THYMUS DEPENDENT IMMUNE COMPETENCE

EFFECTS OF AGEING TUMOUR-BEARING AND THYMIC HUMORAL FUNCTION

ADA M. KRUISBEEK

THYMUS

DEPENDENT

IMMUNE COMPETENCE

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STELLINGEN

-1-

In het kader van het onderzoek naar de oorzaken van storingen in cellulaire immuniteit tijdens veroudering is tot nu toe de mogelijke "feed-back" rol van de thymus op de aanwezigheid van voorloper T cellen in milt en beenmerg verwaarloosd.

-2-

De corticosteroid-resistente, immunologisch competente T cellen in de thymus vertegenwoordigen niet de directe voorloper cellen van periphere T cellen. Daarom dient rekening te worden gehouden met de mogelijkheid dat deze cellen een rol spelen bij het T cel differentiatieproces in de thymus.

Stutman, O., Cont. Topics Immunobiology, vol. 7, p. 1, 1977

-3-

Thymus "hormonen" dienen eerst dan te worden toegepast bij pogingen tot herstel van gestoorde T cel functies wanneer is vastgesteld dat a de betreffende stoornis te wijten is aan een afname in het aantal functionerende T cellen en b de onrijpe voorloper cellen die onder invloed van thymus factoren kunnen differentieren tot volledig functionerende T cellen nog aanwezig zijn. Gezien het feit dat deze voorloper cellen nog onvoldoende gedefinieerd zijn, lijken behandelingen met thymusfactoren van vele soorten immunologische defecten wetenschappelijk weinig onderbouwd.

-4-

Bij het bepalen van het cellulaire immunologische reactievermogen dient men ook parameters te gebruiken waarbij het fenomeen "genetische restrictie" betrokken is, omdat met name bij anti-virale reacties het herkennen van de door het eigen MHC gecodeerde celmembraan structuren een essentiele rol lijkt te spelen.

Zinkernagel, R.M. et al., J. exp. Med. 147, 882, 1978

-5-

Bepalingen van lymphocyten functies met behulp van in vitro technieken bij het achterhalen van mogelijke immunologische stoornissen dienen vergezeld te gaan van een bepaling van de ratio tussen lymphocyten en macrophagen of monocyten, teneinde te kunnen differentieren tussen abnormale interactie fenomenen en intrinsieke lymphocyten defecten.

-6-

Het testen van granulocyten en monocyten aantallen en functies vormt, gezien hun belangrijke rol bij bacteriele infecties, een noodzakelijke aanvulling op het testen van de functie van verschillende lymphocyten subpopulaties bij de differentiaal diagnose van immunodeficientie syndromen.

-7-

Bij studies naar cytotoxische en cytostatische effecten van macrophagen op tumorcellen wordt dikwijls over het hoofd gezien dat macrophagen ook immunosuppressieve effecten kunnen uitoefenen.

-8-

Aangezien bekend is dat antisera tegen het Thy 1 alloantigeen ook antistoffen tegen andere alloantigenen op T cellen kunnen bevatten, dient hiermee bij het bepalen van aantallen Thy 1 positieve cellen rekening te worden gehouden.

-9-

Experimenten met muis, rat, rhesus aap en hond geven aan dat voor beenmergtransplantatie na totale lichaamsbestraling slechts de helft van het tot nu toe gebruikelijke aantal beenmergcellen nodig is; door toepassing van deze gegevens bij humane beenmergtransplantatie zouden frequentie en ernst van graft-versus-host reacties gereduceerd kunnen worden.

Vriesendorp, H.M. and van Bekkum, D.W.
Exp. Hemat. 6, suppl. 3, 1978

-10-

Het ter beschikking komen van inteelt Mastomys zou de NZB muis als het model voor de bestudering van autoimmuniteit kunnen verdringen.

-11-

De conclusie dat thymusveranderingen primair verantwoordelijk zijn voor het optreden van Myasthenia gravis lijkt op zijn minst voorbarig te zijn.

Kao, I. en Drachman, D.E., Science 195, 74, 1976
Namba, T. et al., Medicine 57, 411, 1978

-12-

De reacties van Nederlandse feministische groeperingen op de artikelen van Renate Rubinstein over het feminisme bewijzen haar gelijk.

-13-

Het gebrek aan vrijheid dat volwassen mensen door bepaalde groeperingen wordt toegestaan om te beslissen over abortus staat in schril contrast tot de grote vrijheid die dezelfde groeperingen anderen bieden wanneer het gaat om beslissingen het geboren leven betreffende.

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE AAN DE RIJKSUNIVERSITEIT TE UTRECHT, OP GEZAG VAN DE RECTOR MAGNIFICUS PROF. DR. A. VERHOEFF, VOLGENS BESLUIT VAN HET COLLEGE VAN DECANEN IN HET OPENBAAR TE VERDEDIGEN OP DINSDAG 19 DECEMBER 1978 DES NAMIDDAGS TE 4.15 UUR

DOOR

ADRIANA MARIA KRUISBEEK
GEBOREN OP 2 OKTOBER 1948 TE ROTTERDAM

W.D. MEINEMA B.V. - DELFT

PROMOTOR :

PROF. DR. C. F. HOLLANDER

PREFACE

It would be ridiculous to pretend that the work described in this thesis has been achieved through efforts of myself alone. I am especially indebted to Tonny J.M. Kröse and Jelly J. Zijlstra for the indefatigable way in which they agreed to repeatedly perform many of the experiments described hereafter. In addition, I want to thank them, Dr. Marie-Jose Blankwater and Fred A. Steinmeier for their friendship and sympathy which undoubtedly must have often been difficult to keep alive.

I have had the advantage of Prof. Dr. C.F Hollander's guidance on many occasions and also his willingness to allow me the freedom to choose certain research directions.

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Last but not least, I would like to mention my parents. I am still grateful for the fact that they stimulated me to enter university and created the conditions for the succesful completion of my studies.

CONTENTS

ABBREVIATIONS

"B mice"	adult thymectomized lethally irradiated and bone marrow reconstituted mice
CFU	colony forming unit
CML	cell mediated lympholysis
CS	control supernatant
CTL	cytotoxic T lymphocyte
Con A	concanavalin A
C *parvum*	*Corynebacterium parvum*
cpm	counts per minute
FCS	foetal calf serum
FTS (= TF)	facteur thymique serique
GvH	graft versus host
HC	hydrocortisone
LAF	lymphocyte activating factor
LD (antigens)	lymphocyte defined
MCF	macrophage culture fluid
2-ME	2-mercaptoethanol
MLC	mixed lymphocyte culture
MLR	mixed lymphocyte reaction
MSV	Moloney sarcoma virus
N (rats)	normal
PEC	peritoneal exudate cells
PFC	plaque forming cells
PG	prostaglandin
PHA	phytohaemagglutinin
RES	reticuloendothelial system
SD (antigens)	serologically defined
SF	serum factor
SRBC	sheep red blood cells
TB (rats)	tumour-bearing
T cell	thymus-dependent lymphocyte
TDF	thymocyte differentiating factor
TdR	radioactive thymidine
TES	thymic epithelial culture supernatant
TF (= FTS)	thymic factor
THF	thymic humoral factor
TL (antigen)	thymic leukemia
Tx	thymectomy (thymectomized)

CHAPTER I

INTRODUCTION

Many immunological dysfunctions are accompanied by increased occurrence of several diseases (e.g., infectious diseases, autoimmune disorders, immune complex diseases and neoplasias). Elucidation of the possible causal relationships between immunologic dysfunction and predisposition for these diseases is essential before attempts at therapeutic manipulation of the immune system can be envisaged. Thus, detailed analysis of immunologic dysfunction and its underlying mechanisms in experimental models is needed. Studies described in this thesis were involved with two such experimental models. These were: age-related decline in normal immune functions and tumour-induced immunosuppression.

A considerable amount of data seems to indicate a causal relationship between the age-related decline in normal immune functions and predisposition for several diseases (for a recent review, see ref. 235). Hence, delay, reversal or prevention of immunosenescence could perhaps delay the onset and/or minimize the severity of certain diseases of the elderly. However, a proper understanding of the basic mechanism(s) responsible for immunosenescence is lacking. Many studies on ageing have revealed that the most striking changes occur in the thymus-dependent lymphocytes (T cells) (177, 235), but the causes of such changes remain unclear. The present studies concerning age-related changes in T cell functions are aimed at defining more precisely the decline in T cell proliferative capacity (Chapter II) and at designing models which could help to find causes for the observed defects in T cell immunocompetence (Chapter III). An introduction to the experiments described in these Chapters will be presented in the first section of this Introduction. Attention will be focused on how T immune competence can be analyzed, which aberrations in T cells and their functions have been demonstrated during ageing and the possible underlying changes in precursor T cells and the thymus.

In the other part of our study on immunologic dysfunctions (i.e., those induced by tumour-bearing), impairment of T cell functions has also been shown to be the main feature in experimental animals (181). Apart from its relevance in relation to age-related aberrations (i.e., the increased incidence of cancer, 56, 319), this study also seemed justified because of the lack of comprehensive studies in the literature. Only information concerning the possible consequences of immunological dysfunction in cancer patients is available (e.g., increased susceptibility to infections) (55, 120, 153, 169, 192, 218, 222, 226,

322), but there is a complete absence of data on the underlying causes of these disorders. Thus, it would seem essential to obtain insight into the basic mechanism(s) responsible for tumour-induced immunosuppression in well characterized experimental models. This requirement was the basis for the experiments concerning the effects of tumour bearing on T cell responses in young animals reported in Chapters IV and V. These experiments are introduced in the second section of this Chapter (1.2) which brings together current views on the effects of tumours on T cell immunocompetence. Since both in our own and some other studies macrophages were shown to strongly interfere with T cell functions in tumour bearers, more specifically the role of macrophages as immunosuppressive elements will be discussed. It was found that macrophages from normal animals could also exert suppressive effects on T cell proliferation, therefore, it was considered necessary to investigate more extensively what conditions are required for macrophages to exert either suppressor or helper effects; experiments on this subject are reported in Chapter VI.

