this cell death represented a dead-end pathway in development, and served no useful purpose (Shortman and Jackson 1974; Fathman et al 1975; Scollay et al 1984). However, a better understanding of the lineage relationships between sub-populations of thymocytes and of the molecular mechanisms that generate T cell receptor (TCR) diversity has radically changed this view.

About 80% of murine thymocytes express both the T cell accessory molecules CD4 and CD8 (Kisielow et al 1975; Mathieson et al 1979; Scollay and Shortman 1983), which on mature T cells are generally mutually exclusive (Dialynas et al 1983b; Swain 1981). It is these double positive cells which die in the thymus (reviewed by Scollay et al 1984; Rothenberg and Lugo 1985). Studies involving reconstitution of irradiated mice (Fowlkes et al 1985; Fowlkes and Mathieson 1985), abrogation of normal T cell development *in vivo* by the administration of monoclonal antibodies (mabs) (Smith 1987; MacDonald et al 1988a; Fowlkes et al 1988), and the generation of mice transgenic for TCR genes (Kisielow et al 1988a and b; Sha et al 1988) have suggested the following lineage in thymus development: Double negative (CD4⁻CD8⁻) cells to double positive (CD4⁺CD8⁺) cells to single positive (CD4⁺CD8⁻ and CD4⁻ CD8⁺) cells. The double negative thymocytes lack expression of the CD3 αβTCR complex (Roehm et al 1984; Bluestone et al 1987), although a subpopulation express the γδTCR (Pardoll et al 1987). A proportion (about half) of the double positive thymocytes express low levels of the

CD3 αβTCR complex (Havran et al 1987; Crispe et al 1987) and, in humans, there is evidence that a small proportion (4-9%) are CD3^{high} (Blue et al 1987). The single positive thymocytes express the same levels of the CD3 αβTCR complex as T cells in the periphery (Snodgrass et al 1985; Cristanti et al 1986). A rapidly cycling CD8$^+$CD3$^-$ single positive population also exists, which is thought to represent the transition between the double negative thymocytes and expression of both accessory molecules (Paterson and Williams 1987; MacDonald et al 1988b). The CD3$^+$ single positive thymocytes possess mature T cell function (Ceredig et al 1982 and 1983; Chen et al 1982) and are functionally and phenotypically indistinguishable from recent emmigrants from the thymus (identified by intrathymic FITC injection, Scollay et al 1978 and 1984; Scollay 1982), whereas the double positives are not functionally mature (Adkins et al 1987).

The fact that the mature single positive thymocytes which leave the thymus are derived from the large double positive population has lead to the suggestion that the cell death one observes in the double positive population reflects thymic selection procedures rather than a dead-end developmental lineage. The diversity of the T cell receptor repertoire is generated by genetic recombination events (reviewed by Kronenberg et al 1986) that produces self-reactive cells, which must be either deleted (Burnet 1962) or suppressed (reviewed by Howard and Mitchison 1975; Nossal 1983). Until recently no conclusive evidence for either deletion or suppression had been presented. Experiments that assay the primary T-cell responses of single positive thymocytes to MHC antigens *in vitro* had shown that these cells can respond to non-self, but not self MHC molecules, indicating that some tolerization of T cells occurs before they leave the thymus, suggesting either clonal deletion or inactivation in the thymus (Good et al 1983; Kruisbeek et al 1981).

Other work, however, favoured the maintenance of tolerance by suppressor T cells in the periphery. Many authors have claimed that some anti-self T cell reactivity can be measured *in vitro* (eg Smith and Pasternack 1978; Glimcher and Shevach 1982; Glimcher et al 1982) and it has been proposed that these self reactive cells are regulated by suppressor cells *in vivo* (Brondz et al 1984). Chen and Splitter (1983) found that $Lyt1^+2^-$ T cells, but not unseparated or $Lyt1^-2^+$ T cells, from nude mice grafted with normal neonatal thymuses gave a strong proliferative response *in vitro* to grafted thymus MHC-type stimulator cells, suggesting that tolerance to self MHC antigens is maintained *in vivo* by $Lyt1^-2^+$ suppressor T cells.

The observation that certain TCR Vβ domains are strongly correlated with reactivity to a particular antigen in the context of a defined MHC molecule (Kappler et al 1987a; MacDonald et al 1988e), and the availability of mabs to such Vβ domains, has made it possible to track the developmental fates of cells known to be self-reactive. In this way, clonal deletion as a mechanism of tolerance induction has now been demonstrated in several systems (Kappler et al 1987b; MacDonald et al 1988c; Kappler et al 1988; White et al 1989). That clonal deletion in the thymus is responsible for tolerance to some antigens does not exclude the possibility that other mechanisms such as suppression also occur.

Clonal deletion in tolerance induction has also been shown using mice transgenic for a TCR reactive to the H-Y antigen in the context of $H-2D^b$, backcrossed to *scid* mice, and hence expressing only the transgenic TCR. In the thymuses of the male transgenic mice (which express the H-Y antigen) there are virtually no single positive $CD8^+$ cells and fewer than normal double positive thymocytes, indicating that tolerance in the thymus is caused by clonal deletion in the double positive population (Kisielow et al 1988a). Evidence that clonal deletion

123

occurs at the stage when T cell precursors are expressing both accessory molecules also comes from experiments in which the deletion of CD8$^+$ single positive thymocytes, identifiable by their Vβ gene usage, is prevented by *in vivo* administration of mab against CD4 (MacDonald et al 1988a; Fowlkes et al 1988) and from histological analysis of where in the thymus cells that express a self-reactive receptor are absent (Hentgartner et al 1988).

The hypothesis I wish to consider in this chapter is that the mechanism of clonal deletion is apoptosis, induced by a ligand binding to the TCR of double positive thymocytes. This suggestion is supported by the work of Smith et al (1989) who show that addition of a mab against CD3 to the culture medium of foetal thymus organ cultures leads to an increase in cell death in the thymus organ culture, and that the dying cells show the characteristic morphology of apoptosis and DNA fragmentation.

This explanation for the death of double positive thymocytes is, however, complicated by the need for positive selection of the T cell repertoire. In 1975 the phenomenon of MHC restriction was discovered with the observation that cytotoxic T cells recognize antigens in association with self class I MHC molecules on the surface of target cells (Zinkernagel and Doherty 1975). Later MHC restriction was demonstrated to apply to T-helper cells also, although these are primarily restricted by class II MHC molecules (Sprent 1978). Experiments with chimaeric mice showed that T cell restriction is governed by the MHC type of the thymus in which T cells develop (Zinkernagel et al 1978; Fink and Bevan 1978). It was therefore postulated that developing T cells are selected in the thymus on the basis of their ability to bind to the MHC molecules of the thymus, such that cells which complete their maturation and exit to the periphery are

primarily restricted to thymus-type MHC antigens. Until recently this theory of positive selection had remained controversial: allo-restricted T cells, whose activity can be measured if animals are primed with antigen on allogeneic antigen presenting cells, were shown to exist (eg Wilson et al 1977; Matzinger and Mirkwood 1978; Ishii et al 1981). Experiments in which thymuses were grafted to nude mice showed that responses were restricted to nude MHC type rather than to that of the graft thymus (Kindred 1978; Zinkernagel et al 1980; Lake et al 1980). Moreover, the conceptual problem of how antigen-specific, MHC-restricted T cells can be selected in the thymus by MHC alone was unresolved.

Both the work with transgenic mice (Kisielow et al 1988b; Sha et al 1988) and the approach of following the fate of cells expressing particular $V\beta$ gene products throughout their development have demonstrated positive selection in the murine thymus. In the case of the 'monoclonal' anti-H-Y transgenic mice produced by von Boehmer's group, female H-2D^b mice (which do not express H-Y) were found to have CD8$^+$ single positives and double positives cells in their thymuses, whereas mice that did not express H-2D^b had double positives but no single positives (Kisielow et al 1988b). This suggests that an interaction between the TCR and MHC is necessary for differentiation from double positive to single positive and that this interaction determines the fate of the cell: since the transgenic TCR interacts with Class I, only CD8$^+$ T cells are produced. In MacDonald's system (reactivity to the Mls^a gene product in the context of I-E correlates with $V\beta6$ expression), the same Class II MHC loci which are required for deletion of $V\beta6^+$ cells in Mls^a-expressing mice, and which restrict Mls^a-reactive T cell clones and hybrids, control the frequency of $V\beta6^+$ cells among mature CD4$^+$ T cells in mice lacking expression of Mls^a (MacDonald et al 1988d). This is

consistent with positive selection in which interaction of the TCR with MHC Class II is required for differentiation into a mature CD4$^+$ single positive cell. The existence of positive selection is also supported by experiments in which the ratio of CD4$^+$ to CD8$^+$ mature thymocytes is skewed in fetal thymus organ culture by addition of hybrid antibodies against both one accessory molecule and CD3 (Zepp and Staerz 1988).

The need for positive selection to allow cells in the periphery to recognise antigen in the context of MHC clearly complicates an interpretation of the role of cell death in T cell development in the thymus. Perhaps all double positive thymocytes are programmed to die, unless rescued by a survival signal if their TCR has appropriate MHC-reactivity, and it is this that accounts for the cell death one observes in the double positive population.

Evidence for cell death in the murine thymus

The initial evidence for cell death in the murine thymus came from 'balance-sheet' studies in which an estimate of thymocyte proliferation, assessed by an autoradiographic analysis of ^{3}H-thymidine (^{3}H-TdR) incorporation, was compared with thymus weight gain and migration from the thymus. The extent of proliferation in the thymus could only be explained if most of the cells died *in situ*, as the population turnover time was about 3 days for thymocytes and yet the thymuses did not get larger and only a small percentage of labelled cells migrated to the periphery (Matsuyama et al 1966; Metcalf and Wiadrowski 1966). Similar work in guinea pigs (Nossal 1964; Murray and Woods 1964) and rats (Goldschneider and McGregor 1968) also showed that most thymocytes die in the thymus and have a lifespan of 3 to 4 days

The extent of thymus cell death has been disputed, however, largely because of the difficulty of producing histological evidence

126

(Borum 1968, Michalke et al 1969; Sainte-Marie and Peng 1971; Poste and Olsen 1973). It was argued that if nearly all thymocytes die within 4 days of their birth then this cell death should be easy to visualize. Thymocyte cell death was demonstrated more conclusively by exploiting the difference in the ability of cells to incorporate the thymidine analogues, ^{3}H-TdR and ^{125}I-uridine (^{125}I-UdR) (Feinendegen et al 1973). ^{3}H-TdR is incorporated about six times more efficiently than ^{125}I-UdR (Baugnet-Mathieu and Goutier 1968) and so if thymocytes are pulse labelled with these precursors the relative rates of decline of the two isotopes will reflect the extent of local reutilization of thymidine, which should in turn mirror the degree of local cell death: If cells migrate, both isotopes should be lost in parallel, but if cells die and the nucleotides are reutilized locally by salvage pathways, then ^{3}H-TdR levels should be maintained about six times more efficiently than ^{125}I-UdR levels. Several studies (Feinendegen et al 1973; Laissue et al 1976; McPhee et al 1979) have demonstrated that about 60% of the injected ^{3}H-TdR is reused locally in the thymus and that the transit time from the last DNA synthesis until migration or cell death is about 3 days (Ernstroem and Larsson 1965). This work was still criticized because of the possible toxicity of ^{125}I-UdR, but this criticism was excluded by McPhee et al (1979) (see Results) who estimated that a minimum of 60% of all thymocytes formed die in the thymus, both in adult mice and in 7-day old mice. Another criticism, that the observed cell death was induced by hydrocortisones released because of the stress of the experiment, was also excluded in McPhee's study, as the same estimate was obtained in adrenalectomised mice.

A final argument in favour of the contention that most thymocytes, and nearly all double positive thymocytes, die *in situ* can be made from the near impossibility of culturing dissociated double

positive thymocytes *in vitro*. In the absence of an intentional lethal stimulus from the experimenter all double positive thymocytes die within about four days in culture (Hopper and Shortman 1976). Where mature single positive cells are obtained from such cultures (eg Blue et al 1985, 1987) it has been argued that these are due to preferential expansion of contaminating single positive thymocytes, and limiting dilution cloning has suggested that very few double positive cells (<2%) can survive (Chen et al 1982 and 1987; Andrews 1985; Ceredig et al 1982). Recent experiments, in which the objection of contamination by single positive thymocytes was circumvented (by taking advantage of the fact that mature T cells bearing Vβ6 are eliminated by tolerance mechanisms in the thymus of Mls[a] positive mice, and by providing CD4$^+$CD8$^+$ thymocytes from such mice with a Vβ6-specific stimulus) have suggested that about 3% of double positive thymocytes are clonable (Howe and MacDonald 1989). This fits well both with the theory that the double positive thymocytes are the pool on which both rounds of repertoire selection are carried out and with data from *in vivo* studies which indicate that about 1% of thymocytes migrate each day and 30% are born each day (Claesson and Hartman 1976; Scollay et al 1980; Scollay et al 1988). Thus most thymocytes die in the thymus.

The fact that this cell death is so difficult to see under the light microscope might be explained if thymocytes die by apoptosis. Apoptosis occurs when death is part of an organised tissue reaction, such as in embryogenesis, and is characterised by lack of scaring and rapid removal of dead cells, which do not elicit an inflammatory response (Wyllie 1981 and 1987). Apoptotic bodies are rapidly phagocytosed by adjacent cells (Duvall et al 1985), and it has been estimated that the half-time for which apoptotic cells remain visible by light microscopy is less than nine hours (Wyllie et al 1980; Ijiri and

Potten 1983). The speed of disappearance of apoptotic bodies from tissues *in vivo* indicates that their plasma membranes may be altered in a way that enhances phagocytosis, and abnormalities have been detected in the sugars exposed on their surfaces (Morris et al 1984; Wyllie 1985).

Apoptotic cells have been described in the thymus under normal conditions (Van Haelst 1967a; Fraker et al 1977; Joel et al 1977; Wyllie et al 1980) and glucocorticoid hormones are well known to induce apoptosis in thymocytes *in vivo* (Van Haelst 1967b; Umansky et al 1981; La Pushin and de Harven 1971) and in thymocytes *in vitro* (Wyllie 1980, Wyllie and Morris 1982).

RESULTS

Development of an assay for apoptosis *in vivo*

Strategy

The difficulty of visualizing cell death in the murine thymus, despite the convincing arguments suggesting that many cells must be dying at any point in time, led me to try to develop a way of detecting and quantifying apoptosis *in vivo*. Such a method would have to identify dying cells before their rapid phagocytosis and destruction and so ideally would rely on detection of a molecule that is expressed or up-regulated early in death. The molecule(s) might be the one whose synthesis is thought to be inhibited by cycloheximide, when it delays death by apoptosis (Williams et al 1990; Duke and Cohen 1986; Pratt and Greene 1976), or the sugars that change on the cell surface (Morris et al 1984; Wyllie 1985). Unfortunately the biochemistry of apoptosis has not been elucidated (Kerr et al 1987; Wyllie 1987) and so such probes are not available.

I tried, unsuccessfully, to raise monoclonal antibodies which distinguish between apoptotic and viable cells, by immunizing rats with either nuclei of apoptotic cells or whole apoptotic cells and screening hybridoma supernatants from a fusion by flow cytometry. The only mabs produced by this protocol which seemed hopeful, in that they stained a higher proportion of dex-treated thymocytes than fresh thymocytes, turned out to recognise a nuclear antigen, and the increase in their staining on the dying cells only reflected changes in plasma membrane permeability on death and hence suggested that my fixation and permeabilization protocol (D.H.Katz, personal communication) was inadequate. In addition, in collaboration with A.H. Wyllie, I immunized a mouse with an FPLC fraction containing endonuclease activity

130

obtained from a rat thymocyte nuclear extract. Hybridomas were screened by dot-blotting onto the nuclear extract, but none of the mabs obtained from this fusion convincingly stained more strongly on frozen sections of rat thymus treated with hydrocortisone *in vivo,* than on frozen sections of untreated rat thymus, or on permeabalized rat thymocytes treated with dex *in vitro* and analysed on the Facs, than on ˙ fresh permeabilized thymocytes.

As the antibody approach proved unsuccessful and it seemed unlikely that one would generate antibodies against something so specific when immunizing so crudely, (particularly given that the molecule must be in the thymus and so an animal would be very likely to be tolerant to it), I decided to try to adapt the DNA fragmentation assay used in CTL-mediated killing (Duke and Cohen 1983) to assess the extent of DNA fragmentation in a tissue at a given time. In principle, then, the plan was to label mice *in vivo* with a radioactive thymidine analogue (^{3}H-TdR or ^{125}I-UdR), to kill the mice and prepare cell suspensions from tissues of interest, and then to determine the degree of DNA fragmentation in the cells by partial lysis with Triton-X100 and centrifugation, to establish what proportion of the radiolabel was in different fractions of chromatin, as separated by size. As DNA fragmentation is a hallmark of apoptosis (Wyllie et al 1984) this approach to quantifying cell death should have the advantage that it measures apoptosis, but not necrosis. For this method to be successful the cells of interest would have be proliferating in order to acquire the radiolabel (fortunately thymocytes are, Metcalf and Wiadrowski 1966), and one would have to be certain that the radiolabel itself was not toxic to the cells (McPhee et al 1979).

Labelling the mice

Four week old male B10 BR mice were labelled with either ^{3}H-TdR ([6-^{3}H]-thymidine, 22Ci/mmol, Amersham) or ^{125}I-UdR ([5-^{125}I] iodo-2'-deoxyuridine, 5Ci/mg, Amersham). Doses were titrated to obtain sufficient counts in the thymus.

Labelling with ^{3}H-TdR

Mice were injected intraperitoneally (i.p.) with the ^{3}H-TdR in 0.4 ml PBS. As most thymocytes have a life span of about 3-4 days (Bryant 1972) and small cortical thymocytes (double positives) are thought to die about 3 days after their last DNA synthesis (McPhee 1979), each mouse was injected every morning for four days, in order for as many cells in the thymus to be labelled as possible, and not just one subpopulation or developmental stage to be labelled. To determine if the labelling was successful the mice were killed, cell suspensions of their thymuses were made and washed twice in MEM-H, and then 5×10^5 cells in 150 µl MEM-H were harvested on a Dynatech microtiter automash cell harvester. Filter paper was dried and each piece was emersed in 3 ml Optiscint scintillation fluid (LKB) and counted on a liquid scintillation counter (LKB Rackbeta 1214).

Fig. 21a shows the counts per 5×10^5 thymocytes after different labelling protocols. The largest increase in labelling achieved was between 50µCi per day per mouse and 100 µCi per day per mouse, and as I wanted to obtain maximum intrathymic labelling with minimum cost, I decided to adopt a dose of 100 µCi per day for future experiments. At the highest doses used labelling appears to be approaching saturation point (for this ratio of Ci/mol) and so one can estimate that at 100µCi per day about 2/3 of possible cells are labelled, assuming that there is not a large dose-dependent variation in counts per labelled cell. Higher

132

counts in the thymus on the day of killing might have been achieved by building up the dose over the four day period, but as far as possible it was preferable for thymocytes born on each day of the labelling period to incorporate equivalent amounts of ^{3}H-TdR and so to be exposed to the same dose of ^{3}H-TdR. This dose (100μCi per day) is considerably more than other investigators have used: McPhee et al (1979) injected 25μCi ^{3}H-TdR i.p. on one occasion only, Denizot et al (1986) injected 25μCi ^{3}H-TdR i.p. on 6 consecutive days (150μCi total) and Ewing et al (1988) injected 100μCi per mouse. Their studies assessed incorporation by autoradiography, but as I intended to count radiolabel in different chromatin fractions, I required higher labelling. The high dose used therefore made it particularly important to show that the ^{3}H-TdR was not itself toxic to the cells.