Before any attempt is made to intervene with deficiencies in T cell function, more detailed information will be required on how normal thymus-dependent immune competence is achieved and maintained. Inasmuch as the thymus is necessary for the maturation of precursor cells into various types of T cells, it appears that the process(es) influencing involution of the thymus may be "the key to ageing of the immune system" as suggested by Makinodan (235). Although the mechanism(s) by which the thymus affects T cell differentiation processes are still not fully understood, there seems to be sufficient evidence to assume that thymic humoral factors are required for T cell maturation (reviewed in ref. 13, 136, 353). As soon as the thymus begins to show involution with age, the level of serum thymic factor(s) decreases in both mice (18, 19) and man (9, 19, 161). One might postulate that this could lead to a deficit in T cell functions. However, possible therapeutical application of thymic hormones to compensate for such deficiencies is still unwarranted, since only marginal in vivo effects have been reported in experimental animals up to now. Furthermore, several factors have been described (13, 136, 353) and it is unknown whether different steps of the T cell differentiation pathway (schematically shown in Figure 1.1, which represents a putative model) are controlled by different thymic factors. Thus, in the framework of the ageing studies described in this thesis, it seemed essential to place effort on more fundamental studies of the humoral function of the thymus. As a source of thymic factors, thymic epithelial culture supernatants (TES) were employed. These experiments are reported in Chapters VII-IX and had the following objectives:

a) to study the influence of TES on several T cell parameters (i.e., T cell markers, proliferative capacity and effector cell function);

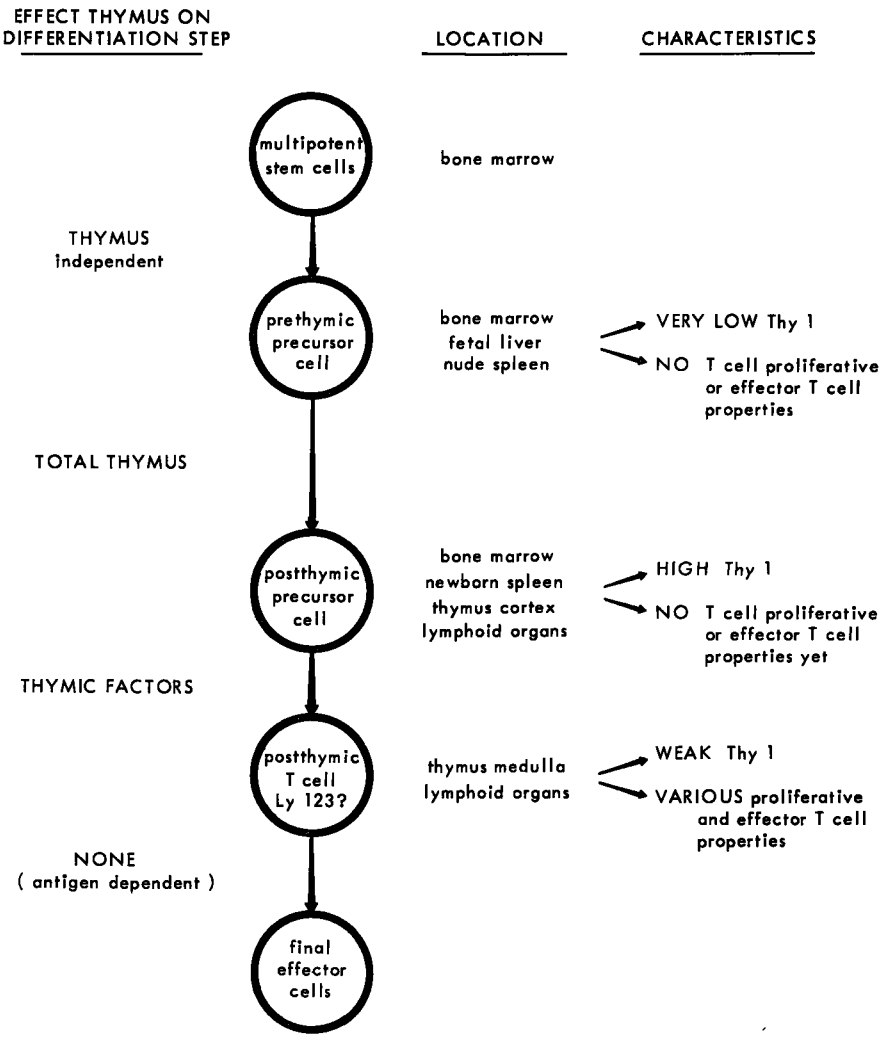

Figure 1.1

Model for the T cell-differentiation pathway.

b) to identify the cell type sensitive to the action of TES through a systematic investigation of the effects of TES on the differentiation steps illustrated in Figure 1.1.

An entry into the area of thymic factors is given in the third part of this introduction (1.3), which compares the different thymic factors with regard to their *in vitro* biological effects and several other factors exhibiting similar effects as thymic factors.

1.1 T CELLS UNDER NORMAL CONDITIONS AND IN AGEING

Demonstration of fully developed specific cellular immune reactions is complicated by the fact that many features of this manifestation of immunity are still not well established. It is known that T cells are the essential elements involved in host defense against viruses, fungi and mycobacteria. In addition, T cells are involved in graft rejection, graft versus host reactivity (GvH), delayed-type hypersensitivity and possibly tumour resistance. However, the exact functions and interactions of the various fully differentiated T cell subsets participating in these phenomena are not clear.

In vivo studies on cellular immune competence in ageing rodents have revealed that delayed type skin reactivity (368), capacity to reject allogeneic skin grafts (241, 346, 347) and GvH reactivity (191, 368) generally show a decrease, although some exceptions have been reported (341, 347, 368). Most of our present knowledge of the consequences of ageing on cellular immune competence, however, has been achieved through in vitro studies of lymphocyte function. Obviously, the limited availability of old animals does not permit extensive in vivo evaluation of several forms of immune competence. In addition, the various compartments of the immune system can be studied separately in in vitro experiments, which in turn opens possibilities for determining the contribution of these separate compartments to the observed aberrations in immune competence.

In order to better understand the causes of the age-related decrease in T cell functions (or in fact of any thymus-related immune deficiency), the following questions must be answered:

1) Are precursor T cells which still have to undergo various differentiation steps present in normal numbers?
2) Are thymic factors which induce these maturation steps present in normal concentrations?
3) Are T cells (as recognized by typical membrane antigens) present in normal numbers in the peripheral lymphoid organs?
4) Can these cells proliferate upon stimulation with well-defined T cell activators?
5) Can these activated cells proceed to terminal differentiation into effector cells (T helper, T suppressor and T killer function)?

Possible approaches to answer these questions with in vitro techniques and the findings reported so far will be dealt with separately in sections 1.1.1 to 1.1.5. Because most of our knowledge on T cell differentiation and its possible age-related defects has been obtained through studies in mice, this intro-

duction is mostly concentrated on studies in mice. Possible changes in other features of T cells, such as humoral T cell derived products, tolerance, memory and genetic restriction will not be discussed as these phenomena have not been investigated with in vitro techniques in ageing mice.

1.1.1 Availability of precursor T cells

The development of T cells can be considered in two steps: (a) differentiation of immigrant stem cells (in the adult, mainly present in the bone marrow but also in the spleen) (33, 98) into immature thymocytes within the thymus; (b) the subsequent progress from immature thymocytes to immunocompetent T cells, either in or outside the thymus.

a) precursor T cells in the bone marrow and the spleen

One of the possible causes for the age-related decrease in T cell functions could be a deficiency in bone marrow progenitors of T cells. A few investigators have found that, given sufficient time (3 to 10 months), marrow from old donors can restore immune functions in young irradiated recipients (150, 246). Approaching the problem from the other direction, it was found (159, 246) that the response of old sublethally irradiated mice to SRBC could not be restored by marrow grafts from young recipients. Both types of studies seem to indicate that availability of T lymphocyte progenitor cells is not the only limiting factor in ageing mice. In the reconstitution attempts in old mice, the old recipient's thymus might have prevented young bone marrow from further differentiation. When a young marrow graft was combined with a young thymus graft (159), the low response of sublethally irradiated old mice to SRBC and T cell mitogens could be restored.

Recently, a different picture of bone marrow precursor T cells emerged from the studies of Tyan (356, 357), who found that the capacity of bone marrow from old donors to repopulate thymuses in young irradiated mice is strongly reduced. In these experiments, there was a much shorter observation period (i.e., 21 days) and the defects could be attributed to both an absolute decrease in the number of progenitor T cells and a diminished proliferative capacity in part of these cells.

The degree of repopulation of thymuses from young irradiated mice was also dependent on the age of the donor when spleen cells were injected (33). The relative precursor content of the spleen strongly declines during the early postpartum period and reaches exceedingly low levels in 42-week-old mice (33).

b) precursor T cells in the thymus and lymphoid organs

The thymus also contains precursor T cells. These cells are considered more differentiated than the precursors found in bone marrow, since they have acquired a high density of the Thy 1 membrane antigen, a specific characteristic of T cells (see 1.1.3). "Immature" thymocytes, present in the cortex of the thymus, are characterized by a high density of Thy 1 and TL antigens (284, 285), corticosteroid sensitivity (4, 44, 45, 47, 48, 83, 173, 174) and minimal immune competence (4, 44, 45, 48, 83, 173). Another population of thymocytes (present primarily in the medulla) contains the immunocompetent T cells of the thymus (199, 284) and represents ~ 10% of the organ. These cells are characterized by the absence of TL and a reduction in the amount of Thy 1 on their surface (199, 284), increased density of H-2 antigen (199, 284) and relative resistance to corticosteroids (4, 44, 45, 48, 83, 173). Thus, the majority (i.e., the corticosteroid sensitive cells) of cells in the thymus possibly still has to undergo further differentiation steps in order to become fully competent T cells.

It has been questioned whether all of these cells represent precursors for peripheral T cells. Nevertheless, the thymus does contain precursor cells which, in vivo, in cooperation with humoral thymic function (provided by thymus transplants in a millipore chamber), can differentiate into cells exhibiting properties of competent T cells (GvH reactivity, skin graft rejection, delayed type hypersensitivity) (339).

It also has been suggested (335, 373) that precursor cells from the thymus migrate to the peripheral lymphoid organs where they undergo further differentiation; there these precursor cells are termed "postthymic" precursor cells. This raises the possibility that a decrease or defect in precursor cells in the thymus or periphery also contributes to immunosenescence with increasing age.

Age-related changes in intrathymic or postthymic precursor cells have not been reported so far. Obviously, age-related thymus involution causes a decrease in the absolute number of thymic cells with increasing age, but whether this phenomenon also represents a decrease in precursor cells is unknown. The functional capacity of the aged thymus has been investigated (156-158) by implanting one thymic lobe from donor mice (ranging in age from one day to 33 months) under the kidney capsule of T-cell-deprived syngeneic young recipients (thymectomized, lethally X-irradiated and bone marrow reconstituted). Recovery of splenic Thy 1- positive cells and repopulation of splenic thymus-dependent areas were not distinctly different in the various groups, although newborn thymuses induced the appearance of Thy 1 positive cells in the spleen more rapidly (156, 157). In contrast,

with advancing age, thymic tissue lost the capacity to restore T cell mitogen reactivity, mixed lymphocyte reactivity and T helper cell function in the spleens of recipient mice (156-158). These results may indicate that a reduction in the number of precursors capable of differentiating into functional T cells occurs with advancing age. However, since total thymuses were grafted, the contribution of diminished thymic humoral function and/or the thymic microenvironment (to be discussed in sections 1.1.2 and 1.3) to the observed reduction in restorative capacity cannot be determined.

In summary, several data from the literature suggest that a decline in the number of precursor T cells both in the bone marrow and spleen and in the thymus contributes to age-related deficiencies in T cell immunocompetence, but more detailed information is still required.

Our own experiments regarding possible precursor T cells in the aged thymus (employing in vitro techniques, presented in Chapter III) suggest that, with advancing age the thymus loses cells sensitive to the action of thymic humoral function.

1.1.2 Influence of thymic factors

Although the mechanism(s) by which the thymus affects T cell differentiation are not fully understood, it seems reasonable to assume that humoral thymic factor(s) are required for T cell maturation (for more details, see section 1.3). Thymus-dependent factors displaying possibly the property to induce T cell maturation can be found in the serum of normal young individuals (reviewed in ref. 13).

Evidence is accumulating that a decrease in circulating thymic factors occurs with increasing age. First, in man serum thymosin-like activity, as measured with the rosette inhibition assay (18), rapidly decreases from the age of 25 onwards (161). Secondly, the level of a human thymus-dependent serum factor measured by a cyclic AMP assay (7), also is decreased with advancing age (9). Thirdly, the serum thymic factor level as measured with the mouse rosette inhibition assay is decreased in both man (from the age of 20 onwards) and mice (from the age of 6 months onwards) (18, 19).

NZB and (NZB x NZW)F1 hybrid mice have a normal level of serum thymic factor at birth, but this level decreases prematurely between the 3rd and 6th week of life (18, 19). Inasmuch as the serum thymic factor seems to be of thymic epithelial origin (19, 89), these findings are in accord with the early abnormalities reported in NZB thymic epithelial cells (93). In this respect, it is of interest that NZB mice display an earlier onset of decline in thymus-

dependent immune functions (92, 172, 223, 274, 324, 345, 380) than many other mouse strains and are often regarded as a model for a premature age-related decrease in immune capacity.

The reduction in serum thymic factor level in normal aged mice and man (9, 19, 161) parallels the decrease in thymus weight which starts in young adults (156). Though this weight loss largely represents loss of cortical lymphocytes, the number of epithelial cells also decreases with advancing age (56, 156). In addition, distinct structural changes occur in the thymus epithelium. In aged mice, formation of small clusters of membrane bordered epithelial cells devoid of thymocytes and containing cysts is observed, in contrast to young thymuses, in which the epithelial cells form a network densely packed with thymocytes (156).

All these findings seem to indicate that thymic secretory activity is diminished with increasing age. On the basis of the expected role of thymic factors in T cell differentiation, this could lead to a deficit in T cell functions, but whether depressed thymic factor production plays a role in the pathogenesis of age-related T cell function deficiencies remains to be proven. It has been suggested that there is a correlation between the premature drop in thymic factor level in NZB mice and the early onset of T cell function deficiencies observed in these mice. Treatment of NZB mice with thymic extracts restores their depressed serum thymic factor level (18, 19) and the number of Thy 1 positive cells in their spleens and lymph nodes (18), delays the formation of anti-nucleic acid antibodies if treatment is started early in life (345), restores Con A responsiveness (348) and mixed lymphocyte reactivity (125) in lymph node cells and the anti-SRBC response of spleen cells (125). Unfortunately, no long term follow up on the effect of thymic extract treatment on T cell abnormalities has been performed.

In summary, the extent of the contribution of and the mechanism by which depressed thymic factor production affect the age-related decrease in cellular immune functions are still unknown.

1.1.3 Identification of T cells

T cells of the mouse have unique surface antigens which distinguish them from the other class of lymphocytes, the B cells, whose differentiation is not dependent upon the thymus. These surface markers are:

a) TL, an early differentiation antigen which in certain TL^+ mouse strains is found only on immature corticosteroid sensitive thymocytes (i.e., the cortical cells) and not on immunocompetent medullary thymocytes and periph-

eral T cells (69, 285). TL alloantigens can be assayed by serological means and are a set of 4 antigens (TL 1, 2, 3, 4) which are expressed singly or in certain combinations only on thymocytes of strains which normally express them, but may be expressed anomalously on thymic lymphoma cells from strains whose thymocytes lack these antigens (51). It is likely, but not definitely established, that at least some cells of the TL negative thymocyte and peripheral T cell population stem from cortical TL positive progenitor cells (69, 106, 374). Effects of ageing on the presence of this marker have not been reported. Its significance as a differentiation marker remains difficult to understand, as it does not occur in all mouse strains.

b) Thy 1, a differentiation alloantigen which is present on all T cells in varying amounts. Thy 1 antigens have two allelic alternatives, Thy 1.1 and Thy 1.2 and are present on thymocytes and on all of the peripheral thymus-dependent lymphocytes (285). By employing alloantisera to Thy 1 in either cytotoxicity tests or in immunofluorescent techniques, the number of T cells can be determined.

The relative number of Thy 1^+ cells in mouse spleens and lymph nodes remains the same with advancing age (123, 164, 241, 246, 329, 330) when measured with a cytotoxicity technique, but some exceptions have been noted (52, 62) when immunofluorescence was employed. In NZB mice, however, a decrease in both the relative and absolute number of Thy 1^+ cells has been reported (326, 330). In contrast, others (154) found that while the relative number of Thy 1^+ cells in the NZB spleen was reduced, the absolute number remained the same. That a normal number of T cells does not necessarily represent a normal capacity to proliferate and develop into functional end cells has already been shown in ontogeny studies (256, 325). *This subject will be discussed in the following Chapters.*

c) Lyt 1, 2 and 3 lymphocyte specific differentiation alloantigens are found on most Thy 1^+ cells and are also recognized by alloantisera (for a recent review, see ref. 69). Unique combinations of these cell surface antigens are expressed by thymocytes and distinct subpopulations of peripheral T cells.

So far, T cells can be divided into 4 subpopulations (69) on the basis of the following phenotypes (identified by means of cytotoxicity tests): TL^+ + Lyt 123^+, the cortisone-sensitive thymocytes, TL^- Lyt 1^+ 23^-, representing approximately one-third of the cortisone resistant thymocytes and of peripheral T cells, TL^- Lyt 1^- 23^+, representing $\sim$ 5% of the peripheral T cells and of cortisone-resistant thymocytes, and the TL^- Lyt 123^+ cells, representing the putative common precursor cell of the Lyt 1^+ 23^- and Lyt 1^- 23^+ cells and present on $\sim$ 50% of peripheral T cells and corti-

sone-resistant thymocytes. The Lyt 1^+ 23^- cell population is considered to contain helper T cells (67-69, 73), whereas the Lyt 1^- 23^+ population contains cells which can develop into both alloreactive cytotoxic effector cells and cells with the capacity to suppress humoral and cell-mediated immune responses (39, 67, 68, 72, 107, 175, 311, 355).

Both cell types (Lyt 1^- 23^+ and Lyt 1^+ 23^-) are programmed for their specific functions (69); therefore, possible changes in their organ distribution and proportion during ageing could result in changes in the above-mentioned functions. Age-related differences in the proportion of these two T cell subsets have not been reported. As a consequence, one must deal with functional changes without being able to correlate these with possible changes in the numbers of certain subpopulations.

d) Other markers, i.e., markers on T cells which are much less well-defined. In this category, the immunoglobulin-like determinants (238), the receptors for the Fc part of antigen-antibody complexes (327) and other Lyt markers (Lyt 5 and Lyt 6) (379) should be mentioned, but no ageing studies have yet been reported.

In summary, the scant knowledge of the quantitative aspects of various T cell subsets during ageing stems from studies on Thy 1^+ cells and seems to indicate that, at least with regard to their relative number no changes occur. However, some exceptions have been noted in normal mice (52, 62) as well as in NZB mice (52, 154, 326), so this evidence cannot yet be regarded as conclusive.

1.1.4 Proliferative capacity of T cells in vitro

a) Mitogen-induced T cell proliferation

Nonspecific T cell mitogens are often used to determine the proliferative capacity of T cells. The term "nonspecific" is used as opposed to "antigen-specific" and is in fact misleading, since it is well known that the most commonly used T cell mitogens PHA and Con A stimulate distinct T cell subpopulations (256, 325, 328), although there is considerable overlap. The extent of proliferation is usually determined by measuring the incorporation of radioactive thymidine.

With regard to the effect of ageing on peripheral T cell mitogen responses, a decline has generally been reported (164, 167, 221, 239, 241, 243, 246, 290). This was recently attributed to a relative decrease in the number of responsive cells (1, 166, 206 and Chapter II). Others have suggested that increased interferon production by aged stimulated T cells

(152) could result in growth inhibition (147), and might be responsible for the observed defects. It could be added that the results of Meredith and Walford (242) seem to indicate that the extent of the age-related decrease in proliferative capacity in mice is under the influence of the H-2 gene region.

b) T cell proliferation induced by allogeneic cells

T cells can also be specifically stimulated to proliferation by allogeneic lymphocytes in the mixed lymphocyte reaction (MLR). This T cell response is induced by the lymphocyte defined (LD) determinants present on the stimulator cells and coded for by genes of the major histocompatibility complex. It has been suggested that at least 2 different subsets of T cells participate and synergize in this reaction (80, 349, 350): one obtained from the recirculating lymphoid pool (as found in lymph nodes) and one mainly present in the thymus and spleen. Similar interactions among T cell subsets were demonstrated for the cell populations mediating GvH reactivity (64-66).