Fig. 21b shows the weights of the thymuses in the different groups. There is no decrease in weight at the higher doses, suggesting that these doses of ^{3}H-TdR were not toxic over the time of exposure. In addition, thymocytes from one mouse in each group were stained with mabs against CD4 and CD8. No difference in ratios of double positives to double negatives to single positives were found (approx. 73% CD4$^+$CD8$^+$, 9% CD4$^-$CD8$^-$, 4% CD8$^+$ and 14% CD4$^+$) between the different groups. One would expect double positives to be particularly susceptible to any potential toxin, so the fact that ratios were not changed also suggested that the ^{3}H-TdR was not toxic. The ratios I found in these mice are perhaps skewed towards double negatives; for example Havran et al (1987) reported 82% CD4$^+$CD8$^+$, 3% CD4$^-$CD8$^-$, 12% CD4$^+$ and 3% CD8$^+$ in 12 week old mice. This discrepancy may reflect the younger age (4 weeks) of my mice.
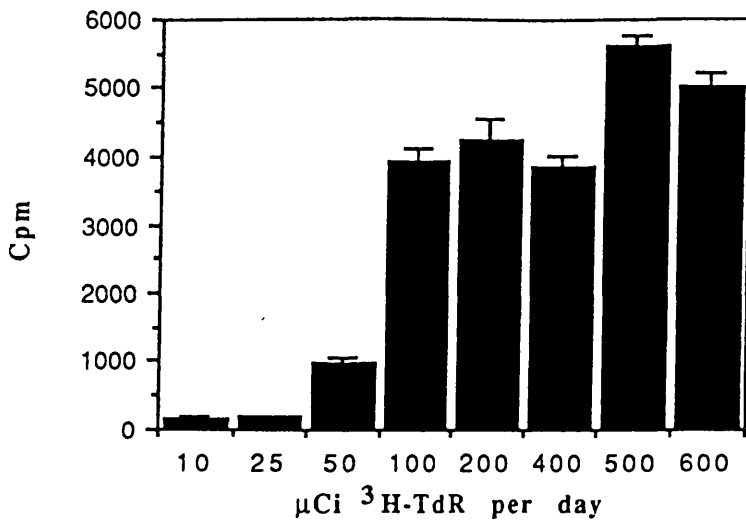
133

Fig. 21a

Cpm in $5x10^5$ thymocytes after daily injection of ^{3}H-TdR in a dose range from 10μCi to 600μCi per day for 4 days. Each point is the mean of cpm from 3 mice, and the value for cpm for each mouse was a mean of 10 readings, with $\sigma_n < 5\%$ of the mean.
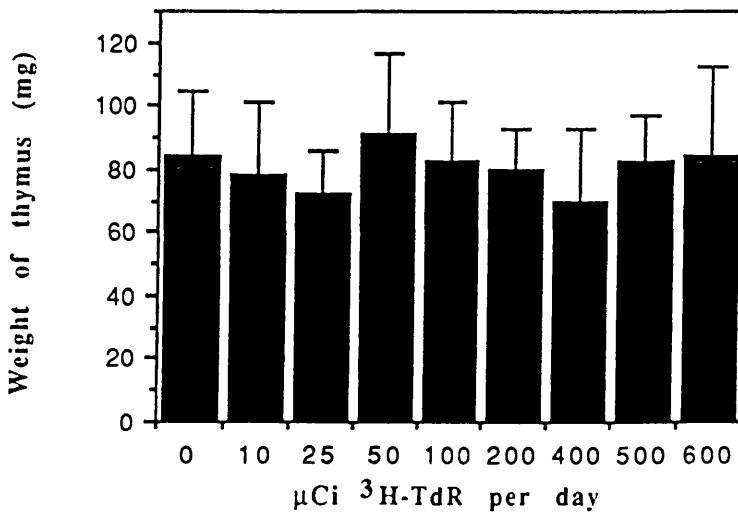
Fig. 21b

Fig. 21b

Weights of thymuses of mice which have been given a daily injection of ^{3}H-TdR in a dose range from 10μCi to 600 μCi for 4 days. Each point is a mean of 3 mice.

134
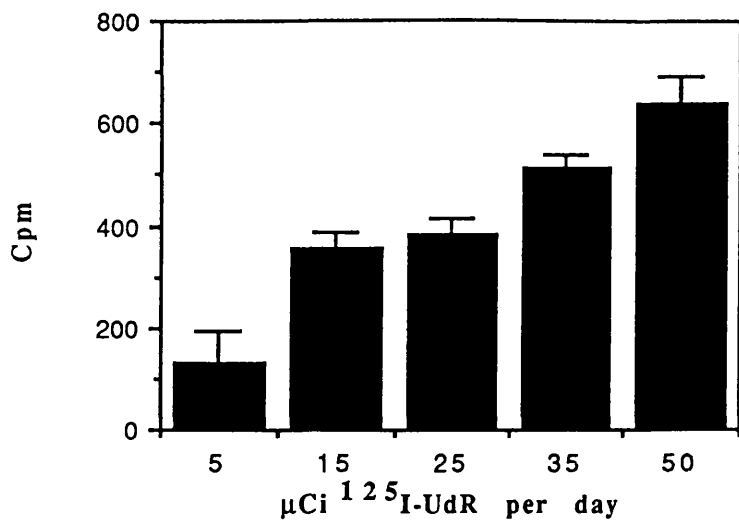
Fig. 21c

Cpm in 5×10^5 thymocytes after daily injection of ^{125}I-UdR in a dose range from 5μCi to 50μCi per day for 4 days. Each point is the mean of cpm from 3 mice, and the value for cpm for each mouse was a mean of 10 readings, with $\sigma_n < 5\%$ of the mean.

Labelling with ^{125}I-UdR

Mice were injected i.p. with ^{125}I-UdR in 0.4 ml PBS at doses ranging from 5 to 50 μCi per day for 4 days. Mice were killed and thymocytes prepared as above, but samples were counted on a gamma counter (Wallace LKB 80000 γ sample counter). Fig. 21c shows the cpm per 5×10^5 thymocytes from mice injected with the different doses. Thymocytes are particularly sensitive to damage, and the induction of apoptosis, by γ-irradiation (Sellins and Cohen 1987), and so although cpm per thymus was still increasing with dose of ^{125}I-UdR in the dose range assayed, it seemed advisable to stay within the doses that other investigators had shown to be non-toxic. McPhee et al (1979) injected mice with either 25μCi 3H-TdR, or 25μCi 3H-TdR together with 25 μCi ^{125}I-UdR, and showed that there was no difference in kinetics of the decline of βcpm between the two groups and hence that 25μCi ^{125}I-UdR was not causing cell death. As in the case of labelling with 3H-TdR, there was no difference in the mean weight of thymuses between the groups of mice which had been labelled with the different doses, or in the percentages of the different thymocyte subpopulations, as defined by accessory molecule expression (data not shown).

Distribution of the radiolabel and the kinetics of its decline

Fig. 22a and b show the relative labelling of cells in the thymus, spleen and lymph nodes of mice labelled with 100μCi ^{3}H-TdR and 35μCi ^{125}I-UdR, respectively.

Fig. 23a and b show the decline of the two radiolabels in thymus and lymph nodes, respectively. Mice were labelled with one isotope only, because of the difficulty in counting two isotopes simultaneously in one sample, and hence the differences shown are from different mice of the same age, sex and strain. ^{125}I-UdR decline is much more

marked than ^{3}H-TdR decline in the thymus, suggesting that cells are dying and not migrating from the thymus. The difference is too great to be accounted for by the shorter half-life of ^{125}I than ^{3}H. The lesser decline in ^{3}H-TdR in the thymus must be due to local reutilization and not to incorporation of free precursor in the blood, as free precursor levels drop to <1% of their initial value within 1 day after injection (McPhee et al 1979, Feinendegen et al 1973).

Feinendegen et al (1973) calculated local TdR-reutilization (and hence cell death) based on the assumption that for each part of ^{125}I-UdR reutilized there would be approximately 5 parts ^{3}H-TdR reutilized. They give the amount of free TdR that is reutilized per unit time, in per cent of the amount of TdR that is, per unit time, liberated from the tissue DNA, (R), by the following equation:

$$R = \frac{1.2\ (\beta - \alpha)}{1.2\beta - 0.2\alpha} \cdot 100$$

where α is the regression coefficient for ^{3}H-TdR (proportion of ^{3}H-TdR liberated from tissue DNA during time) and β is the regression coefficient for ^{125}I-UdR. From Fig. 23a it can be estimated that the gradient of the approximate-best fit (by eye) of decline between days 2 and 5 for ^{3}H-TdR is 10.67, and for ^{125}I-UdR is 23. At day 4, α=21.34, and β=46. Therefore, R=58%. As a minimum estimate this coincided reasonably with other studies. McPhee et al (1979) estimated 61%; Feinendengen et al (1973) estimated 67%; Joel et al (1977) estimated only 28%.

In the case of the lymph nodes one would expect little cell death, (except after an infection, see Chapter 3), but one might expect cells to migrate. Fig. 23b shows that initially there was a decline in both radiolabels, suggesting some cell migration. If R is calculated from the slope of the first four days, it is 47%, which is surprisingly high and indicates thymidine reutilization and cell death. After the first four

137

days both values plateau, and R calculated from days 5-9 is 0, suggesting that no cell death occurred in the lymph nodes during that period. McPhee et al also found high thymidine reutilization in the spleen, estimated at 52%, compared to 61% for the thymus. They did not calculate a value for lymph nodes, but their graphs were not dissimilar to Fig. 23b, with an initial decline in both labels which then levels off. The fact that high levels of cell death were recorded in the spleen and lymph nodes is perhaps a little disconcerting, as it inclines one either to doubt the assay, or to doubt the contention that there is something special about cell death in T cell development in the thymus. The only other explanation I can see for the difference in the loss of the two radiolabels, if it is not due to selective reutilization of ^{3}H-TdR after cell death, is that ^{125}I-UdR is itself killing the cells in which it becomes incorporated, and this has been ruled out by McPhee et al (1979), and I could find no evidence for it myself.

Assay for DNA fragmentation

The assay frequently used to measure the degree of DNA fragmentation in CTL-mediated killing (eg Duke et al 1983; Gromkowski et al 1988; Ostergaard et al 1989, see Chapter 4) relies on pre-labelling targets with a radioactive DNA precursor and then assessing what percentage of the total incorporated radiolabel which is found in intact chromatin or in fragmented chromatin. This is achieved by measuring cpm in the culture supernatant, and then lysing the cells with 0.2% Triton-X100 in PBS and centrifuging the lysate at 15000g for 15 minutes in the cold, to separate intact from fragmented chromatin, and counting the supernatant. The sum of these two measurements, as a fraction of total incorporated counts, gives the percentage fragmentation (see Materials and Methods). This protocol was applied to thymocytes and

lymph node cells which had been labelled <u>in vivo</u> (with 100μCi ^{3}H-TdR per day for 4 days, or 35μCi ^{125}I-UdR per day for 4 days). Mice were killed, cell suspensions made and washed 2 x in MEM-H and aliquots of 5 x 10^5 cells were either harvested to obtain total incorporation, or lysed in 1 ml 0.2% Triton-X100 in PBS, centrifuged and the supernatants and pellets counted. When ^{3}H-TdR was the precursor used, 100μl supernatant was counted in 2 ml Aquasol (LKB) and pellets were also counted in 2 ml Aquasol to which was added 100μl MEM-H. The use of ^{125}I-UdR had the advantage that no scintillation fluid was required, making comparisons between the different fractions more straight forward. The counts from thymuses and lymph nodes of mice labelled with ^{125}I-UdR, and the estimated percentage DNA fragmentation, are given in Tables 1a and 1b respectively. Tables 1c and 1d show the equivalent data from mice labelled with ^{3}H-TdR, but total cpm when the cells were harvested are not given because these were counted using different scintillation fluid and are not directly comparable.

For ^{125}I-UdR labelled mice, the mean(%DNA fragmentation in the thymus) = 10.74, (σ_n = 1.47), and the mean(%DNA fragmentation in the lymph nodes) = 5.48, (σ_n = 1.43) Assuming that both means are of normally distributed, independent populations, one can test the hypothesis that there is no difference between the means (Hoel 1976). This hypothesis was rejected with 99.5% certainty, suggesting that the difference observed in the DNA fragmentation in thymus and in lymph nodes is statistically significant. In the case of ^{3}H-TdR labelled mice the mean(%DNA fragmentation in the thymus) = 9.82, (σ_n = 1.30), and the mean(%DNA fragmentation in the lymph nodes) = 6.24, (σ_n = 0.91). This difference is also statistically significant.

Are these values for DNA fragmentation reasonable? If one assumes that all but 3% of double positive thymocytes are going to die

within 3 days of their birth, that 73% of thymocytes in these mice are double positive, and that the mean time from the onset of a cell's death to its total disappearance is 9 hours, then one might predict that % DNA fragmentation at any instant in time would be: $(73-3) \times \frac{9}{72} \times 100 = 8.75\%$.

Clearly one has a problem in trying to assess the accuracy of an assay of this kind, because one has nothing with which to compare it directly. In order to check its sensitivity, therefore, I decided to see if the assay would pick up the increase in DNA fragmentation in the thymus that one would predict to occur on *in vivo* administration of hydrocortisones (Umansky et al 1981, Soffer et al 1952, Compton et al 1987).

Mice were injected with 35μCi ^{125}I-UdR per day for 4 days and at the time of the last injection were also given 1mg hydrocortisone. The mice were killed at various times after their last injection and %DNA fragmentation in the thymus and lymph nodes was determined as described previously. Figure 24 shows the kinetics of DNA fragmentation. Increased DNA fragmentation was also detected in the lymph nodes, but this is not surprising: pyknotic nuclei have been observed in lymph nodes on glucocorticoid treatment (La Pushin and de Harven 1971); activated T cells seem to be glucocorticoid sensitive (Compton et al 1987) whereas single positive thymocytes are not (MacDonald et al 1989); and single strand nicks in DNA of cells in lymph nodes can be detected as an early event after injection of hydrocortisone to mice (H.R. MacDonald, personal communication).

140
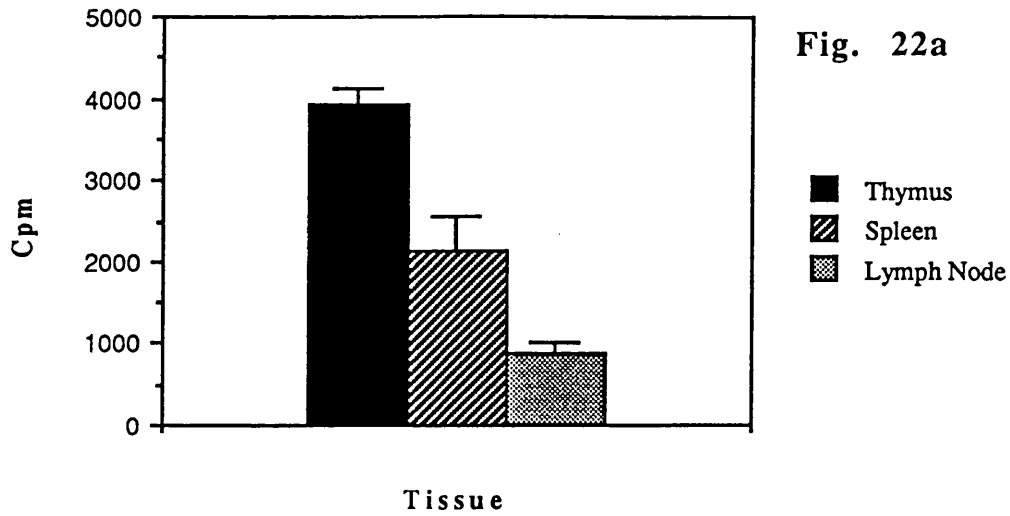
Fig. 22a

Fig. 22a

Cpm in 5×10^5 cells taken from thymus, lymph nodes and spleen of mice injected i.p.with 100μCi ^{3}H-TdR daily for 4 days. Each point is a mean of 3 mice, and the cpm of each tissue of each mouse was a mean of 10 readings with $\sigma_n <$ 5% of the mean.
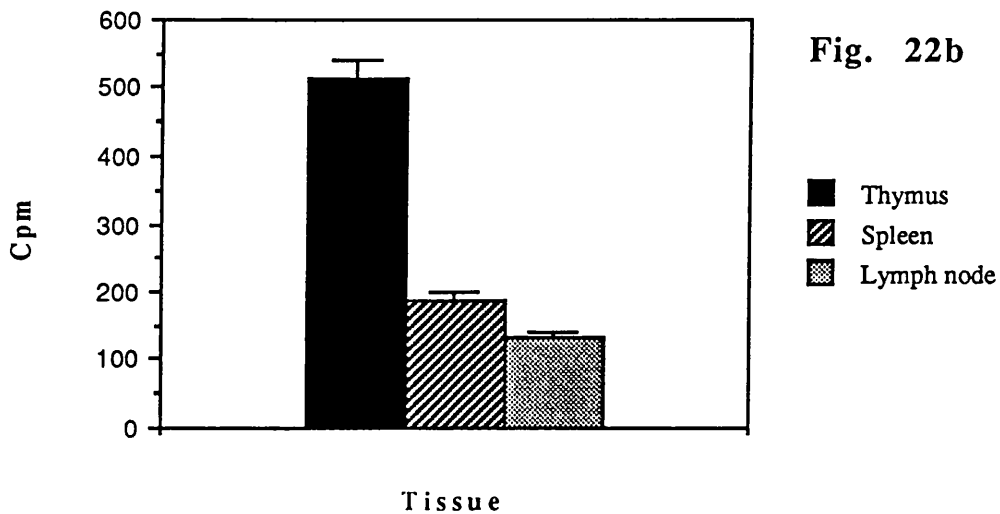
Fig. 22b

Fig. 22b

As above, but mice were labelled with daily i.p. injection of 35μCi ^{125}I-UdR for 4 days.
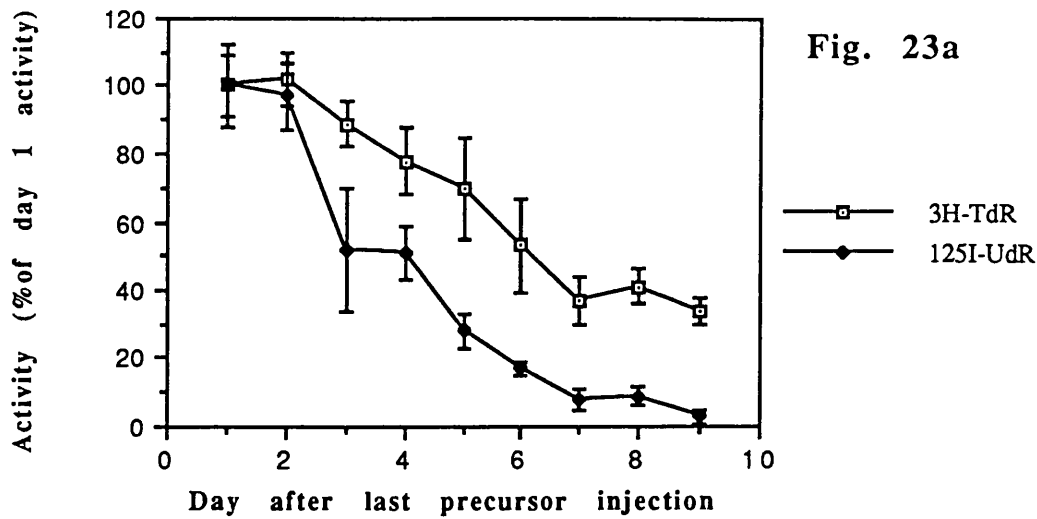
Fig. 23a

Fig. 23a

Decline of radiolabel in thymocytes of mice labelled with either daily injection for 4 days of either 100 μCi ^{3}H-TdR or 35μCi ^{125}I-UdR, expressed as a percentage of cpm on first day after labelling finished. Each point is a mean of 10 readings.
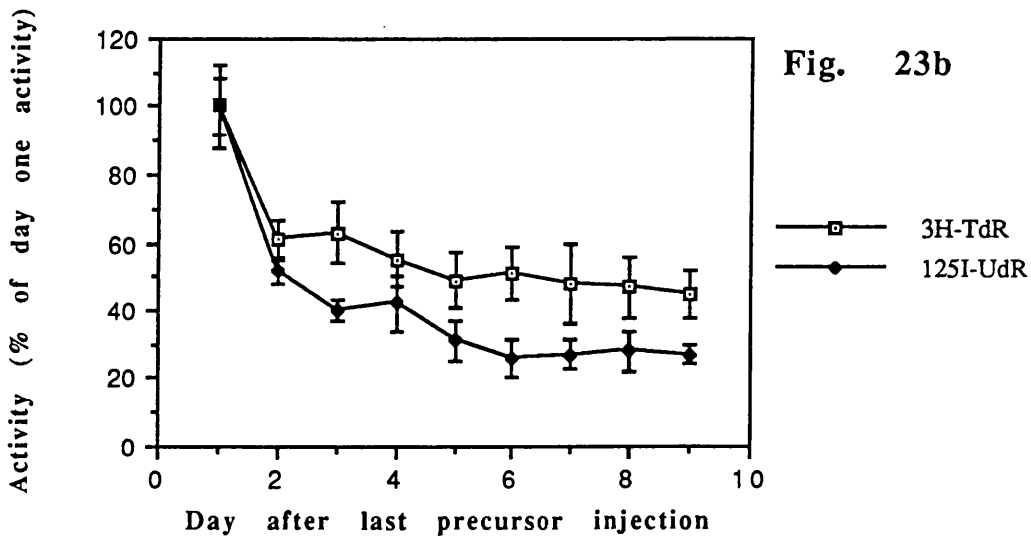
Fig. 23b

Fig. 23b

As above, but for cells taken from the lymph nodes.

Table 1a: Thymuses of ^{125}I-UdR labelled mice.

Total mean cpm (cell harvester)(σ_n)	Mean cpm in supernatant (σ_n)	Mean cpm in pellet (σ_n)	% DNA fragmentation
554 (26)	66.5 (9)	452 (24)	12.8
576 (19)	54 (8.7)	460 (18.7)	10.5
340 (10.2)	42 (12)	306 (15)	12.0
479 (22.9)	52 (6)	532 (47)	8.9
501 (38)	61 (9)	579(42)	9.5

Table 1a: Counts in thymocytes, harvested (column 1) or lysed in 0.2% Triton-X 100 (column 2 and 3). Each row represents a different mouse and each point is a mean of 10. Each measurement was made on 5 x 10^5 thymocytes. % DNA fragmentation is calculated from the value in column 2 divided by the sum of the values in column 2 and column 3, for that mouse (total cpm from harvester is given as a check on expected total counts).