With regard to responses in MLR of spleen and lymph node cells, most authors report an age-related reduction (3, 200, 241, 244, 245, 260, 290, 329), which again could be attributed to fewer cells capable of response (245). Meredith et al. (244) reported that the capacity of lymph node cells to synergize with thymocytes is also diminished in old mice, while the synergizing capacity of thymocytes remains the same.

In conlusion, proliferative responses of peripheral T cells decrease with age, probably due to the fact that fewer T cells can be stimulated by mitogens or alloantigens (1, 166, 206). It seems that this deficiency occurs despite the fact that the relative number of T cells (i.e., Thy 1 positive cells) remains the same (see section 1.1.3b). Apparently, the T cell differentiation pathway does proceed to the stage of acquiring the Thy 1 marker but aberrations occur in acquiring proliferative capacity. Other possibilities are: a) the determination of the number of Thy 1 positive cells has been performed incorrectly or with methods not sufficiently sensitive; it should be noted in this respect that only when immunofluorescence techniques were used, a decline in the relative number of Thy 1 positive cells has been found (52, 62); b) the subpopulation of Thy 1 positive cells which responds to mitogens or alloantigens is diminished, while a simultaneous increase in the number of Thy 1 positive non-responder cells occurs, c) T cell proliferation is inhibited by suppressor T cell function which seems to increase during ageing (133, 236, 308, 309 and section 1.1.5c).

Only a few authors have studied age-related changes in thymocyte proliferation induced by allogeneic cells and these have found either no changes (122) or an increase (244). The latter is to be expected, since thymus involution begins with depletion of cortical thymocytes, i.e., enrichment in responsive cells (as observed in corticosteroid-treated mice). Our own data (to be presented in Chapter III) indicate that large differences among thymocyte responses of individual animals occur with increasing age, some animals exhibiting increased responses, others comparable or decreased responses. When cell pools are used (as in the above mentioned reports), such differences would be neutralized, which may explain why no effect of ageing was sometimes found (122).

1.1.5 Effector functions of T cells

Various functions of T cells can be determined in vitro, i.e., helper cell function, the capacity to synergise with B cells in antibody responses and with T cells in the generation of killer T cells, killer cell function, the capacity to kill allogeneic cells or syngeneic tumour cells after appropriate in vivo or in vitro stimulation and suppressor cell function, the capacity to suppress the response of other cell types after specific or nonspecific stimulation.

a) Helper T cells. The nature and function of the helper T cells and their age-related changes were recently reviewed by Blankwater (42). Direct evidence for deficient helper T cell function has not yet been obtained, but the finding that both in vitro and in vivo responses to T cell-dependent antigens are more affected by ageing than responses to T cell-independent antigens suggests that helper T cell function is more affected by ageing than B cell function (42).

Helper T cells also are involved in the generation of cytotoxic effector cells in mixed lymphocyte cultures (68, 70, 80, 363). It has been demonstrated that these cells do not themselves exhibit significant cytotoxicity either alone or in combination with killer T cells (68, 70) and that they also express the Lyt 1 antigen (68). These helper T cells, stimulated to proliferate by LD antigens, are probably responsible for the proliferative response measured in MLR (see 1.1.4b), whereas killer T cells are mainly stimulated by the serologically defined (SD) antigens (coded for by the SD determinants) which also determine target cell specificity to the greatest extent (11).

No age-related changes in this type of helper T cell function have been reported, although it has been suggested that their capacity to synergize with precursor killer cells is diminished (121, see 1.1.5b).

b) Killer T cells. On in vitro exposure of lymphoid cells to allogeneic cells (in an MLC), also cytotoxic effector T cells are produced (36, 140, 141 364). These killer cells can specifically kill target cells carrying one or more of the same alloantigens (mainly SD-antigens) as those present on the allogeneic stimulator cells. This process is usually determined by measuring the amount of ^{51}Cr released from the appropriate labelled target cells. As mentioned in the previous section, killer cells are stimulated by the SD antigens. Both the precursor and the final effector cells can be recognized by the presence of the Lyt 23 antigens (39, 67, 68, 72, 107, 175, 311, 355). In addition, the fully differentiated effector cells, at least in some strains, carry the Lyt 6 marker (379).

Only a few authors have reported that in vitro generation of killer T T cells in spleen MLC decreases (154, 310) with increasing age. It has been suggested (154, 310) that this decrease is due to a decrease in the number of antigen-sensitive precursor killer cells or to a loss of T helper and T killer synergism (121). Others found no change (382) but, in this report, the oldest age-group studied was 14 months and lymph node instead of spleen cells were used as responder cells.

In vivo immunisation with allogeneic cells also leads to induction of specific killer cells (54) whose activity can be tested in vitro. Production of splenic killer cells has been found to be decreased with advancing age (23, 142, 341) and could be attributed to both a reduction in the number of precursor cells and to a decrease in proliferative capacity (142). Thus, both the in vivo and in vitro studies seem to indicate a deficiency in the generation of killer T cells.

c) Suppressor T cells. An often used method for determination of suppressor T cell function in vitro is the following, rather nonphysiological model. Spleen cells are activated with Con A and subsequently added to MLC or cultures of spleen cells and SRBC. Various authors have demonstrated that the subsequent proliferation (288) or production of antibodies (96, 97) is severely suppressed, suggesting that Con A stimulates the development of suppressor cells for these functions. These suppressor cells (both the precursors and the final effector cells) express the Lyt 23 antigens (39, 72, 107, 175, 311, 355), i.e., the same markers as do killer T cells. Whether cytotoxicity and suppression are manifestations of two distinct Lyt 23^+ subclasses is unclear. Another type of suppressor cell was reported more recently: antigen-activated (in this case, SRBC) Lyt 1^+ helper cells induced suppressor cells in a nonactivated Lyt 123^+ population (73), which consequently suppressed in vitro anti-SRBC antibody production.

It is not known whether changes in the number of suppressor T cells occur with increasing age. Information on this point would contribute to understanding the role of the postulated accelerated loss of suppressor cells in various immunologic disorders in NZB mice (345, for a recent review, see ref. 344), a suggestion recently confirmed (73) by the demonstration of a lack of Lyt 123^+ cells in NZB mice, which therefore cannot develop feedback inhibition. In strains of mice free of overt autoimmune phenomena, an increase in suppressor T cells has been suggested as one of causes for a decline in immune functions (133, 236, 308, 309), but direct estimates of the number of suppressor cells have not been reported.

1.1.6 Concluding remarks

An overview of the influence of senescence on thymus-dependent immune parameters has been presented. The indications are that T cells with the capacity to proliferate and further differentiate efficiently into effector cells are gradually lost with increasing age. Most authors have reported that these defects cannot be attributed to an overt loss of Thy 1 positive cells (123, 164, 241, 246, 329, 330). The observed functional defects may be explained either by shifts in the proportion of Thy 1 positive responder and nonresponder T cells or, alternatively, by defects in interactions between the different T cell subpopulations rather than by intrinsic defects in particular subpopulations only (124).

The loss of the regulatory role of the thymus in T cell differentiation is considered to be one of the keys, if not the only key, to T cell ageing (177, 235). However, in view of recent findings which indicate a loss of T cell progenitor cells in ageing bone marrow and spleen (33, 356, 357), it would appear that not only the thymus affects T cell ageing. Apart from directing attention to the bone marrow, these findings also distort the picture of the ageing T cell system as sketched above, since, theoretically, a decrease in bone marrow precursors should also lead to a quantitative decrease in peripheral T cells. This hypothesis is in contradiction with the generally observed normal level of T cells (123, 164, 241, 246, 329, 330) as determined by cytotoxicity assays. Perhaps the exceptions provide a clue to this controversy, i.e., by using immunofluorescence techniques rather than cytotoxicity tests, a few authors have reported a definite decrease in the relative number of splenic T cells (52, 62); also a decrease in relative number of peripheral T cells was found in the prematurely ageing NZB mice (154, 326, 330). Alternatively, the Thy 1 positive cells still found in ageing mice represent long lived T cells which are no longer fully competent.

What emerges from all of the above mentioned studies is that ageing probably results in both a decrease in the number of precursor T cells in the bone marrow and development of defects in the regulation of differentiation by the thymus. The latter conclusion is substantiated by the studies of Hirokawa et al. (156-158) which indicated that, even in combination with young bone marrow (i.e., when transplanted into young recipients), old thymuses fail to reconstitute T cell functions in thymectomized recipients. It should be realized that changes in thymic influence and in haemopoietic cells during ageing might be related to each other. In thymectomized mice, haemopoietic bone marrow cells show reduced numbers of colony forming cells (CFU-s) and a lower proliferative rate (386-388), although these results could not be confirmed with 'nude' mice, using other strain combinations (281). The reduced CFU-s content of thymectomized mice can be restored by thymic transplantation or in vitro thymus hormone treatment (388). Thus, the thymus might also provide a feedback mechanism for the regulation of lymphopoiesis (359), but a direct effect of thymus deprivation on T lymphocyte precursors in the bone marrow has not yet been demonstrated. Such a feedback mechanism also could explain why old bone marrow in combination with young thymus (i.e., when transplanted into young irradiated recipients) succeeds in restoring various immune functions, while young bone marrow in combination with old thymus (i.e., when transplanted into old irradiated recipients) does not (159, 246). On the basis of these assumptions, the relationship between the thymus and bone marrow during ageing appears to be a promising area for future investigation.

1.2 T CELLS IN TUMOUR BEARING ANIMALS

Since the suggestion was made that the immune system represents a natural host defense mechanism to oppose development and spread of neoplastic cells (58), most immunologically oriented cancer research has been directed towards answering the question as to how the immune system affects the development and growth of tumours. There has been far less experimental scrutiny of the opposite process, viz., how the presence of a tumour affects the immune system. The high incidence of tumours in old age (56, 319) justified a separate investigation into this problem. Patients with advanced cancer have long been known to have suppressed delayed type hypersensitivity responses to various antigens (120, 218, 226, 322) as well as depressed humoral immune responses (222). Whether such depressed cellular and humoral responses precede or are a consequence of the disease is often uncertain. In the case of cancer patients, however, the demonstration of immunodepression has frequently been associated

with poor prognosis (153). In addition, high susceptibility to infections is one of the most common causes of death in cancer patients (55, 169, 192). Thus, insight into the mechanisms of cancer-related immunosuppression could contribute to the treatment of these side effects in patients.