Mean(%DNA fragmentation) = 10.74%, σ_n = 1.47

Table 1b : Lymph nodes of ^{125}I-UdR labelled mice.

Total mean cpm for 5x10^5 cells (harvester) (σ_n)	Mean cpm in supernatant from 3x10^6 cells (σ_n)	Mean cpm in pellet from 3x10^6 cells(σ_n)	% DNA Fragmentation
164 (7)	61.4 (3)	919.5 (67)	6.3
147.5 (15)	34.5 (9.1)	799.5 (54)	4.1
201 (32.9)	59.3 (4.7)	981 (63)	5.7
199.1 (27)	82 (9.3)	1002.5 (78.2)	7.6
157 (8.9)	37 (6.7)	961.4 (84)	3.7

Table 1b: Counts in lymph node cells, harvested (column 1) or lysed in 0.2% Triton-X 100 (column 2 and 3). Each row represents a different mouse and each point is a mean of 3 readings. % DNA fragmentation is calculated as above.

Mean(% DNA fragmentation) = 5.48%, σ_n = 1.43

Table 1c: Thymuses from ^{3}H-TdR labelled mice.

Mean cpm in supernatant (σ_n)	Mean cpm in pellet (σ_n)	% DNA Fragmentation
474.6 (29)	3531.6 (211.4)	11.8
416.7 (31.9)	4524 (197)	8.4
373.1 (12)	3463 (421.1)	9.7
536.9 (46.8)	4475.1(217)	10.7
409.3 (35.1)	4379.4 (316.2)	8.5

Table 1c: Counts in thymocytes, lysed in 0.2% Triton-X 100. Each row represents a different mouse and each point is a mean of 10. Each measurement was made on 5×10^5 thymocytes. % DNA fragmentation is calculated from the value in column 1 divided by the sum of the values in column 1 and column 2, for that mouse.
Mean(%DNA fragmentation) = 9.82%, σ_n = 1.30

Table 1d: Lymph nodes from ^{3}H-TdR labelled mice.

Mean cpm in supernatant (σ_n)	Mean cpm in pellet (σ_n)	% DNA Fragmentation
348.9 (22.6)	5256 (314.1)	6.2
315 (67.5)	4917 (210.7)	6.0
307.1 (73.2)	3587 (476.1)	7.9
261 (36)	4861 (295)	5.1
241.8 (27)	3791 (127.4)	6.0

Table 1d: Counts in lymph node cells, lysed in 0.2% Triton-X 100. Each row represents a different mouse and each point is a mean of 3. Each measurement was made on 3×10^6 cells. % DNA fragmentation is calculated from the value in column 1 divided by the sum of the values in column 1 and column 2, for that mouse.
Mean(%DNA fragmentation) = 6.24%, σ_n = 0.91
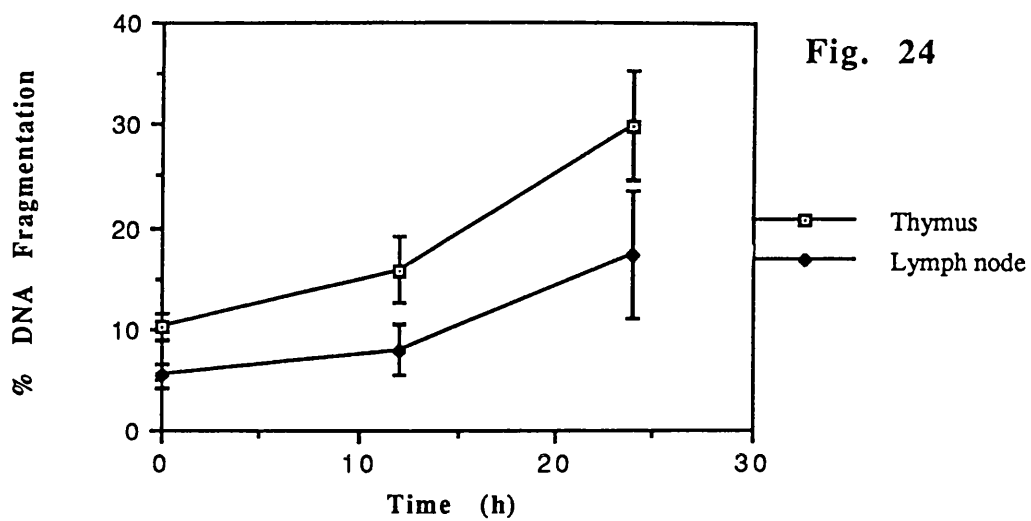
Fig. 24

Fig. 24
DNA fragmentation in thymus and lymph nodes of mice after administration of hydrocortisone. Mice were labelled daily for 4 days with 35μCi ^{125}I-UdR and at the time of the last injection also given 1 mg hydrocortisone, and killed at time intervals after hydrocortisone injection. Each point is the mean of 3 mice and the value for each mouse was a mean of 10 readings, with σ_n < 5% of the mean.

Effect of mab anti-CD3 on thymocytes *in vivo*

Smith et al (1989) have shown that addition of a mab against CD3 to fetal thymus organ cultures caused extensive apoptosis and DNA fragmentation within 18 h, and that the majority of the dying cells (83%) were of double positive phenotype. The same antibody causes proliferation in mature T cells (Bluestone et al 1987), but not in immature thymocytes (Havran et al 1987; Finkel et al 1987), although it does induce a transitory increase in cytoplasmic Ca^{2+} concentration in immature thymocytes (Finkel et al 1987). As changes in intracellular Ca^{2+} concentration can induce apoptosis in thymocytes (Wyllie et al 1984; McConkey et al 1988, 1989a and b), it seems possible that engaging the TCR-CD3 complex might, in immature thymocytes, signal the cells to die, and that this might be the mechanism of clonal deletion of self-reactive T cell precursors in the thymus.

Thymocyte DNA Fragmentation induced by mab against CD3 *in vivo*

Mice were labelled with ^{125}I-UdR as described previously and at the time of their last injection, were also injected with 0.25 mg protein-A-purified 2C11 (hamster IgG, anti mouse CD3, Leo et al 1987). The mice were killed 24 h later and the DNA fragmentation in the thymus, lymph nodes, and spleen was determined. Control mice were injected with either PBS or with 0.25 mg affinity-purified GK1.5. GK1.5 is a rat IgG anti mouse CD4 mab (Dialynas et al 1983a) and was used as a negative control, as CD4 is present on most thymocytes, and no hamster anti mouse IgG directed against a thymocyte cell surface molecule was available to me. Fig. 25 shows the %DNA fragmentation. Injection of 2C11 dramatically increased % DNA fragmentation in the thymus, from about 10% in control mice, to 47% in the treated mice, while it had no

measurable effect on DNA fragmentation in the lymph nodes. The fact that GK1.5 did not increase DNA fragmentation suggests that the effect of 2C11 is not due to a non-specific mechanism such as opsonisation or antibody-dependent killing involving other cells.

It would be very surprising if 2C11 were killing cells which did not express CD3, and so one would expect it to be acting on the CD3^{dull} double positive thymocytes, which make up about half of double positives, or on the CD3$^+$ single positives. To determine which cells are dying, I stained thymocytes from 2C11-treated and normal mice with antibodies against CD4 and CD8. Fig 26a shows the Facs profile of staining with anti CD4 (FL2) and anti CD8 (FL1) on thymocytes from a 2C11-treated mouse 24 h after injection. Fig. 26b shows the equivalent profile from an untreated mouse. 2C11 treatment reduced the proportion of double positive thymocytes from about 75% to about 37%, and the proportions of the other three subpopulations defined by accessory molecule expression increased correspondingly, suggesting that all but the double positive thymocytes were resistant to death induced by 2C11 treatment.

Unfortunately one could not check that it was actually the CD3-expressing double positives which die, because after *in vivo* antibody treatment, 2C11 would be bound to CD3 on the cell surface and so mask the molecule in a staining assay. However, the antibody treatment did kill about the same proportion of double positives as express CD3. Fig. 26c shows staining with 2C11 on double positive thymocytes from an untreated mouse. The double positive thymocytes were purified by two rounds of panning with antibodies against CD4 and CD8 (YTA 156 and 2.43, Sarmiento et al, 1981). This protocol enriched the double positive population to 91% purity (see Fig. 26d) and so the 5.4% CD3^{high} cells that one can see in Fig. 26c are probably contaminating CD4$^+$ single positives

147

(6.2%). Thus one can assume that the ratio of CD3$^-$: CD3^{dull} double positive thymocytes in these mice is 45.4 : 49.3 (ie 52% of double positives express CD3).

If all CD3$^+$ double positives were induced to die by the 2C11 treatment one would expect about 39% DNA fragmentation. The discrepancy of 8% between this figure and the 47% mean DNA fragmentation measured could be due to the fact that not all of the approximately 10% of thymocytes that die in the non-treated mice necessarily express CD3, and so in the treated mice one might observe the death of all CD3$^+$ double positives and in addition some death due to positive selection or to failure to assemble functional TCR genes. The high standard deviation (5.6) and small population size (3) in the DNA fragmentation assay mean that one cannot conclude too much from these data about DNA fragmentation in positive selection.

The antibody staining data does, however, inspire confidence in the assay for apoptosis. Where no increase in DNA fragmentation was found there were no changes in the pattern of CD4$^+$ and CD8$^+$ expression. At the time of making cell suspensions from the mice I also stained cells with Propidium Iodide (PI) (as a measure of plasma membrane integrity and hence viability). In the 2C11 treated mice 11% of thymocytes were stained as opposed to 6% in control mice. This suggests that the DNA fragmentation assay gives information that one would not obtain from more conventional assays of cell death. The difference between the two estimates of death may be due to the fact that DNA fragmentation can proceed loss of plasma membrane integrity (Duvall and Wyllie 1986), but may also be because the apoptotic cells and apoptotic bodies have already been phagocytosed, hence the reduced accessory molecule cell surface staining. As a final check on the assay, I prepared DNA from mice treated with 2C11 and from untreated mice,

148

and electrophoresed this on an agarose gel (See Fig. 27). The only track in which a ladder is seen is that containing the DNA from the thymus of a 2C11-treated mouse. Shi et al (1989) have also shown that a electrophoretic ladder is obtained from thymocytes treated with anti-CD3 antibody *in vivo*.

Apoptosis in immature thymocytes *in vitro*

As nearly all double positive thymocytes die rapidly in culture when dissociated, the effect of a potentially lethal stimulus such as an antibody against CD3, and also the effect of anything which might prolong survival in the presence of such a stimulus, are difficult to study. One can always argue that the cells would have died anyway despite the stimulus, and any surviving cells can be attributed to contamination by other thymocytes subsets. I attempted to look at the apoptosis induced by mab anti-CD3, and at agents which might prolong survival, in double positive thymocytes in culture. Double positives were prepared either by panning as described previously, which gave 91% double positives (See Fig. 26d), or by using thymocytes from embryonic day 20 (E20) embryos, which contained 93% double positives, and less than 3% single positives (See Fig. 28) The double positives from the fetal thymus expressed the same levels of CD3 as those from an adult, 4 week-old mouse (about 50% were CD3^{dull}) and were indistingiushable from those from an adult mouse in my experiments, and so I shall consider all ages together.

The effect of 2C11 on double positive thymocytes in culture

Double positive thymocytes were prepared and incubated either on 2C11-coated flat bottom 96 well plates, or on GK1.5-coated plates, or in

the absence of an antibody, at a density of 5×10^5 cells ml^{-1} in 200μl. In addition, the protein synthesis inhibitor cycloheximide and the nuclease inhibitor aurintricarboxylic acid (ATC) were added to some wells. At various time points wells were analysed for percentage viability by their ability to exclude PI, measured on the Facs. No difference was seen between thymocytes incubated with GK1.5 or with no antibody (data not shown), but cells given 2C11 did die more quickly. Fig. 29 shows a typical experiment. Initially, at t=14 (See Fig. 29a), there was no difference between wells containing mab anti-CD3 and those without it, and cycloheximide and ATC enhanced survival to a similar degree in both instances. By the third day in culture (Fig. 29b), however, there was a marked difference in the extent of cell death in the presence or absence of 2C11. About 20% more cells died when incubated with 2C11 and both inhibitors almost completely negated this difference. By day 4 in culture (Fig. 29d) almost all cells incubated with 2C11 were dead and the inhibitors were no longer effective at enhancing survival of these cells.

Although these data indicate that 2C11 does induce death in immature thymocytes *in vitro*, its effect is clearly much slower than in vivo, where Shi et al (1989) were able to observe a DNA 'ladder' 14 h after antibody administration, and I found disappearance of $CD3^+CD4^+CD8^+$ cells after 24 h. In fetal organ culture, increased apoptosis and DNA fragmentation were observed 18 h after incubation with anti-CD3 antibody (Smith et al 1989). It does not seem likely that this difference is due simply to dose of antibody, as the dose of 2C11 I used (pre-incubation of plates with 2μg ml^{-1}) was sufficient to cause proliferation of T cell hybridomas (data not shown). Other investigators have found that little anti-CD3-induced DNA fragmentation occurs when double positive thymocytes are dissociated *in vitro*, despite the fact that

150

these are the cells believed to be responsible for the fragmentation seen on *in vivo* antibody treatment (Tadakuma et al, 1990). The discrepancy seems unlikely to be the result of a slower death programme as such, since these cells die very quickly in 10^{-6} M dex (See Fig. 30). Thus one is tempted to speculate that, in addition to triggering through CD3, some other interaction, which is present in the thymus *in vivo*, and in organ culture, may be necessary to induce apoptosis in double positive thymocytes. The TCR-transgenic mice (Kiselow et al 1988; Sha et al 1988) have indicated that both positive and negative selection are dependent on a ligand-TCR interaction, and unless the difference between the two outcomes is determined simply by the affinity of the interaction, then other molecules must be involved. If treating a thymus with mab anti CD3 mimics the induction of clonal deletion, then, it is not surprising that other interactions supplied by the thymic environment are also required.

That cycloheximide and ATC prolong survival suggests that this cell death is an active process and that DNA fragmentation is causal. Other interpretations of the action of these inhibitors are discussed in Chapter 3.
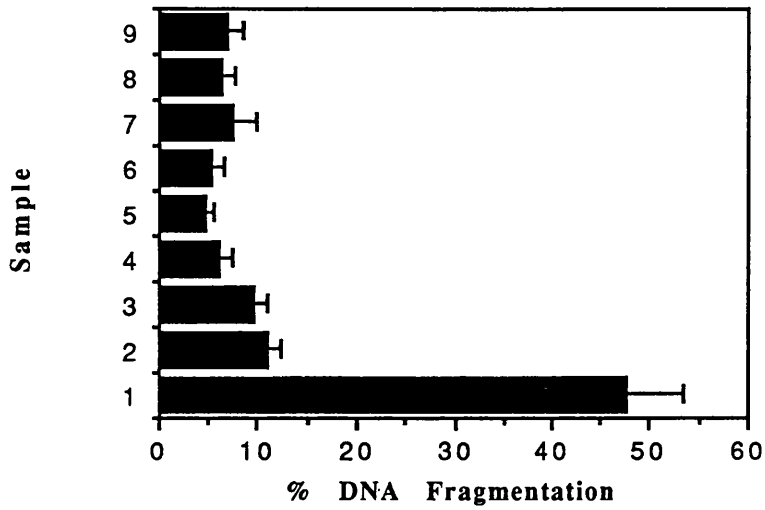
Fig. 25

Number	Sample
1	Thymus + 2C11
2	Thymus + GK1.5
3	Thymus + PBS
4	Lymph node + 2C11
5	Lymph node + GK1.5
6	Lymph node + PBS
7	Spleen + 2C11
8	Spleen + GK1.5
9	Spleen + PBS

Fig. 25

% DNA fragmentation in thymus, lymph nodes, and spleen of ^{125}I-UdR labelled mice, injected with PBS, or with 0.25 mg 2C11 or GK1.5. Each point is a mean of 3 and standard deviations are given.

152
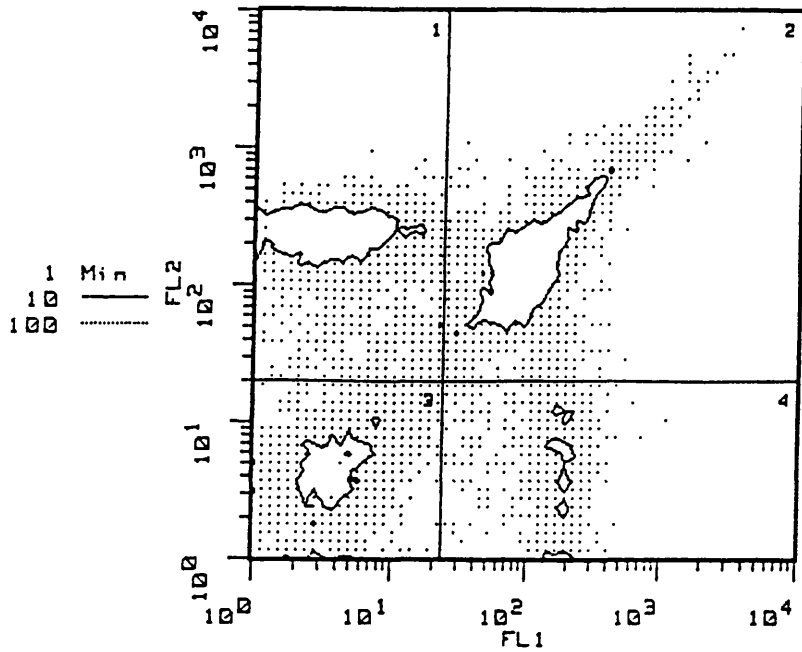
Fig. 26a

Subpopulation	%
CD4$^+$ single positive	31.2
CD8$^+$ single positive	10.03
CD4$^+$CD8$^+$ double positive	36.7
CD4$^-$CD8$^-$ double negative	22.07

Figure 26

Fig. 26a   Facs profile of staining with directly conjugated anti CD4 (FL2) and anti CD8 (FL1) (Beckton   Dickinson) on mouse thymocytes 24 h after i.p. injection of 0.25 mg 2C11.

Fig. 26b
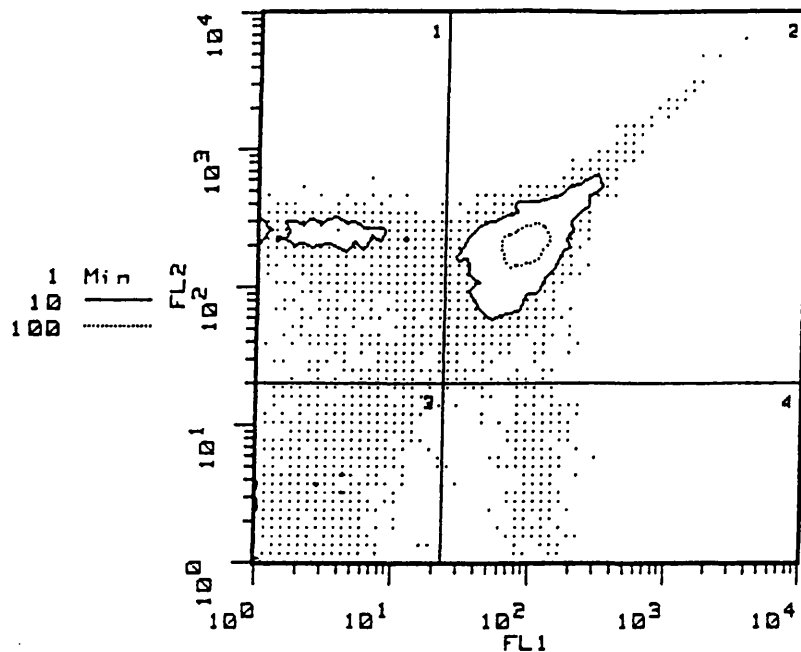
Subpopulation	%
CD4$^+$ single positive	14.19
CD8$^+$ single positive	3.02
CD4$^+$CD8$^+$ double positive	74.32
CD4$^-$CD8$^-$ double negative	8.47

Figure 26

Fig. 26b   Facs profile of staining with directly conjugated anti CD4 (FL2) and anti CD8 (FL1) (Beckton   Dickinson) on mouse thymocytes 24 h after i.p. injection of 200 µl PBS.
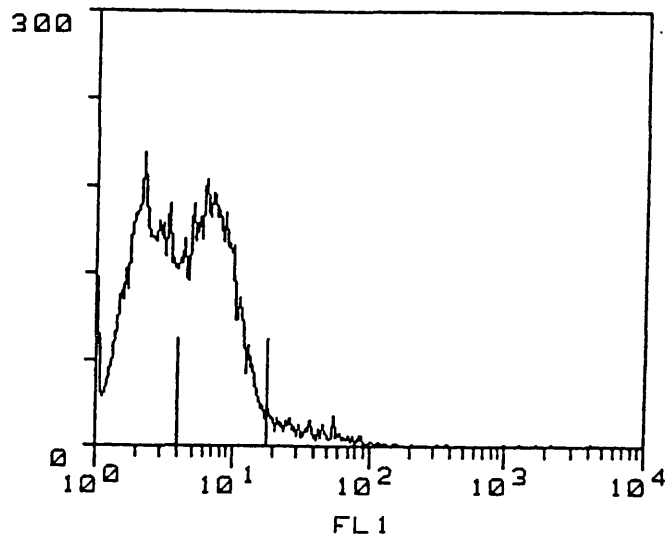
Fig. 26c

Subpopulation	%
CD3⁻	45.4
CD3 dull	49.3
CD3high	5.4

Figure 26

Fig. 26c Facs profile of staining with directly conjugated 2C11 (anti CD3) on mouse thymocytes enriched for double positives to 91% purity with two rounds of panning with YTA 156 and 2.43 (See Fig. 26d).

Fig. 26d
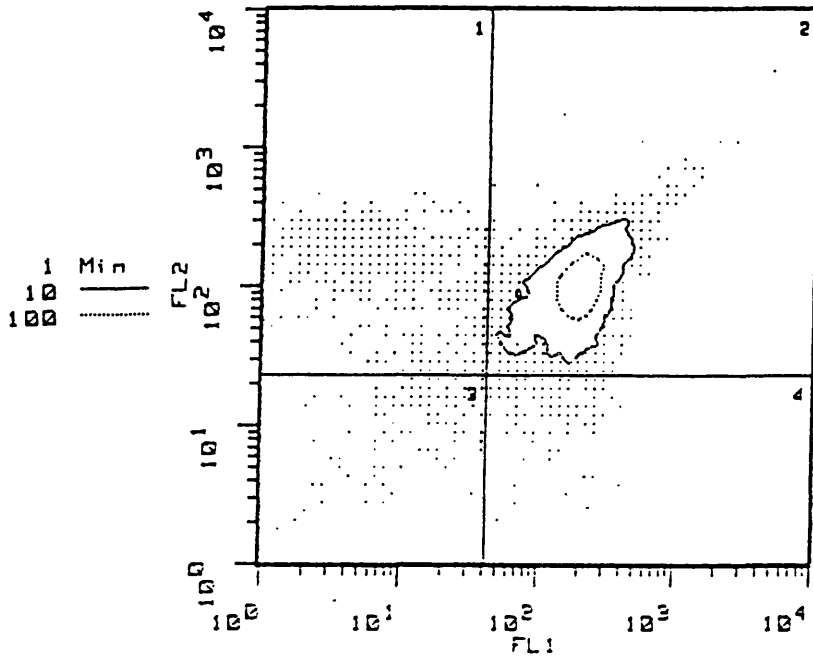
Subpopulation	%
CD4$^+$ single positive	6.29
CD8$^+$ single positive	1.21
CD4$^+$CD8$^+$ double positive	91.16
CD4$^-$CD8$^-$ double negative	1.34

Figure 26

Fig. 26d   Facs profile of staining with directly conjugated anti CD4 (FL2) and anti CD8 (FL1) (Beckton   Dickinson) on mouse thymocytes, enriched for double positives with two rounds of panning with YTA 156 and 2.43.

Fig. 27
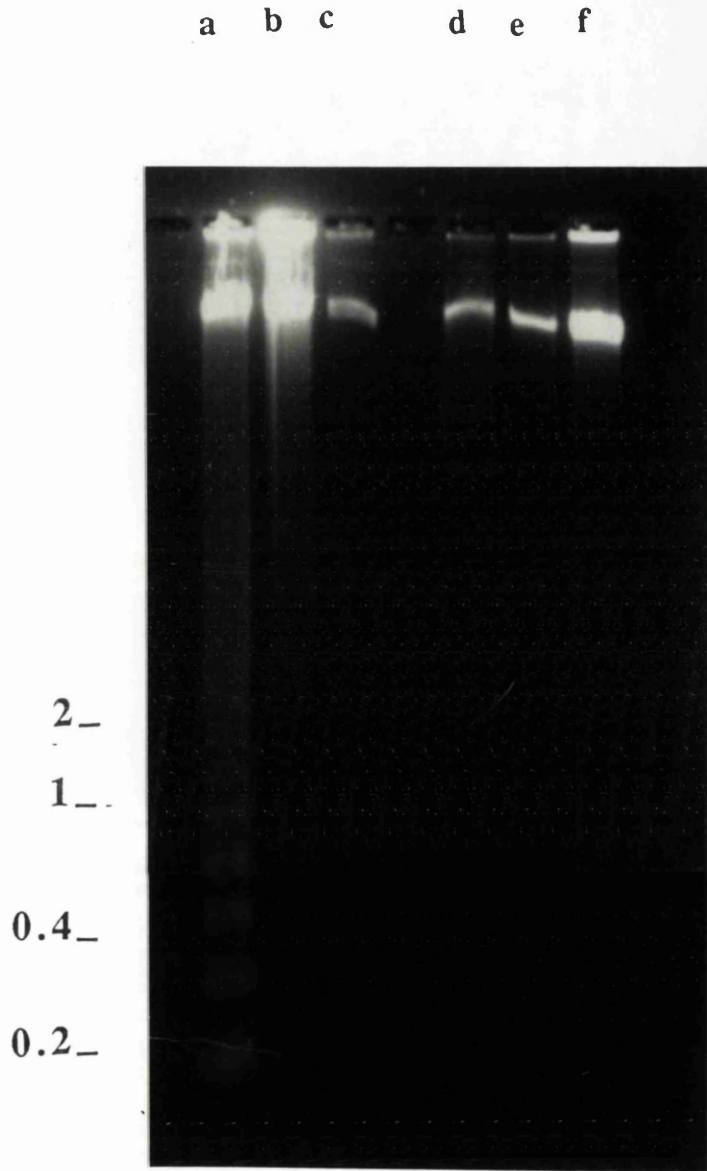
Fig. 27
Ethidium bromide stained 1% agarose gel showing DNA prepared from mice 24 h after injection with 0.25 mg 2C11 (tracks a-c) or 0.25 mg GK1.5 (tracks d-f). Size is given in kilobases.

Track a   thymus + 2C11
Track b   spleen + 2C11
Track c   lymph node + 2C11
Track d   thymus + GK1.5
Track e   spleen + GK1.5
Track f   lymph node + GK1.5

In other gels where loading was equivalent, no ladder was seen in any track but a.

157
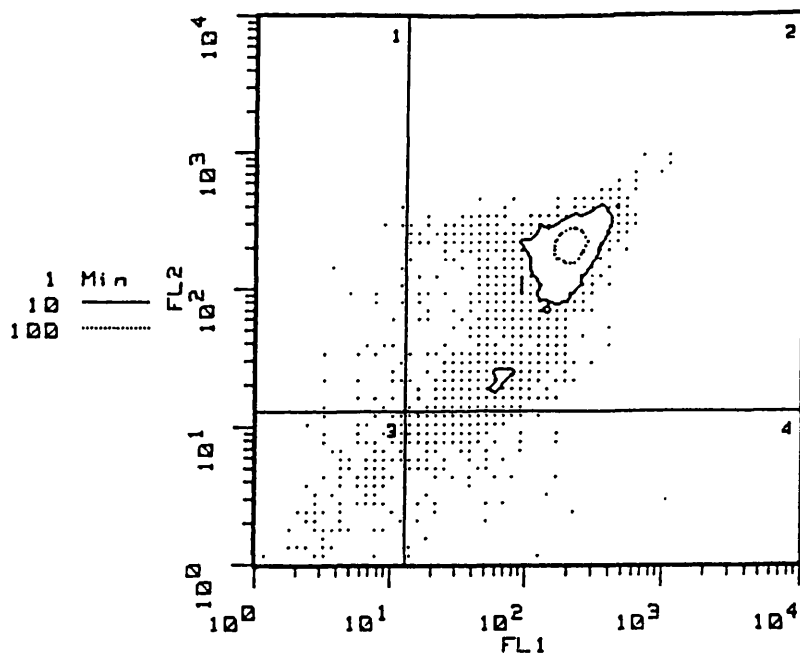
Fig. 28

Subpopulation	%
CD4$^+$ single positive	0.54
CD8$^+$ single positive	2.49
CD4$^+$CD8$^+$ double positive	93.37
CD4$^-$CD8$^-$ double negative	3.6

Figure 28

Fig. 28   Facs profile of staining with directly conjugated anti CD4 (FL2) and anti CD8 (FL1) (Beckton   Dickinson) on E20 mouse thymocytes.
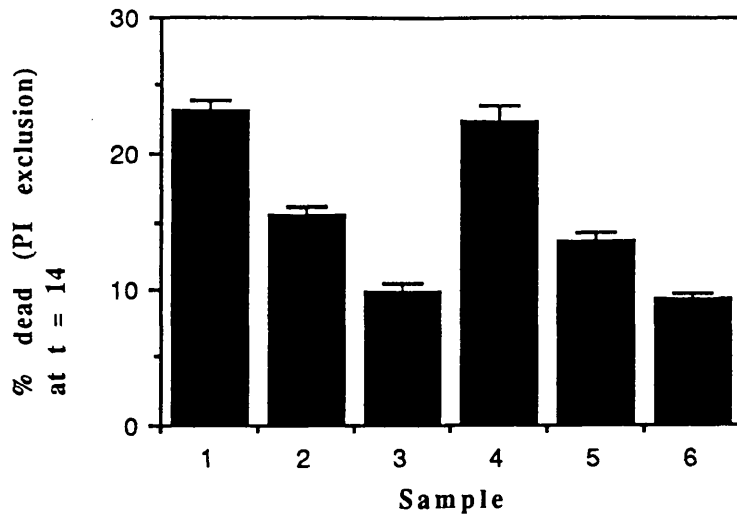
Fig. 29a
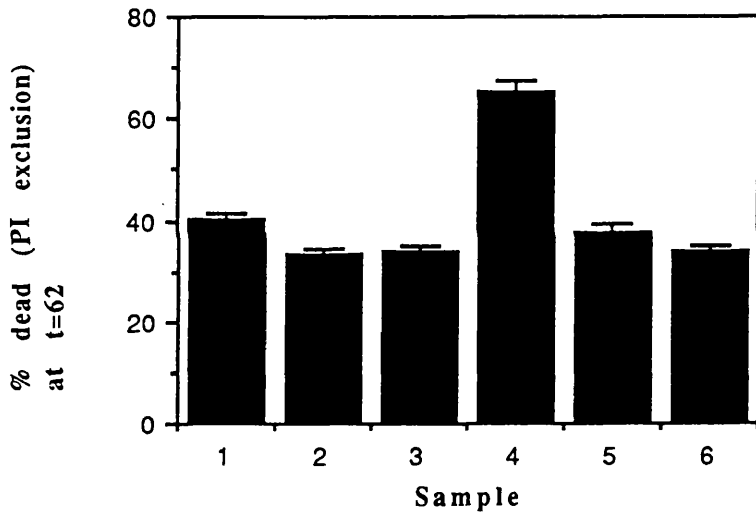
Fig. 29b

Number	Sample
1	double positives only
2	+ 2 µgml^{-1} cycloheximide
3	+ 30 µgml^{-1} ATC
4	+ 2C11
5	+ 2C11 + 2 µgml^{-1} cycloheximide
6	+ 2C11 + 30 µgml^{-1} ATC

Fig. 29

% dead thymocytes, determined by PI exclusion on the facs, after culture in conditions stated for 14 h (**Fig. 29a**) and 62 h (**Fig. 29b**). Each point is a mean of 5.
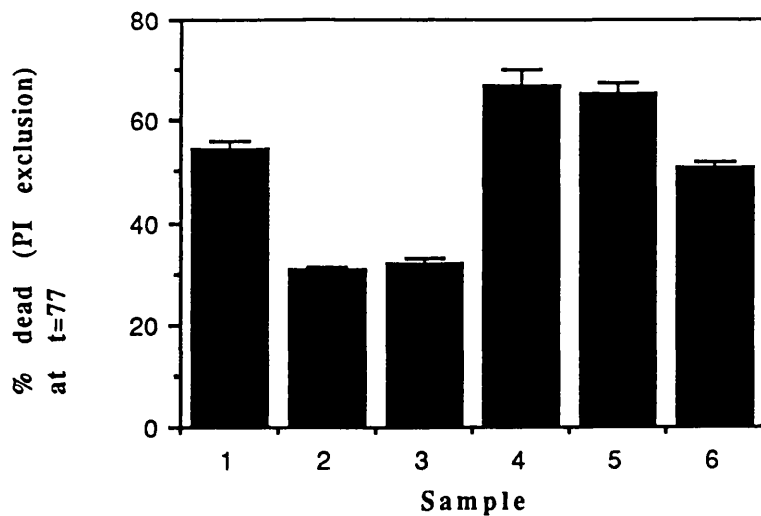
Fig. 29c
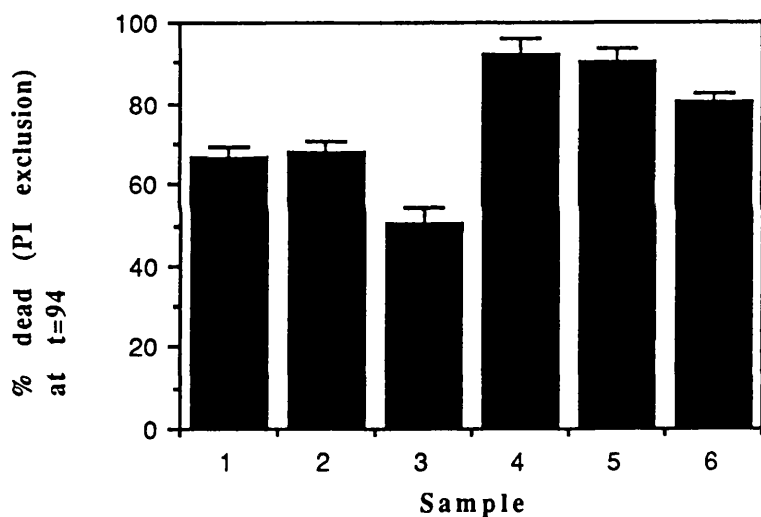
Fig. 29d

Number	Sample
1	double positives only
2	+ 2 μgml^{-1} cycloheximide
3	+ 30 μgml^{-1} ATC
4	+ 2C11
5	+ 2C11 + 2 μgml^{-1} cycloheximide
6	+ 2C11 + 30 μgml^{-1} ATC

Fig. 29

% dead thymocytes, determined by PI exclusion on the facs, after culture in conditions stated for 77 h (**Fig. 29c**) and 94 h (**Fig. 29d**). Each point is a mean of 5.
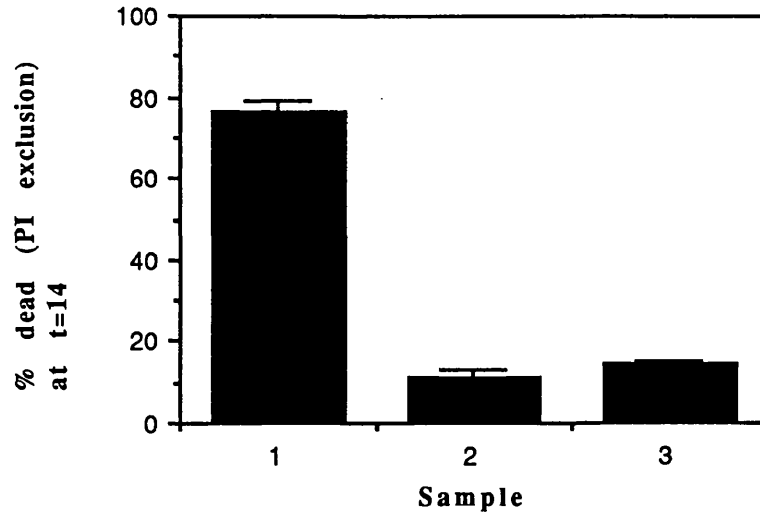
Fig. 30

Number	Sample
1	+ 10^{-6}M dex
2	+ 10^{-6}M dex + 2μg ml^{-1} cycloheximide
3	+ 10^{-6}M dex + 30μg ml^{-1} ATC

Figure 30

Death of double positive thymocytes incubated in 10^{-6}M dex with or without cycloheximide and ATC, as determined by their loss of ability to exclude PI, measured on the Facs. Each point is a mean of 5.

DISCUSSION

From the point of view of understanding clonal deletion, an attractive explanation for the death of thymocytes induced by mab anti CD3 *in vivo* is that by binding to CD3^{dull} double positive thymocytes, the antibody induces an increase in [Ca^{2+}]$_i$, which directly triggers apoptosis. This is supported by the observations that an elevation of [Ca^{2+}]$_i$, induced by a calcium ionophore, is necessary and sufficient to induce apoptosis in immature thymocytes (Kizaki et al 1989; McConkey et al 1989) and that antibodies to CD3 increase [Ca^{2+}]$_i$ to similar levels in immature thymocytes as they would in mature T cells (Finkel et al 1987). Thus it is possible that the antibody mimicks the action of a self-peptide + MHC in triggering the cell to commit suicide.

There are, however, several problems with this interpretation: (1) It has not been shown conclusively that it is the CD3-expressing double positives cells that die in response to anti-CD3 treatment; the reduction in the proportion of double positive thymocytes shown by Facs analysis might conceivably be due to down regulation of accessory molecule expression induced by the antibody treatment (eg Blue et al 1987b) and not due to preferential death of that subpopulation. (2) Neither this study nor published data (Smith et al, 1989; Shi et al 1989) have excluded the possibility that the action of anti-CD3 in promoting double positive cell death is indirect, via another CD3$^+$ cell type (double negative CD3$^+\gamma\delta$TCR$^+$ or single positive CD3$^+\alpha\beta$TCR$^+$) that might either produce something that is toxic to immature thymocytes or stop making something that is essential for their survival.
(3) There is no known case in which a T cell is activated physiologically by a ligand binding exclusively to CD3, without also engaging the $\alpha\beta$TCR. It has recently been demonstrated that, in immature thymocytes,

162

binding to the $\alpha\beta$TCR and signal transduction through CD3 are uncoupled (Finkel et al 1989). Cross-linking with an antibody to CD3 produced a Ca^{2+} signal of similar magnitude in immature and mature T cells, but a pan anti-$\alpha\beta$TCR mab produced a markedly smaller Ca^{2+} in immature thymocytes than in mature T cells, as did mabs that recognize specific Vβ gene products. This difference is corroborated by the observation that *in vivo* administration of mab KJ16 (anti-Vβ8) prevented the emergence of mature single positive thymocytes expressing the KJ16 epitope, but did not reduce the proportion of, or cause the death of, KJ16^{+} double positive thymocytes (McDuffie et al 1986).

The first objection seems merely formal, but the second is perhaps supported by the delayed death response to anti-CD3 mab *in vitro*. One might argue that this is because of the absence of a mediator produced by other thymocyte subpopulations. Tadekuma et al (1990) also observed a reduced response to mab anti-CD3 *in vitro*, as assessed by measurement of DNA fragmentation, in a population of Facs-sorted double positive thymocytes. However, the kinetics of death were not altered when I did not enrich for double positive thymocytes (data not shown) and so it seems more likely that the difference is due to the disruption of the thymic microenvironment by dissociation of the cells, as rapid cell death has been reported in fetal thymus organ culture (Smith et al 1989). Earlier work from Owen's laboratory did suggest that mab anti-CD3 in fetal thymus organ culture abrogated T cell development by interfering with a putative 'nurse' cell function of $\gamma\delta^+CD3^+$ double negative thymocytes (Owen et al 1988 and 1989). Kyewski et al (1989) have demonstrated reduced production of mature T cells after *in vivo* treatment with antibodies against CD3 or TCR. They attributed this to interference in the interactions of the double positive

thymocytes with dendritic cells and epithelial cells and not to cell death, although they did not look for evidence of apoptosis immediately after antibody treatment. It would be interesting to look at the ability of mab anti-CD3 to induce apoptosis *in vivo* during fetal thymus development, where one could rule out the possibility that the action of the antibody results from its effect on other $CD3^+$ cell types, since Finkel et al (1989) have shown that there are very few $\gamma\delta^+CD3^+$ cells until birth and $CD3^+\alpha\beta TCR^+$ single positives are absent until E18 and infrequent until after birth (Kisielow et al 1984; Ceredig et al 1983).

The observation that both stimulation with antigen and with antibodies to CD3 cause apoptosis in T cell hybridomas also supports the view that the action of mab anti-CD3 is direct (Ashwell et al 1987; Mercep et al 1989 and 1990; Shi et al 1989; Sussman et al 1988). T cell hybridomas are the product of a fusion between antigen-specific peripheral T cells and a thymoma cell line, and, as such, their response to antigen may be representative of the response of an immature thymocyte. Shi et al (1989) have shown that cyclosporin A, which is known to interfere with the process of deletion of autoreactive clones and thereby to cause autoimmune disease (Sakaguchi and Sakaguchi 1988; Gao et al 1988) inhibited cell death induced by mab anti-CD3 in T cell hybridomas *in vitro* and in the thymus *in vivo*, suggesting a common mechanism of induction of death. T cell hybridomas, therefore, seem to be an appropriate model with which to study the mechanism of thymocyte cell death, having the advantage of being a homogeneous population.

The third objection remains unanswered and implies that the phenomenon observed on injection of anti-CD3 mab *in vivo* and in fetal thymus organ culture may have little relevance to T cell development, or at least may not give much information about at which

164

developmental stage self-reactive cells are deleted. Such experiments may give insight into the mechanism of death distal to receptor occupancy, but double positive thymocytes have been shown to be particularly sensitive to the induction of apoptosis by numerous other lethal stimuli, such as glucocorticoids (Wyllie 1980), calcium ionophores and phorbol esters (Kizaki et al 1989), environmental contaminants such as TCDD (McConkey et al 1988) and γ-irradiation (Sellins and Cohen 1987), any of which could be used to study the mechanism of death.