In the early seventies, it also became apparent from studies on animal models that tumours exerted immunosuppressive effects, especially on thymus dependent immune functions (see e.g. 40, 232, 298, 299, reviewed in 181). This strengthened our opinion that, because of our interest in disturbances in T cell immune competence and their possible mechanism(s), the effects of tumour bearing on T cell functions should also be investigated in the framework of our ageing studies.

As in the ageing studies, most knowledge of the effects of tumours on the immune system of experimental animals stems from in vitro studies of lymphocyte function, but these have so far been restricted mainly to proliferative capacity of T lymphocytes. It became apparent during the course of our studies that instead of intrinsic defects in the T cells themselves, changes in the macrophage population were responsible for the observed defects (see below). Also in the studies described in this thesis, macrophages were implicated as the suppressive elements in tumour bearers (see Chapters IV and V). Therefore, literature data on suppressive effects of macrophages on T cell functions in tumour bearers will be summarized in this section. The possible mechanisms of the suppressive effects of macrophages will also be discussed. A survey of the helper and suppressor functions of macrophages in normal mice and rats is found in the introduction and discussion of Chapter VI.

1.2.1 Suppressed T cell proliferation in tumour-bearing animals: Role of macrophages

Spleen cells from mice bearing tumours that were induced by inoculation with Moloney sarcoma virus (MSV) respond poorly if at all to T cell mitogens such as PHA and Con A (185). This defect can be fully restored by a number of methods for depleting cell suspensions of macrophages: passing spleen cells over a rayon column (185), pretreatment with iron powder and a magnet (186), or treatment with carrageenan (189), a substance which is specifically toxic for macrophages (217). Further, the suppressor activity is resistant to 2500 rads of X-irradiation (189) and to treatment with anti-Thy 1 antiserum and complement (186, 189). These data indicate that the MSV spleen cells contain a subpopulation of radioresistant, phagocytic, carrageenan-sensitive cells, probably macrophages or monocytes, which could suppress the proliferative response of T

lymphocytes to mitogens. Proliferative responses induced by allogeneic cells also were found to be suppressed by macrophages in MSV spleens (110). Subsequent reports concerning studies of tumour bearing rats (131, 361) and mice (276) demonstrated that splenic macrophages also suppressed responses to mitogens and alloantigens in other tumour models.

With regard to effects of tumour bearing on specific antitumour responses, few reports have appeared so far. In both mice (189) and rats (131, 261), T cell proliferative responses of spleen cells from tumour bearers to tumour-associated antigens were strongly impaired. It has been shown that these depressed responses can be restored by removal of adherent or phagocytic cells (131, 189, 261), indicating that again macrophages were responsible for the observed defects.

These findings have recently been confirmed in patients: it was reported that the decreased proliferative responses of peripheral blood lymphocytes of some cancer patients could be reconstituted by passing the cells through a Sephadex G-10 column (which removes monocytes) (37), by pretreatment of the cells with carrageenan (63) or by depleting them of adherent cells (381).

Analysis of the above data indicates that in vitro T cell proliferative capacity in tumour bearing mice, rats and humans is fully developed, but its expression is prevented by the presence of suppressor macrophages. This information may contribute to the understanding and treatment of immune deficiencies in cancer patients, which were initially considered to be due to intrinsic defects in the T cell population.

1.2.2 Possible suppressive mechanisms of action of macrophages in vitro.

Incorporation of labelled thymidine, the appearance of blast-like cells and the generation of killer T cells were reduced in mouse MLC by suppressor macrophages from MSV spleens (109, 110). Thus, it seems likely that a major mechanism of action of suppressor macrophages in vitro is inhibition of lymphocyte proliferation with consequent limitation of the expansion of an antigen stimulated clone of lymphocytes. Malignant lymphoma cell lines, which are rapidly proliferating cells which do not need to be stimulated to proliferate, are also sensitive to the suppressive effects of macrophages (178, 261).

A number of investigators have reported that macrophages exert their suppressive effects on lymphocyte proliferation through the production of suppressive factors (60, 194, 205, 367). There are several ways in which macrophage-derived factors could inhibit thymidine uptake or lymphocyte proliferation.

a) Production of thymidine

It has been suggested that the decrease in TdR-incorporation caused by macrophages is due to macrophage-derived cold thymidine, resulting from ingestion and degradation of DNA released from dying cells (263, 264). Thus competition of cold thymidine with radioactive DNA precursors would lead to decreased TdR uptake, which, however, does not reflect true suppressed proliferation. More recently, these findings were confirmed by others (323), who demonstrated that the "macrophage-derived suppressive factor" originally described by Calderon et al. (60) was also thymidine. In addition, it was found that part of the thymidine was probably synthesized and released by the macrophages (323).

In the light of all these findings, caution must be exercised in evaluating the nature of suppressive effects of macrophage-derived soluble factors. In order to be seriously considered as a factor or cell which may have immunosuppressive activity in vitro, any suppressive substance or cell type which inhibits labelled thymidine uptake must be shown to actually inhibit cell proliferation. These conditions were fulfilled in some studies (110, 183), indicating that the macrophages in MSV spleens actually inhibited cell proliferation, in addition to inhibiting labelled thymidine uptake.

In order to avoid tissue culture artifacts such as competition between labelled and unlabelled thymidine, one could: 1) use high concentrations of thymidine of low specific activity (as shown in ref. 109 and in the studies described in Chapter IV); 2) wash the proliferating cells in which labelled thymidine uptake is to be assessed prior to pulse labelling, in order to remove competing extracellular thymidine pools (as was also performed in the studies described in Chapter IV).

b) Production of prostaglandins

Depressed T cell mitogen responses of peripheral blood lymphocytes from patients with Hodgkin's disease could be restored to normal values by inhibition of prostaglandin E2 (PGE2) production through addition of indomethacin to the cultures or removal of glass adherent cells (144). These findings suggest that PGE2 production by monocytes was responsible for the suppressive effects observed in these patients. It was subsequently demonstrated (143) that the amounts of PG of the E series produced in cultures of normal human peripheral leucocytes by monocytes ($> 10^{-8}$ M) are sufficient to inhibit T cell mitogen responses. These data have been confirmed by others (216), who additionally showed that normal mouse peritoneal macrophages also synthesize and release PGE. No data are known concerning

PG production by the suppressor macrophages in the tumour models described in section 1.2.1.

It seems appropriate at this point to refer to the experiments of Plescia et al. (275) which suggest that tumour cells can exert immunosuppressive effects through production of PGE when added to lymphocyte cultures. In view of the fact that many tumours contain large numbers of macrophages, there is the possibility that these findings were also due to macrophage-derived PGE.

c) Production of arginase

Production of the enzyme arginase has been found to be markedly increased in macrophages cultured for 24 h (214). As a result of this elevated arginase production, arginine has been shown to be completely depleted from cell-free supernates of mouse MLC which were suppressed by the addition of excess numbers of macrophages (214). Complete reversal of the suppression was accomplished by the addition of arginine to the nonadherent cells after 2.5 days of culture and by removal of the adherent macrophages. These results were recently confirmed by Webb et al. (370), who demonstrated that depletion of arginine from the culture medium was a major factor in the suppression of mitogen responses in tumour bearing rat spleen cells. The response of normal rat spleen cells could also be enhanced by adding arginine, indicating that the response of normal spleen cells is apparently limited by the availability of arginine (370).

It remains to be investigated whether in the macrophage-induced suppression in other tumour models (section 1.2.1), arginine depletion is also involved.

1.2.3 Evidence that macrophages can suppress immune responses *in vivo*

It is rather difficult to relate the above *in vitro* findings to the role of macrophages in regulating the immune response *in vivo*. The main approach taken to evaluate the *in vivo* role of macrophages has been to administer agents such as, e.g., *Corynebacterium parvum* (*C. parvum*) which can stimulate the reticuloendothelial system (RES) and cause systematic activation and migration of macrophages into the lymphoid and circulatory systems. *C. parvum* induces splenic suppressor macrophages in mice (184, 306) which inhibit *in vitro* T cell proliferation. In addition, it has been shown that *in vivo* protection against syngeneic tumours by immunization with irradiated tumour cells is strongly decreased by pretreatment with *C. parvum* (321, 378) and that this is due to a di-

minished capacity to generate cytotoxic effector cells (182). Although these observations by no means prove that the interference with tumour resistance (182, 321, 378) is due to macrophages, similarities between the in vivo and in vitro observations are striking.

Similar correlations between in vivo and in vitro findings were reported for mice suffering from graft versus host disease (GvH). The depressed in vitro response of GvH spleen cells to SRBC (315) could be corrected by removal of phagocytic cells (316). In vivo responses to SRBC (41,253) or Keyhole Limpet Haemocyanin (41) were also strongly suppressed in GvH mice. In addition, allogeneic skin graft survival time was found to be prolonged in GvH mice (219). Thus, GvH disease appears to be another example of a situation in which suppressed in vivo responses are correlated with suppressed in vitro responses mediated by macrophages. Such a correlation has not yet been reported within one model for tumour-bearing animals.

1.2.4 Concluding remarks

The evidence discussed in this section supports the hypothesis that macrophages exert an immunosuppressive effect on T cell function in tumour bearing animals, at least in vitro. Various mechanisms through which macrophages exert their suppressive effects were discussed, but no conclusive evidence for preferring one over the other has been obtained so far. Under several experimental conditions associated with RES stimulation, suppressed T cell proliferation by suppressor macrophages in vitro could be correlated with evidence for immunosuppression in vivo. Thus, one way in which tumours cause immunosuppression might be through a mechanism by which tumour cells or tumour cell products activate the reticuloendothelial system, which in turn leads to suppression of immune responses requiring lymphocyte proliferation. The only way to answer the question as to whether tumour bearing-induced macrophages also exert immunosuppressive effects in vivo is to investigate the effect of in vivo macrophage depletion (e.g., with carrageenan, ref. 170, 171) on in vivo immune parameters in tumour bearing animals.

In summary, unlike in age-related deficiencies of T cell function, the tumour-associated decrease in thymus-dependent immune competence was not due to intrinsic changes in the T cells themselves.