If signal transduction and receptor occupancy are not linked in double positive thymocytes, then it is perhaps more likely that these are the cells on which positive selection operates. Presumably, most of the cells which die when treated with anti-CD3 mab have not reached the stage at which they would be deleted were they self-reactive. As tolerance induction has been shown to occur at the stage of double positives, however, there must be a small population in which binding of ligand to the TCR does trigger a Ca^{2+} signal via CD3, but perhaps these were too few to be detected in Finkel's study. (Alternatively, the theory that ligand-binding to the TCR results in deletion of self-reactive clones by apoptosis triggered through an increase in $[Ca^{2+}]_i$ might be wrong). Schneider et al (1989) have shown that in Mls[a+] neonatal mice some single positive self-reactive Vβ6^{+}CD4^{+} cells do leave the thymus, but that they die in the periphery within a few days of birth, suggesting that the signal for self-reactive cells to die may be a very late event in T cell development.

The work presented here provides evidence that growth factor dependent cell lines die actively on withdrawal of their growth factor, and that DNA fragmentation is causal in their death. In addition it suggests that immature thymocytes die by the same mechanism on ligand-binding to their surface CD3, and hence implicates apoptosis in thymic tolerance induction.

The consensus of opinion on the mechanism of apoptosis seems to be that an increases in $[Ca^{2+}]_i$ signals new gene expression which leads to the activation of nucleases already present in the cell, which cleave the chromatin into nucleosome sized pieces, and this kills the cell. The molecular biology remains obscure, but two strategies might enable one to identify genes involved in the process:

(1) Subtractive hybridisation of cDNA libraries prepared from viable cells and from cells initiating apoptosis. A growth factor dependent cell line would be a convenient source of mRNA from which to prepare libraries because cells would be clonal, and hence any unique sequences in the library from apoptotic cells would be directly related to withdrawal of the growth factor. Cycloheximide blocking studies might facilitate the identification of an optimal time window after growth factor withdrawal from which to prepare the library.

(2) An alternative approach might be to look for mammalian homologues of the *C. elegans ced* genes. *Ced-4* is cloned and sequenced, and *ced-3* and *nuc-1* are likely to soon be available. Unfortunately the nematode genome contains many more C and G residues than the vertebrate genome, and so a direct search for homologues by screening a mammalian cDNA library with nematode probes is unlikely to succeed, even at very low stringency hybridisation. However, once the

166

sequence of the nematode genes are available one might screen mammalian cDNA libraries with short oligonucleotide probes, synthesized to compensate for the discrepancy in base usage between the two genomes.

Adkins, B. Mueller, C., Okada, C.Y., Reichart, R.A., Weissman, I.L. and Spangrude, G.J. (1987) Early events in T-cell maturation Ann. Rev Immunol 5, 325-65

Albertson, D., Sulston, J.E. and White, J.G. (1978) Cell cycling and DNA replication in a mutant blocked in cell division in the nematode Caenorhabditis elegans. Dev Biol 63, 165-178

Allbritton, N.L., Nagler-Anderson, C., Elliot, T.J., Verret, C.R. and Eisen, H.N. (1988) Target cell lysis by cytotoxic T lymphocytes that lack detectable hemolytic perforin activity. J. Immunol. 141, 3243-3248

Allbritton, N.L., Verret, C.R., Wolley, R.C. and Eisen, H.N. (1987) Calcium ion concentration and DNA fragmentation in target cell destruction by murine cloned cytotoxic T lymphocytes. J. Exp. Med. 167, 514-527

Alt, F.W., Blackwell, T.K., DePinho, R.A., Reth, M.G. and Yancopoulos, G.D. (1986) Regulation of genome rearrangenment events during lymphocyt differentiation. Immunol Rev 89, 5-30

Andrews, P., Shortman, K., Scollay, R., Potworowski, E.F., Kruisbeek, A.M., Goldstein, G., Trainin, N. and Bach, J.-F. (1985) Thymus hormones do not induce proliferative ability or cytolytic function in PNA+ cortical thymocytes. Cell Immunol 91, 455

Arrand, J.E. (1985) Preparation of nucleis acid probes. In Nucleic acid hybridisation. Ed, Hames, B.D. and Higgins, S.J. IRL Press (Oxford, Washington D.C.)

Ashwell, J.D., Cunningham, R.E., Noguchi, P.D. and Hernandez, D. (1987) Cell growth cycle block of T cell hybridomas upon activation with antigen. J. Exp Med 165, 173

Askonas, B.A., Williamson, A.R. and Wright, B.G. (1970) Selection of a single antibody-forming cell clone and its propogation in syngeneic mice. P.N.A.S. (USA) 67, 1398

Askonas, B.A. and Williamson, A.R. (1972) Factors affecting the propogation of a B cell clone forming antibody to the 2,4-dinitrophenyl groups. Eur J Immunol 2, 487

Avery, L. and Horvitz, H.R. (1987) A cell that dies during wild-type C. elegans development can function as a neuron in a ced-3 mutant. Cell 51, 1071-1078

Bachvaroff, R.J., Ayvazian, J.H., Skupp, S., and Rapaport, F.T. (1977) Specific restriction endonuclease degradation of DNA as a consequence of immunologically mediated cell damage. Transplantation Proceedings IX, 807-811

Basten, A., Brink, R.A., Mason, D.Y., Crosbie, J. and Goodnow, C.C. (1989) Self tolerance in B-cells from different lines of lysozyme double-transgenic mice. In F. Melchers et al (ed) Progress in Immunology VII, Springer-Verlag, Berlin.

Bauer, E.A., Silverman, N., Besiek, D.F., Kronberg, A. and Deuel, T.F. (1986) Diminished response of Werner's syndrome fibroblasts to growth factors PDGF and FGF. Science 234, 1240-1243

Baugnet-Mathieu, L. and Goutier, R. (1968) Mechanisms responsible for the low incorporation into DNA of the thymidine analogue 5-iodo-2'-deoxyuridine. Biochem. Pharmacol. 17, 1017

Bell, E., Marek, L.F., Levinstone, D.S., Merrill, C., Sher, S., Young, I.T. and Eden, M. (1978) Loss of division potential in vitro: aging or differentiation? Science 202, 1158-1163

Berger, N.A. (1985) Symposium: cellular response to DNA damage: the role of poly(ADP-ribose). Rad. Res. 101, 4-15

Berger, S.J., Sudar, D.C. and Berger, N.A. (1986) Metabolic consequences of DNA damage: DNA damage induces alterations in glucose metabolism by activation of poly(ADP-ribose) polymerase. Biochem. Biophys. Res. Commun. 134, 227-232

Bishop, C.J., Moss, D.J., Ryan, J.M. and Burrows, S.R. (1985) T lymphocytes in infectious mononucleosis. II. Response in vitro to interleukin-2 and establishment of T cell lines. Clin. exp. Immunol. 60, 70-77

Blakely, A.K., Gorman, H.,, Ostergaard, H., Svoboda, C., Lui, J., Young, J. D-E and Clark, W.R. (1987) Resistance of cloned cytotoxic T lymphocytes to cell-mediated cytotoxicity. J. Exp. Med. 166, 1070

Blue, M.-L., Daley, J.F., Levine, H., Craig, K.A. and Schlossman, S.F. (1987a) Identification and isolation of a $T4^+T8^+$ cell with high T3 expression in human thymus: a possible late intermediate in thymocyte differentiation. J. Immunol 139, 1065

Blue, M.-L., Hafler, K.A., Craig, H., Levine, H. and Schlossman, S. (1987b) Phosphorylation of CD4 and CD8 following T cell triggering. J. Immunol 139, 3949

Blue, M.-L., Daley, J.F., Levine, H. and Schlossman, S.F. (1985) Class II major histocompatability complex molecules regulate the development of the $T4^+T8^+$ inducer phenotype of cultured human thymocytes. P.N.A.S. (USA) 82, 8178

Bluestone, J.A., Pardoll, D., Sharrow, S.O. and Fowlkes, B.J. (1987) Characterization of murine thymocytes with CD3-associated T-cell receptor structures. Nature 326, 82-84

Blumenthal, T. and Landers, T.A. (1973) The inhibition of nucleic acid-binding proteins by aurintricarboxylic acid. Biochem. and Biophys. Res. Communications 55, No.3, 680-86

Bogden, J.M., Eastman, A. and Bresnick, E. (1981) A system in mouse liver for repair of O^6-methyl guanine lesions in methylated DNA. Nucleic Acids Res. 9, 3089-3101

Borum, K. (1968) Pattern of cell production and cell migration in mouse thymus studied by autoradiography. Scand J. Haemat 5, 339

Boydston, W.R. and Sohal, G.S. (1979) Grafting of additional periphery reduces embryonic loss of neurons. Brain Res 178, 403-410

Bradley, L.M. and Mishell, R. (1982) Differential effects on the function of subpopulations of helper T lymphocytes. Eur. J. Immunol. 12, 91-94

Brock, M.A. and Hay, R.J. (1971) Comparative ultrastructure of chick fibroblasts in vitro at early and late stages during their growth span.J Ultrastructure Res 36, 291-311

Bronchud, M.H., Potter, M.R., Morgenstern, G., Blasco, M.J., Scarffe, J.H., Thatcher, N., Crowther, D., Souza, L., Alton, N.K., Testa, N.G. and Dexter, T.M. (1988) In vitro and in vivo analysis of the effects of recombinant human granulocyte colony-stimulating factor in patients. Br. J. Cancer 58, 64-69

Brondz, B.D., Abronina, I.F., Zaiceva, M.B., Filatov, A.V. and Chervonsky, A.V. (1984) Specific suppressor T cells immune to antigens of the H-2 complex: receptors, clonal structure, genetic restriction and antigenic markers. Immunol Rev 80, 29-76

Brooks, R.F. (1985) The transition probability model: successes, limitations and deficiencies. In Temporal Order (l.Rensing, N.I.Jaeger, eds). Berlin: Springer, 1985

Brooks, R.F. and Riddle, P.N. (1988) Differences in growth factor sensitivity between individual 3T3 cells arise at high frequency: possible relevance to cell senescence. Exp Cell Res 174, 378-387

Bryant, B.J. (1972) Renewal and fate in the mammalian thymus. Mechanisms and inferences of thymokinetics. Eur J Immunol 13, 576-589

Burmer, G.C., Rabionovitch, P.S. and Norwwod, T.H. (1984) Evidence for differences in the mechanism of cell cycle arrest between senescent and serum-deprived human fibroblasts: heterokaryon and metabolic inhibitor studies. J Cell Physiol 118, 97-103

Burnet, F.M. (1962) Role of the thymus and related organs in immunity. Brit Med J. 2, 807-811

Buttyan, R., Zakeri, Z., Lockshin, R., and Wolgemuth,D. (1988) Cascade induction of c-fos, c-myc, and heat shock 70K transcripts during regression of the rat ventral prostrate gland. Molecular Endocrinology 2, 650-657

Carlstedt-Duke, J. and Gustafsson, J-A (1987) Structure and function of the glucucorticoid receptor. J. Steroid Biochemistry 27, 99-104

Cedar, H. (1988) DNA methylation and gene activity. Cell 53, 3-4

Ceredig, R., Glasebrook, A.L. and MacDonald, H.R. (1982) Phenotypic and functional properties of murine thymocytes. I Precursors of cytolytic T lymphocytes and interleukin-2 producing cells are contained within a subpopulation of "mature" thymocytes as analysed by monoclonal antibodies and flow microfluorimetry. J. Exp Med 155, 358-379

Ceredig, R., Dialynas, D.P., Fitch, F.W. and MacDonald, H.R. (1983) Precursors of T-cell growth factor producing cells in the thymus: Ontogeny, frequency and quantitative recovery in a subpopulation of

phenotypically mature thymocytes defined by monoclonal antibody GK1.5. J. Exp Med 158, 1654-1671

Ceredig, R., MacDonald, H.R. and Jenkinson, E.J. (1983) Flow microfluoremetric analysis of mouse thymus development in vivo and in vitro. Eur J. Immunol 13, 185-190

Chen, W.-F., Ewing, T., Scollay, R.and Shortman, K. (1987) Growth of single T cells and single thymocytes in a high efficiency filler-cell free microculture system. Thymus

Chen, B.P.P. and Splitter, G.A. (1983) Transplantation tolerance: Lyt1^{+}2^{-} helper T cells require a second proliferation signal to overcome Lyt1^{-}2^{+} suppressor T cell activity. J Immunol 131, 57-63

Chen, W.-F., Scollay, R. and Shortman, K. (1982) The functional capacity of thymus subpopulations: limit dilution analysis of all precursors of cytotoxic T lymphocytes and all T cells capable of proliferation in subpopulations separated by the use of peanut agglutinin. J. Immunol. 129, 18-24

Cidlowski, J.A. and Cidlowski, N.B. (1981) Regulation of glucocorticoid receptors by glucocorticoids in cultured Hela cells. Endocrinology 109, 1975-1982

Claesson, M.H. and Hartman, N.R. (1976) Cytodynamics in the thymus of young adult mice: A quantative study on the loss of thymic blast cells and non-proliferative small thymocytes. Cell Tissue Kinetics 9, 273-291

Clark, W., Ostergaard, H., Gorman, K. and Torbett, B. (1988) Molecular mechanisms of CTL-medisted lysis: a cellular perspective. Immunol. Rev. 103, 37-52

Claude, P., Hawrot, D.A., Dunis, R.B.,and Campenot, R.B. (1982) Binding, internalization and retrograde transport of ^{125}I-nerve growth factor in cultured rat sympathetic neurons. J Neurosci 2, 431

Cohen, J.J. and Duke, R.C. (1984) Glucocorticoid activation of a calcium-dependent endonuclease in thymocyte nuclei leads to cell death. J. Immunol. 132, 38-42

Cioco, R.F., Bhogal, S. and Thorbecke, G.J. (1983) Relationships of germinal centres in lymphoid tissue to Immunological Memory. J Immunol 131, 2254-2257

Collin, R. (1906) Recherches cytologiques sur le developpement de la cellulue nerveuse. Nevraxe 8, 181-303

Compton, M.M., Lu-Ann, M., Cidlowski, C. and Cidlowski, J.A. (1987) Glucocorticoid action in the immune system. J. Steroid Biochem 27, 201-208

Costlow, M.E., Pui, C.H. and Dahl, G.V. (1982) Glucocorticoid receptors and sensitivity in childhood acute lymphocytic leukaemia. Cancer Res. 42, 4801-4811

Cowan, W.M., Fawcett, J., O'Leary, D. and Stanfield, B. (1984) Regressive events in neurogenesis. Science 225, 1258-1265

171

Cowan, W.M. and Wenger, E. (1968) The development of the nucleus of origin of centrifugal fibers to the retina in the chick. J Comp Neurol 133, 207-240

Crispe, I. N., Shimonkeviotz, R.P., Husmann, L.A., Kimura, J. and Allison, J.P. (1987) Expression of T cell antigen receptor β-chains on subsets of mouse thymocytes. Analysis by three-colour flow cytometry. J. Immunol 139, 3585

Cristani, A., Colantoni, A., Snodgrass, R. and von Boehmer, H. (1986) Expression of T cell receptors by thymocytes: in situ staining and biochemical analysis. EMBO J. 5, 2837

Danielsen, M., Northrop, J.P. and Ringold, G.M. (1986) The mouse glucocorticoid receptor: mapping of functional domains by cloning, sequencing and expression of wild-type and mutant receptor proteins. EMBO J. 5, 2513

Davies, A,M, (1988) Role of neurotrophic factors in development. Trends Genet 4, 139-1144

Denizot, F., Wilson, A., Battye, F., Berke, G. and Shortman, K. (1986) Clonal expansion of T cells: A cytotoxic T-cell response in vivo that involves precursor cell proliferation, P.N.A.S. (USA) 83, 6089-6092

Dennert. G., Hyman, R., Lesley, J. and Trowbridge, A. (1980) Effects of cytotoxic monoclonal antibody specific for T200 glycoprotein on functional lymphoid cell populations. Cell. Immunol. 53, 350-364

Dennert, G., Landon, C. and Nowicki, M. (1988) Cell-mediated and glucucorticoid-mediated cell lysis do not appear to share common pathways. J. Immunol. 141, 785-791

Dennert, G. and Podack, E.R. Cytolysis by H-2 specific T killer cells. Assembly of tubular complexes on target membranes. (1983) J. Exp Med 157, 1483-1495

Dexter, T.M., Garland, J.M., Scott, D., Scolnick, E. and Metcalf, D. (1980) Growth of factor-dependent hemopoietic precursor cell lines. J. Exp. Med. 152, 1032-1047

Dialynas, D.P., Quan, Z.S., Wall, K.A., Pierres, A., Quintas, J., Loken, M.R., Pierres, M. and Fitch, F.W. (1983a) Characterization of the murine T cell surface molecule designated L3T4, identified by monoclonal antibody GK1.5: similarity of L3T4 to the human Leu3/T4 molecule. J. Immunol 131, 2445-1451

Dialynas, D.P.,Wilde, D.B.,Marrack, P., Pierres, A.,Wall, K.A.,Havran, W., Otten, G., Loken, M.R., Pierres, M., Kappler, J.and Fitch, F.W. (1983b) Characterization of the murine antigenic determinant designated L3T4a, recognised by monoclonal antibody GK1.5: Expression of L3T4a by functional T cell clones appears to correlate primarily with class II MHC antigen-reactivity. Immunol Rev. 74, 29-56

Drescher-Lincoln, C.K. and Smith, J.R. (1984) Inhibition of DNA synthesis in senescent-proliferating human cybrids is mediated by endogenous proteins. Exp Cell Res 153, 208-217

172

Duke, R.C., Chervenak, R. and Cohen, J.J. (1983) Endogenous endonuclease-induced DNA fragmentation: An early event in cell-mediated cytolysis. P.N.A.S. (USA) 80, 6361-6365

Duke, R.C. and Cohen, J.J. (1986) IL2 Addiction : Withdrawal of growth factor initiates a suicide program in dependent T cells. Lymphokine Research 5, No. 4, 289-299

Duvall, E. and Wyllie, A.H. (1986) Death and the cell. Immunology Today 7, No.4, 115-9

Duvall, E., Wyllie, A.H. and Morris, R.G. (1985) Macrophage recognition of cells undergoing programmed cell death (apoptosis). Immunology 56, 351-358

Ellis, H.M. and Horvitz, H.R. (1986) Genetic control of programmed cell death in the nematode C. elegans. Cell 44, 817-828

Ernstroem, U. and Larsson, B. (1969) Thymic export of lymphocytes three days after labelling with tritiated thymidine. Nature 222, 279

Ewing, T., Egerton, M., Wilson, A., Scollay, R. and Shortman, K. (1988) Subpopulations of CD4⁻CD8⁻ murine thymocytes: differences in proliferation rate in vivo and proliferative rate in vitro. Eur. J. Immunol 18, 261-268

Farzeneh, F. Zalin, R., Brill, D. and Shall, S. (1982) DNA strand breaks and ADP-ribosyltransferase activation during cell differentiation. Nature 300, 362-366

Fathman, C.G., Small, M., Herzenberg, L.A. and Weissman, I.L. (1975) Thymus call maturation. II. Differentiation of three "mature" subclasses in vivo. Cell Immunol 15, 109

Fink, P.J. and Bevan, M.J. (1978) H-2 antigens of the thymus determine lymphocyte specificity. J. Exp Med 148, 766-775

Finkel, T.H., McDuffie, M., Kappler, J.W., Marrack, P. and Cambier, J.C. (1987) Both immature and mature T cells mobilize Ca^{2+} in response to antigen crosslinking. Nature 332, 179

Finkel, T.H., Marrack, P., Kappler, J.W., Kubo, R.T. and Cambier, J.C. (1989) αβT cell receptor and CD3 transduce different signals in immature T cells. J. Immunol 142, 3006-3012

Foerster,I., Gu, H. and Rajewsky, K. (1989) Repertoire selection in B cell subpopulations. In F. Melchers et al (ed) Progress in Immunology VII, Springer-Verlag, Berlin.