1.3 THYMIC FACTORS AND THEIR IN VITRO EFFECTS

Ever since the crucial role of the thymus in the development of the immune system was recognized (248, 250), immunologists have been engaged in a search for an endocrine function of the thymus. This interest was based on the demonstration that grafting a thymus enclosed in a cell-impermeable Millipore chamber (268) or pregnancy (267) restores immune competence in neonatally thymectomized (neoTx) mice in a way similar to thymus grafting. These experiments were initially criticized, since others demonstrated that the pore size of the chambers used (0.45 μm) was not completely cell-impermeable (74, 151, 336). However, they were repeated with strictly cell-impermeable 0.22 μm pore size chambers and the conclusions were confirmed (266, 337). In addition, similar results were obtained with chambers containing an epithelial thymoma (337), a finding which disclosed the role of the thymic epithelial cells in thymic humoral function . Restoration of immune competence involved graft versus host reactivity (337, 339), skin allograft rejection (268, 337, 339) and anti-SRBC responses (220, 339). More recently, it has been shown that thymus grafts in millipore chambers can also lead to restoration of the circulating thymic factor (TF) level (see below) (89), disappearance of weakly Thy 1-positive precursor T cells in athymic nu/nu mice (hereafter referred to as nude mice) (292) and repair of the depressed CFU response of adult Tx mice (117).

Convincing results with regard to in vivo restoring effects of thymic factors derived from extracts or serum on thymus related immune deficiencies are scarce (for a recent review, see 42). In addition, it seems that literature data on restoration of immune responses by thymic factors are difficult to reproduce (42, 91). Studies performed in our institute in which nude mice, adult Tx and "B mice" were treated with different thymic factors according to regimens as reported in the literature (85, 168, 255) have not given positive results (M.J. Blankwater and I. Betel, unpublished observations). It should be realized that such in vivo experiments might be hampered by the facts that: a) a very long treatment schedule may be required, since reconstitution with free thymus grafts also takes 10 weeks to be completed (90, 249, 338); b) the use of crude heterologous extracts makes the results difficult to interpret because of the risk of antigenic stimulation; c) the half-life of serum thymic factor (19) and thymosin (88) when injected into mice is relatively short. Consequently, successful restoration with serum thymic factor was observed only when the factor was bound to carboxymethyl cellulose, an insoluble material providing a depot preparation (19). However, only restoration of Thy 1^+ cells in adult mice was reported (19), without data on functional restoration; and d) it has not

yet been conclusively demonstrated which is (are) the target cell(s) for the action of thymic factors. Thus, the choice of the appropriate animal model cannot be rationalized.

Most of the present knowledge of the biological effects of thymic factors comes from *in vitro* studies. These studies can be divided into those involved with T cell markers, T cell proliferative capacity and effector T cell functions. In sections 1.3.3., 1.3.4, and 1.3.5, the effects of several thymic factors with regard to their capacity to modify these three T cell parameters *in vitro* will be summarized. The methods by which these parameters can be analysed have already been introduced in sections 1.1.3, 1.1.4 and 1.1.5.

1.3.1 Introduction to the putative target cells for the action of thymic factors

Although the target cell(s) for the action of thymic factors have not yet been precisely identified, three different types of target cells can be postulated on the basis of *in vivo* studies performed in mice: prethymic precursor cells, intrathymic and postthymic precursor cells. An attempt will be made to fit the *in vitro* data on thymic factors into a scheme (Figure 1.1, p. 13) representing the possible interrelationships among these target cells. This scheme is based on the following observations in mice.

Step A (from stem cell to prethymic precursor cell): The fact that weakly Thy 1-positive cells are found in the spleen of nude mice (291-293), even in nude mice born from homozygous nu/nu parents (231), indicates that the generation of this cell type is thymus-independent. It can be regarded as being under a negative thymic influence, i.e., it is not present in normal mice and disappears under thymic humoral influence (291).

Step B (from prethymic precursor cell to postthymic precursor cell): The *in vivo* studies of Stutman (331-335, 339, 340) and others (229, 273) indicate that, for prethymic precursor cells, humoral thymic influence is *not* sufficient to induce maturation to competent T cells. In contrast, traffic through an intact total thymus is a prerequisite for further differentiation.

These prethymic precursor cells are found in the bone marrow, in foetal liver before day 14-16 of embryonic life and in the nude mouse spleen (335, 340). The prethymic precursor cell in the nude mouse spleen is identified by the presence of low amounts of Thy 1 antigen which can be detected only with sandwich immunofluorescence techniques (231, 291-293) or with rabbit antimouse brain antiserum (300) and not with cytotoxicity or direct immunofluorescence techniques.

Step C (from postthymic precursor cell to postthymic T cell): Many data from in vivo experiments indicate that humoral thymic influence (as provided by thymus transplants in cell-impermeable millipore chambers) can confer upon postthymic precursor cells several proliferative and functional properties associated with mature T cells (see e.g., 333-335, 338-340).

These postthymic precursor cells are found in different quantities in spleen, blood, bone marrow, lymph nodes and neonatal spleen and can be easily killed by treatment with anti-Thy 1-antiserum and complement (333, 334), thus being distinguishable from the prethymic precursor.

Cortical thymocytes (i.e., most of the corticosteroid sensitive cells) can also be regarded as belonging to the category of postthymic precursor cells on an operational basis (339), but will be dealt with separately in the present review under the heading "intrathymic" precursors. These intrathymic, corticosteroid sensitive precursor T cells are, however, different from the peripheral, postthymic precursor cells in that they express the TL surface antigens (198, 199, 285), whereas peripheral precursor cells do not. The relevance of TL antigens as differentiation markers is, however, questionable, as they do not occur in all mouse strains (see section 1.1.3a). It has been shown (335, 373) that at least part of these immature corticosteroid sensitive thymocytes migrate to the periphery where they undergo further maturation. Hence, the bulk of peripheral T cells would be derived from cortical thymocytes (335, 373). These migrating thymocytes have already lost the TL antigens before leaving the thymus (284). Peripheral postthymic precursors disappear within 30-60 days after neoTx (338, 339), which explains why thymus grafts in millipore chambers at 30 days or later after neoTx are no longer effective in restoring immune functions (338, 339).

In summary, the above data fit in with the concept of Stutman (reviewed in 335) which states that a subpopulation of T cell precursors, termed postthymic, is present in the lymphoid and haemopoietic tissues of adult and newborn mice. These cells are defined by their thymus dependency for renewal and represent the most likely candidates for the action of the humoral influence of the thymus. On the other hand, the early embryonic haemopoietic tissues and the nude mouse spleen contain a population of cells, termed prethymic, that is "insensitive" to the humoral function of the thymus and requires contact with the thymus microenvironment for its further differentiation.

1.3.2 Introduction to the thymic factors to be discussed

Factors prepared from thymic extracts are: a) thymosin (136) isolated according to the procedure described in ref. 161 and originally characterized by its capacity to enhance lymph node DNA synthesis after in vivo injection (134); b) THF (thymic humoral factor) (353), isolated according to the procedure described in ref. 203 and initially recognized by its capacity to restore in vitro GvH reactivity of neonatally Tx mouse spleen cells (352); c) thymopoietin I and II, isolated on the basis of their capacity to block neuromuscular transmission (138). It was later shown that these products were capable of inducing the expression of various T cell markers in normal bone marrow and spleen cells (31, 139, 301), thus introducing them into immunology.

In addition to thymic extracts, two thymus-dependent serum factors have been described: a) TF (thymic factor) (13), a factor in mouse serum which disappears after thymectomy (15, 16) and induces Thy 1 antigen on rosette-forming spleen cells from adult Tx mice. This factor also has been shown to occur in human (17, 19), calf, sheep, pig and rat serum (19) and is, since it was synthesized, also referred to as "FTS" (i.e., facteur thymique serique) (20); b) SF (serum factor), a thymus-dependent factor in human serum, identified on the basis of its capacity to increase intracellular cAMP levels in mouse thymocytes (7).

Thymic factor(s) are secreted by the epithelial cells in the thymus (78, 89, 155, 165, 176, 193, 237, 337, 338). Hence, another putative source of thymic factors is represented by supernatants from thymic epithelial cultures (TES) which were originally reported by Pyke and Gelfland (282) to be capable of inducing E rosettes in human bone marrow cells and later by Papiernik et al. (270) to induce Thy 1 antigen on spleen cells from adult Tx mice. Chapters VII to IX of this thesis report the effects of such supernatants on T cell proliferation and T cell function and also on some T cell markers.

1.3.3 Effects of thymic factors on T cell differentiation antigens

a) *Prethymic precursor cells*

With thymosin, it was demonstrated that Thy 1 antigen (21) could be induced in Thy 1 negative cells (as determined by cytotoxicity) in low buoyant density cells from normal mouse bone marrow or adult Tx mouse spleen. These findings were confirmed by others (196), who additionally demonstrated that Thy 1 antigen also appeared in nude mouse spleen and bone marrow cells as well as in fetal liver cells from 14 or 18-day-old embryos after a 2 hr incubation with thymosin (196). Thymopoietin I and II also can

induce Thy 1 and TL antigens in mouse bone marrow cells (31, 139, 301). In addition, both thymosin and THF induced Thy 1 antigen on normal mouse bone marrow rosette-forming cells (RFC) (88). It should be emphasized at this point that the term prethymic precursor cell is used as an operational definition, i.e., a Thy 1-negative cell (on the basis of cytotoxicity tests) which has not yet undergone thymic influence, derived from haemapoietic precursor cells (33, 98) and capable of repopulating the thymus of irradiated recipients (33, 98).

b) Intrathymic precursors cells (thymocytes)

Apart from being divided into cortisone-sensitive and cortisone-resistant cells (see Chapter 1.1.1b), mouse thymocytes can also be classified on the basis of antigenic properties of their cell membranes. As mentioned previously, a major population, about 90%, possesses TL antigen (in TL positive mouse strains), high levels of Thy 1 antigen and low levels of H-2 (199, 284, 285). The remaining minor population has antigenic properties similar to peripheral T cells, viz., absence of TL, low levels of Thy 1 and high levels of H-2 (199, 284, 285) and is relatively resistant to corticosteroid treatment.

The experiments of Weissman and associates have demonstrated that the cortisone-resistant medullary thymocytes are derived from at least part of the intrathymic pool of cortisone-sensitive cortical precursor cells (372). In addition, these investigators (106, 374) demonstrated that the low Thy 1 positive cells represent progeny of the cells bearing high levels of Thy 1 antigen. Thus, if thymic factors affect thymocyte differentiation in vitro, one would expect that a decrease in the number of Thy 1 positive cells occurs. With thymic epithelial culture supernatant, such an effect could indeed be demonstrated (Chapter IX).