Foerster, I., Vieira, P. and Rajewsky, K (1989) Flow cytometric analysis of cell proliferation dynamics in the B cell compartment of the mouse. Intern Immunol 1, 321-331

Fowlkes, B.J., Edison, L., Mathieson, B.J. and Chused, T.M. (1985) Early T lymphocytes. Differentiaton in vivo of adult intrathymic precursor cells. J. Exp Med 162, 802-822

Fowlkes, B.J. and Mathieson, B.J. (1985) Intrathymic differentiation: Thymocyte heterogeneity and the characterization of early T cell precursors. Surv. Immunol. Res. 4, 96-109

Fowlkes, B.J., Schwartz, R.H. and Pardoll, D.M. (1988) Deletion of self-reactive thymocytes occurs at a CD4+CD8+ precursor stage. Nature 334, 620

Fraker, P.J., Haas, S.M. and Luecke, R.W. (1977) Effect of zinc deficiency on the immune response of the young adult A/J mouse. J. Nutr 107, 1889-1895

Fukushima, Y., Hagiwara, S. and Henkart, M. (1984) Potassium current in clonal cytotoxic T lymphocytes from the mouse. J. Physiol. 351, 645-656

Gaal, J.C., Smith, K.R. and Pearson, C.K. (1987) Cellular euthanasia mediated by a nuclear enzyme: a central role for nuclear ADP-ribosylation in cellular metabolism. TIBS 12, 129-30

Gabrilove, J.L., Jakubowski, A., Scher, H., et al (1988) Effect of granulocyte colony-stimulating factor on neutrophenia and associated morbidity due to chemotherapy for transitional-cell carcinoma of the urothelium. N. Eng. J. Med. 318, 1414-1422

Gallitin, M.W., Weissman, I.L. and Butcher, E.C. (1983) A cell surface molecule involved in organ specific homing of lymphocytes. Nature 304, 30-34

Gasson, J.C. and Bourgeois, S. (1983) New determinants of glucocorticoid sensitivity in lymphoid cell lines. J. Cell Biol 96, 409-415

Gasson, J.C., Ryden, T. and Bourgeois, S. (1983) Role of de novo DNA methylation in the glucocorticoid resistance of a T-lymphoid cell line. Nature 302, 621-623

Gao, E.-K., Lo, D., Cheney, R., Kanagawa, O. and Sprent, J. (1988) Abnormal differentiation of thymocytes in mice treated with cyclosporin A. Nature 336, 176-178

Gey, G.O. and Gey, M.K. (1936) The maintainance of human normal cells in continuous culture. I Preliminary report : cultivation of mesoblastic tumours and normal tissue and notes on methods of cultivation. Amer. J.Cancer 27, 45-52

Giguere, V., Hollenberg, S.M., Rosenfeld, M.G. and Evans, R.M. (1986) Functional domains of the human glucucoticoid receptor. Cell 46, 645-652

Gillis, S. and Smith, K.A. (1977) Long-term culture of tumor-specific cytotoxic T cells. Nature 268, 154-155

Gillis, S., Ferm, M.,M, Ou, W. and Smith, K.A. (1978) T cell growth factor: Parameters of production and a quantitive microassay for activity. J. Immonol. 120, 2027-2031

Glimcher, L.H., Schwartz, R.H., Longo, D.H. and Singer, A. (1982) The specificity of the syngeneic mixed leukocyte response, a primary anti-I

region proliferative response is determined intrathymically. J. Immunol 129, 987-991

Glimcher, L.H. and Shevach, E. (1982) Production of autoreactive I-region restricted T cell hybridomas. J Exp Med 156, 640-645

Glimcher, L.H., Sharrow, S.O. and Paul, W.E. (1983) Serological and functional characterization of a panel of antigen-presenting cell lines expressing mutant I-A class II molecules. J. Exp. Med. 158, 1573-1588

Godowoski, P.J., Rusconi, S., Miesfeld, R. and Yamamoto, K.R. (1987) Glucucorticoid receptor mutants that are constitutive activators of transcriptional activation. Nature 325, 365-368

Goldschneider, I. and McGregor, D.D. (1968) Migration of lymphocytes and thymocyte in rat. II. Circulation of lymphocytes and thymocytes from blood to lymph. Lab Invest 18, 397

Golstein, P. (1987) Cytolytic T-cell melodrama. Nature 327, 12

Golstein, P. and Smith, E.T. (1976) The lethal hit stage of mouse T and non-T cell-mediated cytolysis: differences in cation requirements and characterization of an analytical "cation pulse" method. Eur J Immunol 6, 31

Golstein, P. and Smith, E.T. (1977) Mechanisms of T cell mediated cytolysis: the lethal hit stage. Contemp Top Immunol 4, 273

Good, M.F., Pyke, K.W. and Nossal, G.J.V. (1983) Functional clonal deletion of cytotoxic T-lymphocyte precursors in chimeric thymus produced in vitro from embryonic Anlagen. P.N.A.S. (USA) 80, 3045-3049

Goodman, C.S. and Bate, M. (1981) Neuronal development in the grasshopper. TINS July 1981, 163-169

Goodnow, C.C., Crosbie, J., Afelstein, S., Lavoie, T.B., Smith-Gill, S.J., Brink, R.A., Pritchard-Briscoe, H., Wotherspoon, J.S., Loblay, R.H., Raphael, K., Trent, R.J. and Basten, A. (1988) Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. Nature 334, 676

Gray, D., MacLennan, I.C.M. and Lane, P.J.L. (1986) Virgin B cell recruitment and the lifespan of memory clones during antibody responses to 2, 4-dinitrophenylhemocyanin. Eur J Immunol 16, 641-648

Greenberg, M.E., Hermanowski, A.L. and Ziff, E.B. (1985) Effect of protein synthesis inhibitors on growth factor activation of c-fos, c-myc and actin gene transcription. Mol Cell Biol 6, 1050-57

Gray, D. and Skarvall, H. (1988) B cell memory is short lived in the absence of antigen. Nature 329, 445-447

Gromkowski, S., Brown, T.C., Cerutti, P.A. and Cerottini, J-C, (1986) DNA of human Raji target cells is damaged upon lymphocyte-mediated lysis. J. Immunol. 136, 752-756

Gromkowski, S.H., Brown, T.C., Masson, D. and Tschopp, J. (1988) Lack of DNA degradation in target cells lysed by granules derived from cytolytic T lymphocytes. J. Immunol. 141, 774-778

Groudine, M., Eisenman, R. and Weintraub, H. (1981) Chromatin structure of endogenous retroviral genes and activation by an inhibitor of DNA methylation. Nature 292, 311-317

Gustafsson, J-A, Carlstedt-Duke, J., Poellinger, L., Okret, S., Wikstrom, A-C, Broennegard, M., Gillner, M., Dong, Y., Fuxe, K., Cintra, A., Haerstrand, A. and Agnati, L. (1987) Biochemistry, Molecular Biology, and Physiology of the Glucucorticoid Receptor. Endocrine Reviews 8, 185-234

Hallick, R.B., Chelm, B.K., Gray, P.W. and Orcozo, E.M. (1977) Use of aurintricarboxylic acid as an inhibitor of nucleases during nucleic acid isolation. Nucleic Acids Research 4, No.9, 3055-63

Hamburger, V. (1958) Regression versus peripheral control of differentiation in motor hyperplasia. Am J Anat 102, 365-410

Hamburger, V. (1975) Cell death in the development of the lateral motor column of the chick embryo. J Comp Neurol 160, 535-46

Hamburger, V., Brunso-Brechtold, J.K and Yip, J.W. (1981) Neuronal death in the spinal ganglia of the chick embryo and its reduction by nerve growth factor. J Neurosci 1, 60-70

Hamburger, V. and Levi-Montalcini, R. (1949) Proliferation, differentiation and degeneration in the spinal ganglia of the chick embryo under normal and experimental conditions. J. Exp Zool. 12, 268-84

Hameed, A., Lowrey, D.M., Lichtenheld, M. and Podack, E.R. (1988) Characterization of three serine esterases isolated from human IL-2 activated killer cells. J Immunol 141, 3142-3147

Hameed, A., Olsen, K.J., Lee, M-K, Lichtenheld, M.G. and Podack, E.R. (1989) Cytolysis by Ca-permeable transmembrane channels. Pore formation causes extensive DNA degradation and cell lysis. J. Exp. Med. 169, 765-778

Hanahan, D. (1985) Techniques for transformation of E. Coli. In DNA cloning volume 1. Ed. D.M. Glover. IRL press (Oxford, Wsahington D.C.)

Havran, W.L., Poenie, M., Kimura, J., Tsien, R., Weiss, A. and Allison, J.P. (1987) Expression and function of the CD3-Ag receptor on murine CD4$^+$CD8$^+$ thymocytes. Nature 300, 170-173

Hayflick, L. (1965) The limited in vitro lifetime of human diploid cell strains. Exp. Cell Res. 37, 614-636

Hedgecock, E., Sulston, J.E. and Thomson, N. (1983) Mutations affecting programmed cell deaths in the nematode Caenorhabditis elegans. Science 220, 1277-1280

Hendry, I.A. and Campbell, J. (1976) MOrphometric analysis of rat superior ganglion after axotomy and nerve grpwth factor treatment. J Neurocyt 5, 351-360

Henkart, P.A., Millard, P.J., Reynolds, C.W. and Henkart, M.P. (1984) Cytolytic activity of purified cytoplasmic granules from cytotoxic rat large granular lymphocyte tumors. J Exp Med 160. 75-93

Hentgartner, H., Odermatt, B., Schneider, R., Schreyer, M., Walla, G., MacDonald, H.R., Zinkernagel, R.H. (1988) Deletion of self-reactive cells prior to entering the thymus medulla. Nature 336, 338

Hoel, P.G. (1976) Elementary Statistics 4th Edition. pp190-194. John Wiley and sons Inc, New York.

Hollenberg, S.M., Weinberger, C., Ong, E.S., Cerelli, G., Lebo, R., Thompson, E.B., Rosenfeld, M.G. and Evans, R.M. (1985) Primary structure and expression of a functional human glucucorticoid receptor cDNA. Nature 318, 635-641

Holliday, R. (1984) Th unsolved problem of cellular ageing. Monogr. Develop Biol 17, 60-77

Holliday, R. (1986) The concept of error accumulation, In Genes, proteins and cellular aging, R. Holliday Ed. Van Nostrand Reinhold Company, New York.

Hollyday, M. and Hamburger, V. (1976) Reduction of naturally occurring motor neuron loss by enlargement of the periphery. J Comp Neurol 170, 311-20

Hopper, K. and Shortman, K. (1976) The differentiation of T lymphocytes.III.The behaviour of subpopulations of mouse thymus cells in short-term culture. Cell Immunol 27, 256

Horvitz, H.R. (1988) Genetics of cell lineage pp183-186, in W.B. Wood ed., The nematode Caenorhabditis elegans (Cold spring harbour laboratory, Cold spring harbour, NY)

Horvitz, H.R., Ellis, H.M. and Sternberg, P.W. (1982) Programmed cell death in nematode development. Neurosci Comment 1, 56-65

Howard, J.G. and Mitchison, N.A. (1975) Immunological tolerance. In: Progress in Allergy, eds P.Kallos, B.H.Waksman and A. de Weck. S. Karger, Basel

Howe, R.C. and MacDonald, R.H. (1989) Clonogenic potential of murine CD4+CD8+ thymocytes. J. Immunol 143, 793-797

Howell, D.M. and Martz, E. (1987a) The degree of CTL-induced DNA solubilization is not determined by the human vs mouse origin of the target cell. J. Immunol. 138, 3695-3698

Howell and Martz (1987b) Intracellular roevirus survives cytotoxic T lymphocyte-mediated lysis of its host cell. J. gen. Virol. 68, 2899-2907

Howell, D.M. and Martz, E. (1988) Nuclear disintegration induced by cytotoxic T lymphocytes. Evidence against damage to the nuclear envelope of the target cell. J. Immunol. 140,

Ijiri, K. and Potten, C.S. (1983) Response of intestinal cells of differing topographical and hierarchical status to ten cytotoxic drugs and five sources of radiation. Br J. Cancer 47, 175-185

Iscove, N.N. and Melchers, F. (1978) Complete replacement of serum by albumin, transferrin, and soybean lipid in cultures of lipopolysaccharide-reactive B lymphocytes. J. Exp. Med. 147, 923-33

Ishii, N., Baxevanis, C.N., Nagy, Z. and Klein, J. (1981) Responder T cells depleted of alloreactive cells react to antigen presented on allogeneic macrophages from non-responder strains. J. Exp Med 154, 978-982

Jaattela, M., Saksela, K. and Saksela, E. (1989) Heat shock protects WEHI-164 target cells from the cytolysis by tumour necrosis factors α and β. Eur. J. Immunol. 19, 1413-1417

Jerne, N.K. (1966) Antibody formation and immunological memory. In Gaito, J. (ed) Macromolecules and Behaviour, York University, Toronto, pp151-157

Jiang, S., Peresechini, P.M., Perussia, B. and Toung, J. D-E. (1989) Resistance of cytotoxic lymphocytes to perforin-mediated killing. J. Immunol. 143, 1453-1460

Joel, D.D., Chanana, A.D., Coltier, H., Cronkite, G.P. and Laissue, J.A. (1977) Fate of thymocytes: studies with ^{125}I-iododeoxyuridine and ^{3}H-thymidine in mice. Cell tissue Kinetics 10, 57-69

Johnson, T.E. (1987) Aging can be genetically dissected into component processes using long-lived lines of caenorhabditis elegans. P.N.A.S. (USA) 84, 3777-3781

Johnson, E.M., Gorin, P.D., Brandeis, L.D. and Pearson, J. (1980) Dorsal root ganglion neurons are deatroyed by exposure in utero to maternal antibody to nerve growth factor. Science 210, 916-918

Johnson, T.E. and Wood, W.B. (1982) Genetic analysis of life-span in Caenorhabditis elegans. P.N.A.S. (USA) 79, 6603-6607

Johnson, E.M. and Yip, H.K. (1985) Central nervous system and peripheral nerve growth factor trophic support critical to mature sensory neuronal survival. Nature 314, 751-752

Kalinyak, J.E., Dorin, R.I., Hoffman, A.R. and Perlman, A.J. (1987) Tissue-specific regulation of glucucorticoid receptor mRNA by dexamethasone. J. Biol. Chem. 262, 10441-10445

Kappler, J.W., Wade, T., White, J., Kushnir, E., Blackman, M., Bill, J., Roehm, N. and Marrack, P. (1987a) A T cell receptor Vβ segment that imparts reactivity to a class II major histocompatability complex product. Cell 49, 263-271

Kappler, J.W., Roehm, N. and Marrack, P. (1987b) T cell tolerance by clonal elimination in the thymus. Cell 49, 273

Kappler, J.W., Staerz, U., White, J.and Marrack, P. (1988) Self tolerance eliminates T cells specific for Mls-modified products of the major histocompatability complex. Nature 332, 35

Kenyon, C. (1988) The nematode Caenorhabditis elegans. Science 240, 1448-1453

Kerr, J.F.R. (1971) Shrinkage necrosis: a distinct mode of cellular death. J. Pathol 105, 13-20

Kerr, J.F.R., Harmon, B. and Searle, J. (1974) An electron-microscope study of cell depletion in the auran tadpole tail during spontaneous metamorphosis with special reference to apoptosis of striated muscle fibres. .
ell Sci 14, 571-585

Kerr, J.F.R., Searle, J., Harmon, B.V. and Bishop, C.J. (1987) Apoptosis, in Perspectives on Mammalian Cell Death (ed. Potten, C.S.) pp93-128. Oxford University Press

Kety, S.S. and Schmidt, C.F. (1948) The effects of altered arterial tensions of crbon dioxide and oxygen on cerebral blood flow and cerebral oxygen consumption of normal young men. J Clin Invest 27, 484-92

Kindred, B. (1978) Functional activity of T-cells which differentiate from nude mouse precursors in a congenic or allogenic thymus graft. Immunol Rev 42 60-75

Kirkwood, T.B.L. and Holliday, R. (1979) The evolution of ageing and longevity, Proc. R. Soc. 205B, 531-546

Kisielow, P., Hirst, J.A., Shiku, H., Beverley, P.C.L., Hoffman, M.K., Boyse, E.A. and Oettgen, H.F. (1975) Ly antigens as markers for functionally distinct subpopulations of thymus-derived lymphocytes in the mouse. Nature 253, 219-220

Kisielow, P., Leiserson, W. and von Boehmer, H. (1984) Differentiation of thymocytes in foetal organ culture: Analysis of phenotypic changes accompanying the appearance of cytolytic and interleukin 2-producing T cellsJ. Immunol 133, 1117-1123

Kisielow, P. Bluthman, H., Staerz, U.D., Steinmetz, M. and von Boehmer, H. (1988a) Tolerance in T cell receptor transgenic mice involves deletion of nonmature CD4$^+$CD8$^+$ thymocytes. Nature 333, 742

Kisielow, P., Teh, H.S., Bluthman, H. and von Boehmer, H. (1988b) Positive selection of antigen-specific T cells in thymus by restricting MHC molecules. Nature 335, 728

Kizaki, H., Tadakuma, T., Odaka, C., Muramatsu, J. and Ishimura, Y. (1989) Activation of a suicide process of thymocytes through DNA fragmentation by calcium ionophores and phorbol esteres. J. Immunol 143, 1790-1794

Kocks, C. and Rajewsky, K. (1988) Stepwise intraclonal maturation of antibody affinity through somatic hypermutation, P.N.A.S. (USA) 85, 8206-8210

Koike, T., Martin, D,P, and Johnson, E.M. (1989) Role of Ca2+ channels in the ability of membrane depolarization to prevent neuronal death induced by trophic-factor deprivation: evidence that levels of internal Ca2+ determine nerve growth factor dependence of sympathetic ganglion cells. P.N.A.S. (USA) 86, 6421-6425

Kranz, D.M. and Eisen, H.N. (1987) Resistance of cytotoxic T lymphocytes to lysis by a clone of cytotoxic T lymphocytes. P.N.A.S. (USA) 84, 3375

Kreisman, N.R., Olsen, J.E., Horne, D.S. and Holzman, D. (1986) Developmental increases in oxygen delivery and extraction in immature rat cerebral cortex. Neurosci Abstr 12, 451

Kronenberg, M., Siu, G., Hood, L.E. and Shastri, N. (1986) The molecular genetics of the T-cell antigen receptor and T-cell recognition. Annu Rev Immunol 4, 529-592

Kruisbeek, A.M., Hodes, R.J.and Singer, A. (1981) Cytotoxic T lymphocyte responses in chimeric thymocytes: Self-recognition is determined early in T cell development. J Exp Med 153, 13-26

Kupfer, A. and Dennert, G. (1984) Reorientation of the microtubule organizing center and the Golgi apparatus in cloned cytotoxic lymphocytes triggered by binding to lysable target cells. J. Immunol. 133, 2762

Kupfer, A., Dennert, G. and Singer, S.J. (1985) The reorientation of the Golgi apparatus and the microtubule-organizing center in the cytotoxic effector cell is a prerequisite in the lysis of bound target cells. J. Mol Cell Immunol. 2, 37-49

Kyewski, B.A., Schirrmacher, V. and Allison, J.P. (1989) Antibodies against the T cell receptor/CD3 complex interfere with distinct intra-thymic cell-cell interaction in vivo: correlation with arrest of T cell differentiation. Eur J. Immunol , 857-863

La Pushin, R.W. and de Harven, E. (1971) A study of glucocorticoid induced pyknosis in the thymus and lymph node of the adrenalectomised rat. J. Cell Biol 50, 583-97

Laissue, J.A., Chanana, A.D., Cottier, H., Cronkite, E.P. and Joel, D.D. (1976) The fate of thymic radioactivity after local labelling with 125-Iododeoxyuridine. Blood 27, 511

Lake, J.P., Andrew, M.E., Pierce, C.W. and Braciale, T.J. (1980) Sendai virus-specific, H-2-restricted cytotoxic T lymphocyte responses of nude mice grafted with allogeneic or semi-allogeneic thymus glands. J. Exp Med 152, 1805-1810

Lancki, D.W., Kaper, B.P. and Fitch, F.W. (1989) The requirements for triggering of lysis by cytolytic T lymphocyt cloned II cyclosporin A inhibits TCR-mediated exocytosis but only selectively inhibits TCR-mediated lytic activity by cloned CTL. J Immunol 142, 416-424

Land, H., Parada, L.F. and Weinberg, R.A. (1983) Tumorigenic conversion of primary embryo fibroblasts requires at least two coopertaing oncogenes. Nature 304, 596-602

Landmesser, L. and Pilar, G. (1974) Synapse formayion during embryogenesis on ganglion cells lacking a periphery. J Physiol <u>241</u>, 715

Laster, S.M., Wood, J.G. and Gooding, L.R. (1988) Tumor necrosis factor can induce both apoptotic and necrotic forms of cell lysis. J. Immunol. <u>141</u>, 2629-2634

Lau, L.F. and Nathans, D. (1986) Expression of a set of growth-related immediate early genes in BALB/c 3T3 cells : coordinate regulation with c-fos or c-myc. P.N.A.S. (USA) <u>84</u>, 1182-86

Lawton, A.R. and Cooper, M.D. (1974) Modification of B lymphocyte differentiation by anti-Ig. Contemp Topics Immunobiol <u>3</u>, 193-225

Leibrock, J., Lottspeoch, F., Hohn, A., Hofer, M., Hengerer, B., Masiakowski, P., Thoenen, H. and Barde, Y.-A. (1989) Molecular cloning and expression of brain-derived neurotrophic factor. Nature <u>341</u>, 149-152

Leo, O., Foo, M., Sachs, D,H, Samelson, L.H. and Bluestone, J.A. (1987) Identification of a monclonal antibody specific for a murine T3 polypeptide. P.N.A.S. (USA) <u>84</u>, 1374-1378

Levi-Montalcini, R.(1987) The Nerve Growth Factor 35 years later. Science <u>237</u>, 1154-1162

Levi-Montalcini, R. and Booker, B. (1960) Destruction of the sympathetic ganglion in mammals by an antiserum to a nerve-growth protein. P.N.A.S. (USA) <u>46</u>, 384-391

Linton, P.-J. and Klinman, N.R. (1989) Self-nonself discrimination by precursors of memory B cells In F. Melchers et al (ed) Progress in Immunology VII, Springer-Verlag, Berlin.