It cannot be excluded, however, that also subclass maturation pathways other than the one described here (high Thy 1 cells → low Thy 1 cells) occur (95, 162, 163, 312). In the latter reports, evidence for self-replication of the low Thy 1 positive cells was presented. The results suggested that the low Thy 1 and high Thy 1 subpopulations in the thymus represent largely independent lines of development, rather than having a precursor-product relationship. The possibility exists that different experimental designs reveal different maturation pathways.

c) Postthymic precursor cells

No effects of thymic factors on Thy 1 positive cells in the periphery have been reported.

1.3.4 Effects of thymic factors on mitogen or alloantigen induced lymphocyte proliferation.

a) Prethymic precursor cells

The only reports on the effect of thymic factors (in this case, thymopoietin) on prethymic bone marrow precursors stem from Basch and Goldstein (31, 32), who demonstrated that low-density bone marrow cells showed a small increase in Con A responsiveness after preincubation with thymopoietin, in addition to the earlier reported induction of Thy 1 antigen (31). Others (6) demonstrated that a thymosin-like extract promotes the response of nude mouse spleen cells in MLC.

According to Stutman's theory on T cell maturation *in vivo* (335), nude mice should be totally refractory to restoration with thymic factors, since they are devoid of postthymic precursor cells, the putative target cell for thymic humoral function. This prediction has been confirmed in several *in vivo* studies involving restoration attempts with thymus transplants in cell-impermeable millipore chambers (273, 331, 332, 335), which were indeed unsuccessful. Thus, the finding of an enhanced responder cell capacity in MLR after *in vitro* thymic factor treatment (6) contradicts the expectations based on *in vivo* findings.

b) Intrathymic precursor cells

Incubation of thymocytes with thymosin results in an increase in their responder cell capacity in MLR (6, 81) as measured by TdR incorporation. Similarly, preincubation in THF-containing medium for 1 hr also promotes this reaction in thymocytes (358), but not in cortisone-resistant thymocytes (358). Also TdR incorporation into Con A and PHA stimulated thymocytes can be enhanced by a 24-h preincubation in THF (296). Thus, these authors consider these results to be the consequence of a thymosin- or THF-driven differentiation step of immature thymocytes into more competent T cells. However, an increased response or selective survival of the few responsive cells in the thymus could also explain the results, and this possibility still has to be excluded.

Data on the effects of thymic epithelial culture supernatants on MLR and mitogen responsiveness of thymocytes will be presented in Chapters VII and VIII.

c) Postthymic precursor cells

Both T cell mitogen responsiveness (297) and mixed lymphocyte reactivity (358), as measured by TdR incorporation, have been reported to be

enhanced by preincubation of responder spleen cells with THF. Such findings can be interpreted as suggesting that THF acts on postthymic precursors However, no data are available with regard to the surface markers of the target spleen cells (i.e., are they Thy 1^- or Thy 1^+); furthermore, the results could also be explained by increased responses of already reactive cells. The latter hypothesis could be tested by employing cell separation techniques in order to select a population of nonresponsive cells. Doing this, Basch and Goldstein (31, 32) reported a small increase in the Con A responsiveness of low density spleen cells under the influence of thymopoietin, but the extent of the enhancement was very small and must, therefore, be questioned.

With thymic epithelial supernatants, no effect on T cell proliferative capacity was ever observed in postthymic precursor cells (see Chapters VII and VIII) when unseparated spleen cell suspensions were used.

1.3.5 Effects of thymic factors on effector T cell functions

a) Prethymic precursor cells

Miller et al. (247) reported that normal bone marrow cells preincubated in vitro with a thymosin-like extract could replace T cells in the reconstitution of the anti-SRBC response of lethally irradiated mice, suggesting that helper cell function in bone marrow precursors was induced. GvH reactivity also could be induced in bone marrow cells with thymosin (135), but this proved to be impossible with THF (317). Bone marrow contains both postthymic (339) and prethymic precursor cells (33, 98), but, from the above data (135, 247, 317), it cannot be judged which was the target cell for the observed effects of thymic factors, since no data were provided on the Thy 1 expression of the target cell, i.e., Thy 1^- or Thy 1^+.

Thymosin also induced helper T cell function in nude mouse spleen (6), in contrast to expectations based on Stutman's theory (335 and section 1.3.3a).

b) Intrathymic precursor cells

Thymocytes display poor, if any, helper T cell function for humoral immune responses in vitro(see Chapter VIII) or for the generation of killer cells (70) and can hardly be induced by Con A to exhibit suppressor T cell function (287), unlike spleen cells (96, 97, 288). In addition, only few cytotoxic effector cells can be derived from thymocytes stimulated in mixed

lymphocyte cultures (365) and precursor killer T cells are found only in the cortisone-resistant population (365). This low level of functional T cell capacities is correlated with relatively low numbers (i.e., as compared to peripheral lymphoid organs) of cells with the phenotype of specialized functional T cells, i.e., Lyt 1^+ 23^- cells (containing helper cells) and Lyt 1^- 23^+ cells (containing killer/suppressor cells) (see section 1.1.3c and ref. 69).

No literature data on the effects of thymic factors on thymocyte immune competence can be found. Effects of thymic epithelial culture supernatants on the helper and killer T cell functions of thymocytes are described in Chapters VIII and IX, respectively.

c) Postthymic precursor cells

Only with THF was an effect on postthymic precursor cell function shown: graft versus host reactivity can be induced in spleen cells from neoTx mice (352) and this effect is abolished when the target cells are pretreated with anti-Thy 1 antiserum plus complement (228). Spleen cells from antilymphocyte serum treated mice could also be restored by THF treatment (228), a finding which supports the postthymic nature of the target cell for THF, since postthymic precursor cells are considered to be insensitive to the effect of antilymphocyte serum (334). In addition, THF has been reported to increase the number of cytotoxic spleen cells generated towards a syngeneic tumour (75) in a mixed lymphocyte-tumour cell culture.

1.3.6 Effects of thymic factors on some other parameters

a) Intracellular cyclic AMP level

SF (7), thymopoietin (301) and THF (201) were found to elevate levels of intracellular cyclic AMP in thymocytes. It has been suggested that thymic factors exert their effects via cyclic AMP. This view was originally based on the indirect evidence that some effects of TF, THF and thymopoietin could be mimicked by agents which increase levels of intracellular cyclic AMP (10, 24, 25, 201, 202, 301, 302). Such agents include cyclic AMP, dibutyryl cyclic AMP, theophylline, isoproterenol, prostaglandins (PG) of the E series and polyadenylic uridylic acid (poly[A:U]). The effects of some of these agents can be blocked by their specific inhibitors or antagonists, i.e., flufenamic acid for PG (201), indomethacin for PG (24) and propranolol for isoproterenol (301).

Because the action of several hormones has been shown to be mediated by cyclic AMP (342), the above results were interpreted as permitting the

classification of these thymic factors as hormones. Thymic epithelial culture supernatants were also found to increase the levels of intracellular cyclic AMP in thymocytes (Chapter VIII).

b) Cortisone resistance

As mentioned previously (see 1.3.3b), the cortisone resistant thymocytes are considered by some authors to represent progeny of at least part of the cortisone-sensitive precursor cells in the thymus (106, 372, 374). Thus, it might be expected that incubation of thymocytes with thymic factors (assuming these factors induce T cell differentiation) makes some of these cells more resistant to corticosteroids.

This hypothesis was indeed confirmed for both SF (10) and THF (351), suggesting that these thymic factors confer a property of immunocompetent T cells (i.e., cortisone resistance) on previously immature T cells. Also thymic epithelial culture supernatants were found to increase the number of corticosteroid resistant thymocytes (Chapter IX).

c) Terminal deoxynucleotidyl transferase (TdT)

TdT is a T cell specific enzyme which is found only in bone marrow cells and thymocytes (84, 215). More specifically, it was found to be restricted to the cortisone-sensitive thymocyte population in the rat (29) and it is thought to be a marker for immature T cells. It is absent or present in low concentration in nude mouse bone marrow and disappears with time after thymectomy in normal mice (269).

Incubation of nude mouse bone marrow with thymosin or bone marrow from thymectomized mice has been reported to induce TdT expression (269).

d) Bone marrow colony forming units

In connection with the ageing studies, it has been mentioned above (see 1.1.6) that the thymus also is involved in haemopoiesis. The colony forming capacity (as determined by the number of CFU-s) of bone marrow from thymus-deprived mice is greatly reduced (386, 387). This defect can be overcome by previous *in vitro* treatment with THF (388). So far, data on the effects of other thymic factors on CFU-s are not available.

The relevance of these findings remains questionable because the number of CFU-s in nude mouse bone marrow has been found to be similar to that obtained with normal mouse bone marrow (281). Also the number of cells capable of repopulating the thymus of lethally irradiated was similar in normal and nude mouse bone marrow (281).

1.3.7 Several other factors mediating effects similar to those reported for thymic factors

Before summarizing the above data on the current status of thymic factors, a review of a few other substances which might also be involved in one or more T cell differentiation steps will be given (assuming for the moment that some of the above-mentioned events are indeed manifestations of T cell differentiation). Among these substances are:

a) Agents increasing levels of intracellular cyclic AMP. These were shown to induce the appearance of T cell markers (24, 25, 301, 302), T cell function, i.e., GvH reactivity (201), and T cell proliferative capacity in MLR (202) as well as hydrocortisone resistance in thymocytes (10). It suffices to mention the effects of such products here, since they were previously discussed in more detail elsewhere (see 1.3.6a).

b) Factors released by cultured macrophages. Such factors were originally described by Gery and Waksman (127) and designated "LAF" (lymphocyte activating factor). LAF stimulates DNA synthesis in thymocytes and potentiates their response to T cell mitogens. Its production can be markedly increased by adding various stimulants to the cultured adherent cells (127). Both human blood adherent cells and mouse peritoneal macrophages can be used as a source of LAF (128). Furthermore, it was shown that LAF also enhances MLR and intracellular cyclic AMP levels of thymocytes (265). Similar factors have been described by others (59, 61, 204), who additionally showed that macrophage culture fluid (MCF), apart from the mitogenic principle, also contained factors which increased the anti-SRBC responses of nude mouse spleen cells (61, 204) and B cell proliferation in normal spleen cells (61). The molecular weights (m.w.) of the active moieties (LAF, MCF) reported by the various investigators were in general agreement, ranging from 14,000 to 20,000 daltons (61, 126, 204).