Lipman, M. and Barr, R. (1976) Glucocorticoid receptors in purified subpopulations of peripheral blood lymphocytes. J. Immunol. <u>118</u>, 1977-1981

Lipman, M.E., Yarbro, G.K. and Levethal, B.G. (1978) Clinical implications of glucocorticoid receptor studies in acute lymphoblastic leukaemia. Cancer Res. <u>38</u>, 4251-4256

Loo, D.T., Fuquay, J.I., Rawson, C.L. and Barnes, D.W. (1987) Extended culture of mouse embryo cells without senescence: inhibition by serum. Science <u>236</u>, 200-202

Lowrey, D.M., Aebischer, T., Olsen, K., Lichtenheld, M., Rupp, F., Hengartner, H. and Podack, E.R. (1989) Cloning, analysis, and expression of murine perforin 1 cDNA, a component of cytolytic T-cell granules with homology to complement component C9. P.N.A.S. (USA) <u>86</u>, 247-251

Lui, Y.-J., Joshua, D.E., Williams, G.T., Smith, C.E., Gordon, J. and MacLennan, I.C.M. (1990)

Lui, Y.-J., Oldfield, S. and MacLennan, I.C.M. (1988) Memory B cells in T cell dependent antibody responses colonize the splenic marginal zones. Eur J Immunol 18, 355

Lui, C.-C., Steffen, M., King, F. and Young, J., D.-E. (1987) Identification, isolation, and characterization of a novel cytotoin in murine cytotoxic lymphocytes. Cell 51, 393-403

Lumpkin, C.K., McClung, J.K., Pereira-Smith, O.M. and Smith, J.R. (1986) Existemce of high abundance antiproliferative mRNA's in senescent human diploid fibroblasts. Science 232, 393-395

Lynch, M.P., Nawaz, S. and Gerschenson, L.E. (1986) Evidence for soluble factors regulating cell death and cell proliferation in primary cultures of rabbit ondometrial cells grown on collagen. P.N.A.S. (USA) 83, 4784-4788

MacClennan, I.C.M. and Gray, D. (1986) Antigen-driven selecyion of virgin and memory B cells. Immunol Rev 91, 61

MacClennan, I.C.M., Liu, Y.-J., Joshua, D.E. and Gray, D. (1989) The production and selection of memory B cells in follicles. In F. Melchers et al (ed) Progress in Immunology VII, Springer-Verlag, Berlin.

MacDonald, H.R., Phillips, R.A. and Miller, R.G. (1973) Allograft immunity in the mouse. I Quantitation and specificity of cytotoxic effector cells after in vitro sensitization. J. Immunol. 111, 565

MacDonald, H.R., Hentgartner, H. and Pedrazzini, T. (1988a) Intrathymic deletion of self reactive cells prevented by neonatal anti-CD4 antibody treatment. Nature 335, 174

MacDonald, H.R., Budd, R.C. and Howe, R.C. (1988b) A CD3⁻ subset of CD4⁻ 8⁺ thymocytes: a rapidly cycling intermediate in the generation of CD4⁺CD8⁺ cells. Eur. J. Immunol. 18, 519-523

MacDonald, H.R., Less, R.K., Schhneider, R., Zinkernagel, R.M. and Hentgartner, H. (1988d) Positive selection of CD4⁺ thymocytes controlled by MHC classII gene products. Nature 336, 468

MacDonald, H.R., Glasebrook, A.L., Schneider, R., Lees, R.K., Pircher, H., Pedrazzini, T., Kanagawa, O., Nicolas, J.-P., Howe, R.C., Zinkernagel, R.M. and Hengartner, H. (1989) T-cell reactivity and tolerance to Mls[a]-encoded antigens. Immunol Rev 107, 89-108

MacDonald, H.R., Pedrazzini, T., Schneider, R., Louis, J., Zinkernagel, R.,M. and Hentgartner, H. (1988c) Intrathymic elimination of Mls[a]-reactive (Vβ6+) cells during neonatal tolerance induction to Mls[a]-encoded antigens. J. Exp Med 167, 2005

MacDonald, H.R., Schneider, R., Lees, R.K., Howe, R.C., Acha-Orbea, H., Festenstein, H., Zinkernagel, R.M. and Hentgartner, H. (1988e) T cell receptor Vβ use predicts reactivity and tolerance to Mls[a]-encoded antigens. Nature 332, 40

Main, E.K., Lampson, L.A., Hart, M.K., Kornbluth, J. and Wilson, D.B. (1985) Human neuroblastoma cell lines are susceptible to lysis to natural killer cells but not by cytotoxic T lymphocytes. J. Immunol. 135, 242

Main, E.K., Monos, D.S. and Lampson, L.A. (1988) IFN-treated neuroblastoma cell lines remain resistant to T cell-mediated allo-killing, and susceptible to non-MHC-restricted cytotoxicity. J. Immunol. 141, 2834-2950

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular cloning: A laboratory manual. (Cold Spring Harbor, New york: Cold Spring Harbor Laboratory)

Martin, R.D. (1981) Relative brain size and basal metabolic rate in terrestrial vertebrates. Nature 293, 57-60

Martin, D.P., Schmidt, R.E., DiStefano, P.S., Lowry, O.H., Carter, J.G. and Johnson, E.M., (1988) Inhibitors of protein synthesis and RNA synthesis prevent neuronal death caused by nerve growth factor deprivation. J. Cell Biol 106, 829-844

Martin, G.M., Sprague, C.A. and Epstein, C.J. (1970) Replicative lifespan of cultivated human cells: effects of donor's age, tissue and genotype. Lab Invest 23, 86-92

Martz, E. and Howell, D.M. (1989) CTL: virus control cells first and cytolytic cells second? DNA fragmentation, apoptosis and the prelytic halt hypothesis. Immunology Today 10, 79-86

Maryanski, J.L., Van Snick, J., Cerottini, J.C. and Boon, T. (1982) Immunogenic variants obtained by mutagenesis of mouse mastocytoma P815. III. Clonal analysis of syngeneic cytolytic T lymphocyte response. Eur. J. Immunol. 12, 401

Masson, D. and Tschopp, J. (1985) Isolation of a lytic, pore-forming protein (perforin) from cytolytic T-lymphocytes. J Biol Chem 260, 9069-9072

Masson, D. and Tschopp, J. (1987) A family of serine esterases in lytic granules of cytolytic T lymphocytes, Cell 49, 679-685

Mastrangelo, R., Malandrino, R., Riccardi, R., Longo, P., Ranaletti, F.O. and Jacobelli, S. (1980) Clinical implications of glucocorticoid receptor studies in acute lymphoblastic leukaemia. Blood 56, 1036-1040

Mathieson, B.J., Sharrow, S.O., Campbell, P.S. and Asofsky, R. (1979) An Lyt differentiated thymocyte subpopulation detected by flow microfluorimetry. Nature 277, 478-480

Matsuyama, M., Wiadrowski, M.N. and Metcalf, D. (1966) Autoradiographic analysis of lymphopoiesis and lymphocyte migration in mice bearing multiple thymus grafts. J. Exp Med 123, 559

Matzinger, P. and Mirkwood, G. (1978) In a fully H-2 incompatible chimera, T cells of donor origin can respond to minor histocompatability antigens in association with either donor or host H-2 type. J. Exp Med 147, 84-92

McConkey, D.J., Hartzell, P., Duddy, S.K., Hakansson, H. and Orrenius, S. (1988) 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) kills immature thymocytes by Ca^{2+}-mediated endonuclease activation. Science 242, 256-259

McConkey, D.,J., Hartzell, P., Nicotera, P. and Orrenius, S. (1989) Calcium-activated DNA fragmentation kills immature thymocytes. FASEB 3,1843-1849

McConkey, D.J., Nicotera, P., Hartzell, P., Bellomo, G., Wyllie, A.H. and Orrenius, S. (1989) Glucocorticoids activate a suicide process in thymocytes through an increase in cytosolic Ca^{2+} concentration. Arch Biochem. Biophys 269, 38-42

McKearn, J.P. and Rosenberg, N. (1985) Mapping cell surface antigens on mouse pre-B cell lines. Eur J Immunol 15, 295-298

McDuffie, M., Born, W., Marrack, P. and Kappler, J. (1986) The role of the T-cell receptor in thymocyte maturation: Effects in vivo of anti-receptor antibody. P.N.A.S. (USA) 83, 8728-8732

McIntyre, W.R. and Samuels, H.H. (1985) Triamicolone acetonide regulates glucocorticoid receptor levels by decreasing the half-life of the activated nuclear receptor form. J. Biol. Chem. 260, 418-427

McPhee, D., Pye, J. and Shortman, K. (1979) The differentiation of T lymphocytes. V. Evidence for intrathymic death of most thymocytes. Thymus 1, 151-162

Medawar, P.B. (1952) An unsolved problem in biology, Lewis, London (reprinted in "The uniqueness of the individual", 1957, Methuen, London).

Melchers, F., Erdei, A., Schulz, T. and Dierich, M. (1985) Growth control of activated synchronized murine B cells by the C3d fragment of human complement. Nature 317, 264-66

Mercep, M., Noguchi, P.D. and Ashwell, J.D. (1989) The cell cycle block and lysis of an activated T cell hybridoma are distinct processes with different ca2+ requirements and sensitivity to cyclosporin A. J. Immunol 142, 4085-4092

Mercap, M., Weissman, A.M., Frank, S.J., Klausner, R.D. and Ashwell, J.D. (1990) Activation-driven programmed cell death and T cell receptor $\zeta\eta$ expression. Science 246, 1162-1165

Metcalf, D. (1966) In: The Thymus: Experimental and Clinical Studies, Ciba Foundation Symposium (G.E.W. Wilstenholme and R. Porter eds) p. 242 J.A Churchill Ltd., London

Metcalf, D. (1970) Studies on colony formation in vitro by mouse bone marrow cells. II. Action of colony stimulating factor. J. Cell. Physiol. 76, 89-100

Metcalf, E.M. and Klinman, N.R. (1977) In vitro tolerance induction of bone marrow cells: a marker for B cell maturation. J Immunol 118, 2111-2116

Metcalf, D. and Wiadrowski, M. (1966) Autoradiographic analysis of lymphocyte proliferation in the thymus and in thymic lymphoma tissue. Cancer Research 26, 235

Michalke, W.D., Hess, M.W., Riedwyl, H., Stoner, R.D. and Cottier, H. (1969) Thymic lymphopoiesis and cell loss in new-born mice. Blood 33, 541

Miesfeld, R., Rusconi, S., Godowski, P.J., Maler, B.M., Okret, S., Wikstroem, A-C, Gustafsson, J-A and Yamamoto, K.R. (1986) Genetic complementation of a glucocorticoid receptor deficiency by expression of cloned receptor cDNA. Cell 46, 389

Milarski, K.L. and Morimoto, R.I. (1986) Expression of human Hsp70 during the synthetic phase of the cell cycle. P.N.A.S. (USA) 83, 9517-9521

Miovic, M.L. and Pizer, L.I. (1979) Characterization of RNA synthesized in isolated nuclei of herpes simplex virus type-1 infected cells. J. Virol. 33, 567-70

Mishell, B.B. and Shiigi, S.M. (1980) Selected methods in cellular immunology. (W.H.Freeman & Co, San Francisco)

Moller, G. and Sjoberg, O. (1970) Effect of antigenic competition on antigen-sensitive cells and on adoptively transferred immunocompetent cells. Cell. Immunol. 1, 110

Morgan, B.P., Luzio, J.P. and Campbell, A.K. (1986) Intracellular Ca^{++} and cell injury: a paradoxical role of Ca^{++} in complement membrane attack. Cell Calcium. 7, 399-411

Morris, R.G., Hargreaves, A.D., Duvall, E. and Wyllie, A.H. (1984) Hormone-induced cell death 2. Surface changes in thymocytes undergoeing apoptosis. Am J. Pathol 115, 426-36

Mougneau, E., Lemieiux, L., Rassouldegan, M. and Cuzin, F. (1984) Biological activities of v-myc and rearranged c-myc oncogenes in rat fibroblasts cells in culture. P.N.A.S. (USA) 81, 5758-5762

Muller-Eberhard, H.J. (1988) The molecular basis of target cell killing by human lymphocytes and of killer cell self-protection. Immunol Rev. 103, 87-98

Murray, R.G. and Woods, P.A. (1964) Studies on the fate of lymphocytes. The migration and metamorphosis of in situ labeled thymic lymphocytes. Anat. Rec. 150, 113

Nabholz, M. and MacDonald, H.R. (1983) Cytolytic T lymphocytes. Ann Rev Immunol 1, 273-306

Nemazee, D. and Buerki, K. (1989c) Are autoreactive B cells deleted? In F. Melchers et al (ed) Progress in Immunology VII, Springer-Verlag, Berlin.

Nemazee, D. and Buerki, K. (1989a) Clonal deletion of B lymphocytes in a transgenic mouse bearing anti-MHC class I antibody genes, Nature 337, 562

Nemazee, D. and Buerki, K. (1989b)Clonal deletion of autoreactive B lymphocytes in bone marrow chimeras.   P.N.A.S. (USA) 86, 8039-8043

Nguyen, T.T. and Nabholz, M. (1985) Somatic cell and molecular genet. 11, 593

Nophar, Y., Holtman, H., Ber, R. and Wallach, D. (1988) Dominance of resistance to the cytocidal effect of tumor necrosis factor in heterokaryons formed by fusion of resistant and sensistive cells. J. Immunol. 140, 3456-3460

Norton, P.M. (1989) The Mammalian Heat shock protein: expression and functional role.   PhD   thesis, University of London, 1989.

Norwood, T.H., Pendergras, W.R., Sptague, C.A. and Martin, G.M. (1974) Dominance of the senescent phenotype in heterokaryons between replicative and post-replicative human fibroblast-like cells.   P.N.A.S. (USA) 71, 2231-2235

Nossal, G.J.V. (1964) Studies on the rate of seeding of lymphocytes from the intact guinea pig thymus. Ann New York Acad Sc 120, 171

Nossal, G.J.V. (1983) Cellular mechanisms of immunological tolerance. Ann Rev Immunol 1, 33

Okret, S., Poellinger, L., Dong, Y. and Gustafsson, J-A (1986) Down-regulation of glucucorticoid receptor mRNA by glucucorticoid hormones and recognition by the receptor of a specific binding sequence within a receptor cDNA clone. P.N.A.S. (USA) 83, 5899-5903

Okret, S., Wikstroem, A-C, Gustafsson, J-A and Yamamoto, K.R. (1986) Genetic complementation of a glucucorticoid receptor deficiency by expression of a cloned DNA. Cell 46, 389

Oppenheim, R.W., Chu-Wang, I.-W. and Maderdrut, J.L. (1978) Cell death of motor neurons in the chick embryo spinal cord. III The differentiation of motorneurons prior to their induced degeneration following limb-bud removal. J Comp Neurol 177, 82-112

Oppenheim, R.W., Haverkamp, L.J., Prevette, D., McManaman, J.L. and Appel, S,H, (1988) Reduction of naturally occurring motoneuron death in vivo by a target-derived neurotrophic factor. Science, 240, 919-922

Oppenheim, R.W. and Prevette, D.M. (1988) Reduction of naturally occuring neuronal death in vivo by the inhibition of protein and RNA synthesis.   Soc. Neurosci. Abstract. 14, 368

Opstelten, D. and Osmond, D.G. (1983) Pre-B cells in mouse bone-marrow: Immunofluorescence ststmokinetic studies of the proliferation of cytoplasmic m-chain bearing cells in normal mice.   J Immunol 131, 2635-2640

Orgel, L.E. (1973) Ageing of clones of mammalian cells.   Nature 243, 441-445

Ostergaard, H.L. and Clark, W.R. (1987) The role of Ca^{2+} in activation of mature cytotoxic T lymphocytes for lysis. J. Immunol. 139, 3573-3579

Ostergaard, H.L. and Clark, W.R. (1989) Evidence for multiple lytic pathways used by cytotoxic T lymphocytes. J. Immunol. 143, 2120-2126

Ostergaard, H.L., Kane, K.P., Mescher, M.F. and Clark, W.F. (1987) Cytotoxic T cell mediated lysis without release of serine esterase. Nature 330, 71-72

Owen, J.J.T., Jenkinson, E.J., Kingston, R., Williams, G.T. and Smith, C.A. (1989) The role of the thymus in the development of T cells. In F. Melchers et al (ed) Progress in Immunology VII, Springer-Verlag, Berlin.

Owen, J.J.T., Owen, M.J., Williams, G.T., Kingston, R., Jenkinson, E.J. (1988) The effects of anti-CD3 antibodies on the development of T-cell receptor $\alpha\beta^+$ lymphocytes in embryonic thymus organ cultures. Immunology 63, 639-642

Owen, J.J.T., Wright, P.E., Habu, S., Raff, M.C. and Cooper, M.D. (1977) Studies on the generation of B lymphocytes in fetal liver and bone marrow. J. Immunol 118, 2067]

Palmatier, M.A., Hartman, B.K.,and Johnson, E.M. (1984) Demonstration of retrogradely transported endogenous nerve growth factor in axons of sympathetic neurons. J Neurosci 4, 751

Pardee, A.B. (1974) A restriction point for control of normal animal cell proliferation. P.N.A.S. (USA) 71, 1286-1290

Pardoll, D., Fowlkes, B.J., Bluestone, J.A., Kruisbeek, A., Maky, W.L., Coligan, J.E. and Schwartz, R.H. (1987) Differential expression of two distinct T-cell receptors during thymocyte development. Nature 326, 79-81

Paterson, D.J. and Williams, A.F. (1987) An intermediate cell in thymocyte differentiation that expresses CD8 but not CD4 antigen. J. Exp Med 166, 1603-1609

Pereira-Smith, O.M., Fisher, S.F. and Smith, J.R. (1985) Senescent and quiescent cell inhibitors of DNA synthesis. Membrane-associated proteins. Exp Cell Res 160, 297-306

Peters, P.J., Geuze, H.J., Van der Donk, H.A., Slot, J.A., Griffith, J.M., Stam, N.J., Clevers, H.C. and Borst, J. (1989) Molecules relevant for T cell-target cell interaction are present in cytolytic granules of human T lymphocytes. Eur. J. Immunol. 19, 1469-1475

Pittman, R. and Oppenheim, R.W. (1979) Cell death of motoneurons in the chick embryo spinal cord. J Comp Neur 187, 425-446

Podack. E.R. (1986) Molecular mechanisms of cytolysis by complement and cytolytic lymphocytes. J. Cell Biochem 30, 133-170

Podack, E.R. (1989) Killer lymphocytes and how they kill. Current Opinion in Cell Biology 1, 929-933

Podack, E.R. and Dennert, G. (1982) Assembly of two types of tubules with putative cytolytic function by cloned natural killer cells. Nature 302, 442-445

Podack, E.R. and Konisberg, P.J. (1984) Cytolytic T cell granules. Isolation, Structural, Biochemical and Functional characterisation. J. Exp Med 160, 695-710

Poste, M.E. and Olsen, I.A. (1973) An investigation of the sites of mitotic activity in the guinea-pig thymus using autoradiography and colcemid-induced mitotic arrest. Immunology 24, 691-697

Pratt, R.B. and Greene, R.M. (1976) Inhibition of palatal epithelial cell death by altered protein synthesis. Dev. Biol. 54, 135-145

Raff, M.C., Megson, M., Owen, J.J.T. and Cooper, M.D. (1976) Early production of intracellular IgM by B lymphocyte precursors in mouse. Nature 259, 224-226

Rammensee, H.-G., Kroschewski, R. and Frangoulis, B. (1989) Clonal anergy induced in mature Vβ6$^+$ T lymphocytes on immunizing Mls-1^a mice with Mls-1^b expressing cells. Nature 339, 541-544

Rassoulzadegan, M., Naghashfar, Z., Cowie, A., Carr, A., Grisoni, M., Kamen, R. and Cuzin, F. (1983) Expression of the large T protein of polyoma virus promotes the establishment in culture of 'normal' rodent fibroblast cell lines. P.N.A.S. (USA) 80, 4354-4358

Reynolds, D. (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J.Cell Biol. 17, 208-211

Rich, K.M., Luczczynski, J.R., Osborne, P.A. and Johnson, E.M. (1987) Nerve growth factor protects adult sensory from cell death and atrophy caused by nerve injury. J. Neurocyt 16, 261-268

Roberts, R.A. et al (1987) Metabolically inactive 3T3 cells can substitute for marrow stromal cells to promote the proliferation and development of multipotent haemopoietic stem cells. J. cell. Physiol. 132, 203-214

Roberts, R., Gallagher, J., Spooncer, E., Allen, T.D., Bloomfield, F. and Dexter, T.M. (1988) Heparan sulphate bound growth factors: a mechanism for stromal cell mediated haemopoiesis. Nature 332, 376-378

Roehm, N., Herron, L., Cambier, J., DiGuisto, D., Haskins, K., Kappler, J. and Marrack, P. (1984) The major histocompatability complex-restricted antigen receptor on T cells: Distribution on thymus and peripheral T cells. Cell 38, 577-584

Rolink, A.G., Radaszliewicz, T. and Melchers, F. (1987) The autoantigen-binding B cell repertoires of normal and chronically graft-versus-host diseased mice, J Exp Med 165, 1675-1687

Rosewicz, S., McDonald, A.R., Maddux, B.A., Goldfine, I.D., Miesfeld, R.L. and Longdon, C.D. (1988) Mechanism of glucucorticoid receptor down-regulation by glucucorticoids. J. Biol. Chem. 263, 2581-2584

Ross, E.A.M., Anderson, N. and Micklem, H.S. (1982) Serial depletion and regeneration of the murine hematopoietic system. J. Exp. Med. 155, 432-44

Rothenberg, E. and Lugo, J.P. (1985) Differentiation and cell division in the mammalian thymus. Developmental Biology 112, 1-17

Ruley, H.E. (1983) Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. Nature 304, 602-606

Russell, J.H., Masakowski, V.R. and Dobos, C.B. (1979) Mechanisms of immune lysis. I Physiological distinction between target cell death mediated by cytotoxic T lymphocytes and antibody plus complement. J. Immunol. 124, 1100-1105

Russell, J.H. (1983) Internal disintegration model of cytotoxic lymphocyte-induced target damage. Immunol. Rev. 72, 97-118

Sainte-Marie, G. and Peng, F.-S. (1971) Emigration of thymocytes from the thymus. A review and study of the problem. Rev Canc Biol 30, 51

Sakaguchi, S.,and Sakaguchi, N. (1988) Thymus and autoimmunity. Transplantation of the thymus from cyclosporin A-treated mice causes organ-specific autoimmune disease in athymic nude mice. J. Exp Med 167, 1478-1485

Sanchez, E.R., Toft, D.O., Schlesinger, M.J. and Pratt, W.B. (1985) The 90 kDa non steroid binding phosphoprotein that binds to the untransformed glucucorticoid receptor in molybdate stabilized L-cell cytosol is the murine 90 kDa heat shock protein. J. Biol. Chem. 262, 6992-7000

Sarmiento, M., Dianylis, D.P., Lancki, D.W., Wall, K.A., Lorber, M.I., Loken, M.R. and Fitch, F.W. (1981) Cloned T lymphocytes and monoclonal antibodies as probes for cell surface molecules active in T cell-mediated cytolysis. Immunol Rev 68, 135-169

Schlechhe, J.A., Ginsberg, B.H. and Sherman, B.M. (1982) Regulation of the glucucorticoid receptor in human lymphocytes. J. Steroid Biochem. 116, 69-74

Schmidt, R.E., MacDermott, R.P., Bartley, G., Bertovich, M., Amato, D.A., Austen, F., Schlossman, S.F., Stevens, R.L. and Ritz, J. (1985) Specific release of proteoglycans from natural killer cells during target lysis. Nature 318, 289-291

Schneider, R., Lees, R.K., Pedrazzini, T., Zinkernagel, R.M., Hengartner, H. and MacDonald, H.R. (1989) Postnatal disappearance of self-reactive (Vβ6+) cells from the thymus of Mlsa mice. J. Exp Med 169, 2149

Schwartzendruber, D.C. and Congdon, C.C. (1963) Electron microscope observations on tingible body macrophages in mouse spleen. J. Cell Biol. 19, 641-646

Scollay, R. (1982) Thymus cell migration: cells migrating from the thymus to the peripheral lymphoid organs have a mature phenotype. J. Immunol. 128, 1566-1570

Scollay, R., Bartlett, P. and Shortman, K, (1984) T cell development in the adult murine thymus: changes in the expression of the surface

antigens Ly2, L3T4 and B2A2 during development from early precursor cells to emigrants. Immunol Rev 82, 79-103

Scollay, R., Butcher, E. and Weissman, I. (1980) Thymus migration: Quantitative studies on the rate of migration of cells from the thymus to the periphery in mice. Eur. J. Immunol. 10, 210

Scollay, R. Kochen, M., Butcher, E. and Weissman, I.L. (1978) Lyt markers on thymus cell migrants. Nature 276, 79-80

Scollay, R., Chen W.-F., and Shortman, K. (1984) The functional capabilities of cells leaving the thymus. J. Immunol. 132, 25-30

Scollay, R. and Shortman, K. (1983) Thymocyte subpopulations: An experimental review, including flow cytometric cross-correlations between the major murine thymocyte markers. Thymus 5, 245-295

Scollay, R. and Shortman, K. (1984) Cell trafic in the adult thymus: cell entry and exit, cell birth and death. In Recognition and Regulation in cell mediated immunity, Watson, J.D. and Marbrook, J. eds P3 Marcel Dekker, Inc, New york and Basel.

Scollay. R., Wilson, A., D'Amico, A., Kelly, K., Egerton, M., Pearse, M., Wu, L. and Shortman, K. (1988) Developmentak status and reconstitution potential of subpopulations of murine thymocytes. Immunol Rev 104, 81-120

Searle, J., Kerr, J.F.R. and Bishop, C.J. (1982) Necrosis and apoptosis: distinct modes of cell death with fundamentally different significance. Pathol. Annu. 17, 229-259

Sellins, K.S. and Cohen, J.J. (1987) Gene induction by γ-irradiation leads to DNA fragmentation in lymphocytes. J. Immunol 139, 3199-3206

Sellins, K.S. and Cohen, J.J. (1989) Polyomavirus DNA is damaged in target cells during cytotoxic T lymphocyte killing. J. Virology 63, 572-578

Sha, W.C., Nelson, C.A., Newberry, R.D., Kranz, D.M., Russel, J.H. and Loh, D.Y. (1988) Positive and negative selection of an antigen receptor on T cells in transgenic mice. Nature 236, 73-76

Sheridan, J.W., Bishop, C.J. and Simmons, R.J. (1981) Biophysical and morphological correlates of kinetic change and death in a starved human melanoma cell line. J. Cell Sci. 49, 119-37

Shi, Y., Sahai, B.M. and Green, D.R. (1989) Cyclosporin A inhibits activation-induced cell death in T-cell hybridomas and thymocytes. Nature 339, 625-626

Shinkai, Y., Ishikawa, H., Hattori, M. and Okumura, K. (1988) Resistance of mouse cytolytic cells to pore-forming protein-mediated cytolysis. Eur. J. Immunol. 18, 29-33

Shipley, W.U., Baker, A.R. and Colten, H.R. (1971) DNA degradation in mammalian cells following complement-mediated cytolysis. J. Immunol. 106, 576-579

Shipman, G.F., Bloomfield, C.D., Gaji-Peczulska, K.J., Munch, A.V. and Smith, K.A. (1983) Glucocorticoids and lymphocytes III Effects of glucocorticoid administration on lymphocyte glucocorticoid receptors. Blood 61, 1086-1090

Shortman, K. and Jackson, H. (1974) The differentiation of T lymphocytes. I. Proliferation kinetics and interrelationships of subpopulations of mouse thymus cells. Cell Immunol. 12, 230-246

Sibley, C.H. and Tomkins, G.M. (1974) Mechanisms of steroid resistance. Cell 2, 221-227

Siekevitz, M., Kochs, C., Rajewsky, K. and Dildrop, R. (1987) Analysis of somatic mutation and class switching in naive and memory B cells generating adoptive primary and secondary responses. Cell 48, 757-770

Sidman, C.L. and Unanue, E.R. (1975) Receptor-mediated inactivation of early B lymphocytes. Nature 257, 149-151

Sigal, I.S. and Gibbs, J.B. (1989) Oncogenes. Current Opinion in cell biology 1, 286-290

Sims, J.L., Sinorski, G.W., Catino, D.M. Berger, S.J. and Berger, N.A. (1983) Poly(adenophosphoribose)polymerase inhibitors stimulate unscheduled deoxyribonucleic acid synthesis in normal human lymphocytes. Biochemistry 21, 1813-1821

Skinner, M. and Marbrook, J. (1987) The most efficient cytotoxic T lymphocytes are the least susceptible to lysis. J. Immunol. 139, 985

Smith, L. (1987) CD4+ murine T cells develop from CD8+ precursors in vivo. Nature 315, 232

Smith, J.B. and Pasternack, R.D. (1978) Syngeneic mixed lymphocyte reaction in mice: strain distribution, kinetics, participating cells, and absence in NZB mice. J. Immunol 121, 1889-1892

Smith, J.R. and Whitney, R.G. (1980) Intraclonal variation in proliferative potential of human diploid fibroblasts: stochastic mechanism for cellular aging. Science 198, 366-372

Smith, C.A., Williams, G.T., Kingston, R., Jenkinson, E.J. and Owen, J.J.T. (1989)
Antibodies to CD3/T cell receptor complex induce death by apoptosis in immature T cells in thymic cultures. Nature 337, 181-184

Snodgrass, H.R., Kisielow, P., Kiefer, M., Steinmetz, M. and von Boehmer, H. (1985) Ontogeny of the T-cell antigen receptor within the thymus. Nature 313, 592-595

Soffer, L.J., Grabilove, J.L.and Wolf, B.S. (1952) Effect of ACTH on thymic masses. J. Clinical Endocrinology 12, 690

Somoza, C., Fernandez-Ruiz, E., Jotterand-Bellomo, M., Sanz, E., Nabholz, M. and Silva, A./ (1989) Loss of interleukin 2 dependence in cloned interleukin 2-dependent rat lymphocyte x BW5147 hybridomas is not associated with segregation of a specific pair of rat chromosomes. Eur J Immunol 19, 1177-1181

Spadari, S., Sala, F. and Pedrali-Noy, G. (1982) Aphidicolin: a specific inhibitor of nuclear DNA replication in eukaryotes. TIBS 7, 29-38

Spandidos, D.A. (1985) Mechanisms of carcinogenesis: the role of oncogenes, transcriptional enhancers and growth factors. Anticancer Research 5, 485-498

Spandidos, D.A. and Wilkie, N.M. (1984a) Tumorigenic conversion of early passage rodent cells can be achieved with a single activated human oncogene. In Cancer cells: oncogenes and viral genes vol2, Vande Woude G.F., Levine, A.J., Topp, W.C. and Watson, J.D. eds. New York Cold spring harbour Lab, 1984, pp495-500

Spandidos, D.A. and Wilkie, N.M. (1984b) Malignant transformation of early passage rodent cells by a single mutated human oncogene. Nature 310, 469-475

Spooncer, E., Heyworth, C.M. and Dexter, M.T. (1986) Self-renewal and differentiation of IL3 dependent multipotent stem cells are modulated by stromal cells and serum factors. Differentiation 31, 111-118

Sprent, J. (1978) Role of the H-2 complex in induction of T helper cells in vivo. I Antigen-specific selection of donor T cells to sheep erythrocytes in irradiated mice dependent upon sharing of H-2 determinants between donor and host. J. Exp Med 148, 478-488

Sprent, J. (1978) Role of H-2 gene products in the function of T helper cells from normal and chimeric mice in vivo. Immunol Rev 42, 108-137

Sprent, J. and Basten, A. (1973) Circulating T and B lymphocytes in the mouse II life span Cell Immunol 7, 40

Stanistic, T., Sadlowsky, R., Lee, C. and Grayhack, J.T. (1978) Partial inhibition of castration induced ventral prostrate regression with actinomycin D and cycloheximide. Invest Urol 16, 19-22

Strober, S. (1975) Transplanr Rev 24, 85

Sulston, J.E. (1976) Post-embryonic development in the ventral cord of Caenorhabditis elegans. Phil Trans Roy Soc (Lond) B 275, 287-297

Sulston, J.E. and Horvitz, H.R. (1977) Post-embryonic cell lineages of the nematode Caenorhabditis elegans. Dev Biol 82, 110-156

Sulston, J.E., Scheirenberg, E., White, J.G. and Thomson, N. (1983) The embryonic lineage of the nematode Caenorhabditis elegans. Dev Biol 100, 64-119

Sulston, J.E. and White J.G. (1980) Regulation and cell autonomy during postembryonic development of Caenorhabditis elegans. Dev Biol 78, 577-597

Sussman, J.J., Saito, T., Shevach, E.M., Germain, R.N. and Ashwell, J.D. (1988) Thy-1 and LY-6 mediated lymphokine production and growth inhibition are of a T cell hybridoma require co-expression of the T cell antigen receptor complex. J. Immunol 140, 2520-2526

Stewart, M.L., Grollman, A.P. and Huang, M.-T. (1971) Aurintricarboxylic Acid : inhibitor of initiation of protein synthesis. P.N.A.S. (USA) 68, 97-101.

Svec, F. and Rudic, M. (1984) Glucocorticoids regulate the glucocorticoid receptor in AET-20 cells. J. Biol. Chem. 256, 5984-5987

Swain, S.L. (1981) Significance of Lyt phenotypes: Lyt2 antibodies block activities of T-cells that recognize class I major histocompatability antigens regardless of their function. P.N.A.S. (USA) 78, 7101-7105

Tada, H., Shiho, O., Kuroshima, K., Koyama, M. and Tsukamoto, K. (1986) An improved colorimetric assay for interleukin 2. J. Immunol. Methods 93, 157-165

Tadakuma, T. et al (1990) DNA fragmentation in $CD4^+CD8^+$ thymocytes. Eur J. Immunol (in press)

Townsend, A., R. (1987) Recognition of influenza virus proteins by cytotoxic T lymphocytes. Immunol Res 6, 80-100

Trenn et al (1987) Exocytosis of cytolytic granules may not be required for target cell lysis by cytotoxic T-lymphocytes. Nature 330, 72-73

Trent, C., Tsung, N. and Horvitz, H.R. (1983) Egg-laying defective mutants of the nematode Caenorhabditis elegans. Genetics 104, 619-647

Tschopp, J., Schaefer, S., Masson, D., Peitsch, M.C. and Heusser, C. (1989) Phosphorylcholine acts as a Ca^{2+} dependent receptor molecule for lymphocyte perforin. Nature 337, 272-274

Ucker, D.S. (1987) Cytotoxic T lymphocytes and glucocorticoids activate an endogenous suicide in target cells. Nature 323 62

Umansky, S.R. (1982) The genetic program of cell death. Hypothesis and some applications: transformation, carcinogenesis, ageing. J. theor Biol 97, 591-602

Umansky, S.R., Korol, B.A., Nelipovich, P.A. (1981) In vivo DNA degradation in thymocytes of γ-irradiated or hydrocortisone-treated rats. Biochim Biophys Acta 655, 9-17

Van Ewijk, W. and Van der Kwast, T.H. (1980) J Cell Res 212, 497-508

Van Haelst, U. (1967a) Light and electron microscope study of the normal and pathological thymus of the rat. I. The normal thymus. Z. Zellforsch 77, 534-553

Van Haelst, U. (1967b) Light and electron microscope study of the normal and pathological thymus of the rat.II. The acute thymic involution. Z. Zellforsch Mikrosk Anat 80, 153-182

Vaux, D.L., Cory, S. and Adams, J.M. (1988) Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. Nature 335, 440-442

Ventkataraman, M. Aldo-Benson, M. Borel, M. and Scott, D.W. (1977) Persistance of antigen-binding cells with suface tolerogen: isologous versus heterologous immunoglobulin carriers. J Immunol 119, 1006-1009

Verlardi, A. and Cooper, M.D. (1984) An immunofluorescence analysis of the ontogeny of myeloid, T and B lineage cells in mouse haemopoiesis. J Immunol 133, 672-677

Verret, C.R., Firmenich, A.A., Kranz, D.M. and Eisen, H.N. (1987) Resistance of cytotoxic T lymphocytes to the lytic effects of their toxic granules. J. Exp. Med. 168, 1536

Voris, B.P. and Young, D.A. (1981) Glucocorticoid-induced proteins in rat thymus cells. J. Biol. Chem. 256, 11319-29

Walford, R.L., Jawaid, S.Q. and Naeim, F. (1981) Age 4, 67-70

Whetton, A.D. and Dexter, M.T. (1983) Effect of haemopoietic cell growth factor on intracellular ATP levels. Nature 332, 376-378

Wielckens, K., Delfs, T., Muth, A., Freese, V. and Kleeberg, H.J. (1987) Glucocorticoid-induced lymphoma death: The good and the evil. J. Steroid Biochem. 27, 413-419

White, J., Herman, A., Pullen, M., Kubo, R., Kappler, J.W. and Marrack, P. (1989) The Vβ-specific superantigen staphylococcal enterotoxin B: stimulation of mature T cells and clonal deletion in neonatal mice. Cell 56, 27

Wilkie, N.M. and Spandidos, D.A. (1984) In vitro maliognant transformation of primary cells by active oncogenes. In UCLA Symposium on molecular and cellular biology. Genes and Cancer 17, 471-485

Williams, R.W. and Herrup, K. (1988) The control of neuron number. Ann Rev Neurosci 11, 423-53

Williams, G.T., Smith, C.A., Spooncer, E., Dexter, T.M. and Taylor, D.R. (1990) Haemopoietic colony stimulating factors promote cell survival by suppressing apoptosis. Nature 343, 76-69.

Wilson, D.B., Lindahl, K.-F., Wilson, D.H. and Sprent, J. (1977) The generation of killer cells to trinitrophenyl-modified alloogeneic targets by lymphocyte populations negatively selected to strong alloantigens. J. Exp Med 146, 361-367

Wyllie, A.H. (1980) Glucocorticiod-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature 284, 555-556

Wyllie, A.H. (1981) Cell Death: a new clasification separating apoptosis from necrosis. In Cell Death in Biology and Pathology (eds I.D.Brown and R.A.Lockshin) pp9-34. Chapman and Hall, London and New York.

Wyllie, A.H. (1985) The Biology of cell death in tumours. Anticancer Res 5, 131-136

Wyllie, A.H. (1987) Apoptosis: Cell death under homeostatic control. Mechanisms and Models in Toxicology Arch. Toxicol. Suppl. 11, 3-10

Wyllie, A.H., Kerr, J.F.R., and Currie, A.R. (1980) Cell death: the significance of apoptosis. Int. Rev. Cytol. 68, 251-306

Wyllie, A.H. and Morris, R.G. (1982) Hormone-induced cell death. Purification and properties of thymocytes undergoing apoptosis after glucocorticoid treatment. Am J Pathol 109, 78-87

Wyllie, A.H., Morris, R.G., Smith, A.L. and Dunlop, D. (1984) Chromatin cleavage in apoptosis : association with condensed chromatin morphology and dependence on macromolecular synthesis. J. Pathol. 142, 67-77

Yamamoto, K.R. (1985) Steroid receptor regulated transcription of specific genes and gene networks. Ann. Rev. Genet. 19, 209-52

Yanelli, J.R., Sullivan, J.A., Mandell, G.L. and Engelhard, V.H. (1986) Reorientation and fusion of cytotoxic T lymphocyte granules after interaction with target cells as determined by high resolution cinematography. J. Immunol 136, 337-382

Young, J. D.-E. and Cohn, Z.A. (1986) Cell-mediated killing: a common mechanism? Cell 46, 641-642

Young, J.D., Cohn, Z.A. and Podack, E.R. (1986) The ninth component complement and the pore forming protein (perforin 1) from cytotoxic T cells: structural, immunological, and functional similarities. Science (Wash DC) 233, 184

Yue, C.C., Reynolds, C.W. and Henkart, P. (1987) Inhibition of cytolysin activity in large granular lymphocyte granules by lipids: evidence for a membrane insertion mechanism of lysis. Mol. Immunol 24, 647-653

Zalman, L.S., Wood, L.M. and Muller-Eberhard, H.J. (1986) Isolation of a human erythrocyte membrane protein capable of inhibiting expression of homologous complement transmembrane channels. P.N.A.S. (USA) 83, 6975

Zalman, L.S., Wood, L.M. and Muller-Eberhard, H.J. (1987) Inhibition of antibody-dependent lymphocyte cytotoxicity by homologous restriction factor incorporated into target cell membranes. J. Exp. Med. 166, 947

Zamenhof, S. (1942) Stimulation of cortical cell proliferation by the growth hormone. III Experiments on albino rats. Physiol Zool 15, 281-92

Zawydiwski, R., Harmon, J.M. and Thomson, E.B. (1983) Glucocorticoid-resistant human acute lymphoblastic leukaemic cell-lines with functional receptor. Cancer Res 43, 3865-3873

Zepp, F. and Staerz, U.D. (1988) Thymic selection process induced by hybrid antibodies. Nature 336, 473-475

Zetterberg, A. and Larsson, O. (1985) Kinetic analysis of regulatory events leading to proliferation or quiescence of Swiss 3T3 cells. P.N.A.S. (USA) 82, 5365-5369

Zinkernagel, R.M., Althage, A., Waterfield, E., Kindred, B., Welsh, R.M., Callahan, G. and Princetl, P. (1980) Restriction specificities, alloreactivity, and allotolerance expressed by T cells from nude mice reconstituted with H-2 compatible or incompatible thymus grafts. J. Exp Med 151, 376-399

Zinkernagel, R.M., Callahan, G.N., Althage, A., Cooper, S., Klein, P.A. and Klein, J. (1978) On the thymus in the differentiation of "H-2 self-recognition" by T-cells: evidence for dual recognition? J. Exp Med 147, 882-896

Zinkernagel, R.M. and Doherty, P.C. (1975) H-2 compatible requirement for T cell mediated lysis of target cells infected with lymphocytic choriomeningitis virus. Different cytotoxic T cell specificities are associated with structures coded for in H-2K or H-2D. J. Exp Med 141, 1427-1436