The group of Unanue has termed "their" macrophage-derived factor "TDF" (thymocyte differentiating factor, 34, 35), since they demonstrated that TDF also induces an increase in the expression of H-2D and K antigens and a decreased sensitivity to lysis by anti-TL antiserum plus complement in thymocytes. However, when macrophage culture supernatant was fractionated on Sephadex G-75, it was found that TDF had a different m.w. (i.e., 35,000 to 40,000) than the mitogenic factor which eluted, as previously reported, in a peak with a m.w. of about 15,000 daltons (34). TDF fractions exhibited much less mitogenic activity than LAF or MCF. It seems that TDF induces the

development of cells with the phenotype of mature T cells. Since macrophages in the thymus also produce TDF activity (35), it was speculated that macrophages are implicated in intrathymic T cell differentiation (35), while thymic factors would take care of the prethymic precursor to immature thymocyte differentiation step (35).

The reason for discussing macrophage-derived factors in the context of thymic differentiation is the following. In early thymic explant cultures (i.e., the starting phase of thymic epithelial cultures which represent the source of thymic factors in the study described in this thesis), many macrophages can be observed (see Chapter VII), although these cells disappear later. Thus, there is the possibility that those culture supernatants (TES) also contain LAF, MCF or TDF. Only chemical purification will answer whether TES contains LAF-, MCF- or TDF-like molecules. The fact that TES was shown not to contain a mitogenic principle (Chapters VII and VIII) is evidence that it does not contain LAF or MCF, but theoretically it could contain TDF, which hardly exhibits mitogenic activity (34).

c) Mitogens. T cell mitogens are capable of inducing Thy 1 antigen in previously Thy 1 negative bone marrow cells (82). The PHA-responsive cells of bone marrow are not thymus-dependent (46), in that they recover fully after thymectomy, irradiation and bone marrow reconstitution, whereas PHA responsiveness in other organs is restored only in the presence of a thymus. Furthermore, treatment of bone marrow with anti-Thy 1 does not affect its capacity to respond to PHA (46). Thus, the finding of Thy 1 induction by PHA provides an explanation for the occurrence of thymus-independent PHA-responding bone marrow cells. In PHA or Con A stimulated bone marrow cultures, which initially contained no detectable numbers of Thy 1 positive cells, lymphoblasts which are positive for both Thy 1 and TL antigens appear (82), suggesting that Con A and PHA induce differentiation of prethymic precursor cells. This suggestion has been more recently supported by the studies of Press and co-workers (278-280), who showed that even bone marrow cells from nude mice could respond to PHA (279) and that they acquired T cell surface antigens during the course of their blastogenic response to PHA (280), which requires a much longer culture period to reach optimal levels than that required for spleen and lymph node cells (278).

1.3.8 Concluding remarks

More questions than answers emerge from the above review. Among these questions are the following. Are the changes induced by thymic factors indeed associated with a true "differentiation" to functional T cells? Especially those studies dealing with prethymic precursor cells (21, 31, 88, 139, 196, 301) have mainly yielded information concerning T cell markers, but it is known from ontogeny studies (256, 325) that when a cell expresses a T cell marker this does not necessarily imply that it also displays proliferative and functional T cell properties. Hence, those factors which were shown to induce T cell markers in cells which, as determined by cytotoxicity, were Thy 1 or TL negative (i.e. thymosin, thymopoietin, TF) could tentatively be regarded as substances which are at least involved in step B of the T cell differentiation pathway (Figure 1.1). However, it is unknown whether acquisition of these T cell antigens represents the expression of an irreversible process of further differentiation to functional end cells which does not require further thymic influence. The latter possibility seems unlikely on the basis of, e.g., in vivo studies in nude mice (230), demonstrating that semiallogeneic thymus grafts did fully restore the number of Thy 1 positive cells in spleens and lymph nodes, but that even a year after grafting only moderate levels of T cell proliferative responses were observed.

It is also not known whether the different steps of the T cell differentiation pathway are controlled by different thymic factors, nor whether the development of different T cell functions is influenced by different factors. This brings us to the next question. Are there so many different thymic factors as described above and, if so, what is the functional relevance of all of these different entities? This question is further complicated by the fact that some of these factors are derived from thymus extracts (THF, thymosin, thymopoietin) while others are derived from serum (TF or FTS and SF). So far, chemical purification has clearly shown that the different isolation methods finally yield different products. Concerning the relationships among these products, the following possibilities arise: a) some factors are cleavage products of others but they represent similar biological activities, b) there is a family of factors which act in concert to induce T cell differentiation; c) a number of totally different principles exist and these exert different biological activities independently.

Such differences in biological activities could be manifested at the target cell level (i.e., prethymic, intrathymic and postthymic) as well as at the level of the type of effect (i.e., influences on markers, proliferative capacity and different functional properties). At present, it is impossible to obtain

a clear picture, due to the fact that none of the existing factors has been investigated systematically with regard to its properties on different types of target cells and in different assays for T cell markers or functions. Some (TF or FTS, thymopoietin) have been studied mainly with respect to their effects on T cell markers (15, 16, 19, 31, 139, 301). Other factors (THF, thymosin) were characterized mainly on the basis of their effects on mitogen or alloantigen induced T cell proliferation (6, 81, 297, 358). Only occasionally were effects on in vitro parameters for T cell function reported (GvH reactivity for THF (228, 352), for thymosin, 135; killer T cells: 75 for THF).

With regard to the target cells for thymic humoral function, perhaps some general conclusions can be drawn. The theoretically possible sites of action of thymic factors presented in Figure 1.1 were already discussed (p. 32). On the basis of the observations reviewed above, the following picture emerges:

Step B. Several in vitro data with thymic factors suggest that these can confer some of the properties of mature T cells upon prethymic precursors (markers: 21, 31, 139, 196, 301, 302; functional properties: 6, 135, 247). However, on the basis of numerous in vivo studies (see e.g., 229, 273, 331-334, 339, 340), one should not expect thymic humoral function to induce differentiation in such prethymic cells. Hence, these in vitro findings must be interpreted with caution, even more so because criteria used for evaluation of in vivo restoration (e.g., skin graft rejection time, GvH reactivity, regression of virally induced tumours) represent more biologically relevant phenomena than those used for the determination of "induction" of T cell properties in vitro.

Step C. Both in vitro data with thymic factors (e.g., 6, 10, 75, 81, 228, 296, 351, 352, 358) and in vivo experiments with thymus transplants in millipore chambers (e.g., 333-335, 338-340) suggest that humoral thymic influence can confer upon postthymic precursor cells several marker characteristics and proliferative and functional properties of mature T cells. This consideration is based on the assumption that the same type of (operationally defined) postthymic precursor cells is detected in these in vivo and in vitro experiments. However, it should be realized that interpretation of the in vitro experiments is difficult, since it is impossible to decide whether certain T cell properties were induced (as suggested in 6, 81, 297, 358) or whether simply amplification of already responsive cells has occurred. Nevertheless, the agreement between in vivo and in vitro data provides a firm basis for the postthymic precursor cells as potential target cells for the action of humoral thymic function and also permits the further use of these types of in vitro experiments for thymic factor studies.

The question of induction vs., amplification/enhancement of reactivity, continues to be a major problem in thymic factor studies: in order to detect

true induction, cell populations exhibiting none at all of the properties to be induced should be preferentially used. In the assays used so far for proliferative or functional capacity, this is usually not the case. This requirement has been fulfilled with marker induction (TL, Thy 1) (21, 31, 139, 196, 301), but here we encounter another problem of interpretation: does appearance of T cell markers represent the first step of a further differentiation process to cells which are also functionally active? As stated earlier, this question cannot be answered at this writing, though some suggestion for a correlation between in vitro marker and function induction has been reported (302). In any event, it seems wise at this time to base the working hypothesis concerning the target cell for thymic-hormone-induced differentiation on Stutman's concept (summarized on page 32,33) rather than on in vitro data: the in vivo data leading to the division of precursor T cells into prethymic and postthymic are more conclusive than the in vitro data suggesting induction of function in prethymic precursors with thymic factors.

When we now compare the different thymic factors with regard to their potential target cells, the following tentative conclusions may be drawn. Thymosin, thymopoietin and TF were all shown to exert similar effects (i.e., induction of T cell markers) on prethymic precursors (21, 31, 88, 139, 196, 301, 302), whereas all of the effects of THF reported so far are concerned with intrathymic and postthymic precursor cells (228, 297, 317, 358). In fact, bone marrow cells could not be induced in vitro to express GvH reactivity (317) or MLR (358) by THF. Thymosin, in contrast, induced in vitro GvH reactivity in bone marrow (135) and MLR in nude mouse spleen cells (6). Thus, we could tentatively regard thymosin, thymopoietin and TF as related principles which act on prethymic precursor cells. This possibility is further supported by the observation that in vivo thymosin treatment of thymectomized mice temporarily restores serum TF levels (88). It is more difficult to locate the other thymus-dependent serum factor, SF, in this comparison, since no effects of SF on prethymic cells have been reported. However, the finding that treatment of some immunodeficient patients with thymosin could induce the appearance of previously absent SF (8) suggests that SF might be related to thymosin; it could either be a cleavage product of thymosin or its production might be induced by thymosin. It should be realized, however, that the comparison of all of these different factors is based on observations with partially purified preparations only.

The working hypothesis advanced by Goldstein (137) is that the different components of the crude preparations will, when isolated, be found to possess distinct biological activities on distinct target cells. It is also possible that many of these components represent irrelevant contaminating proteins. This

applies specifically to the extracts thymosin and THF, the isolation of which is still in progress (see, e.g., 137, 203, 354) and has revealed the presence of numerous components. The factors which were actually purified (i.e., TF or FTS and thymopoietin, ref. 20 and 303, 304, respectively) have, unfortunately been studied mainly with respect to their effects on T cell marker induction, so it remains uncertain whether they can also induce functional T cell properties.

The data discussed in this section provide evidence which supports a fundamental role of thymic factors in the generation of several T cell properties. This evidence has been mainly derived from *in vitro* studies which admittedly must be interpreted with caution, but which nevertheless are invaluable for examining aspects of the T cell differentiation pathway that cannot be studied *in vivo*. In addition, this overview also shows how many gaps in the present knowledge about thymic factors and T cell differentiation still have to be filled up. It can be expected that the information derived from *in vitro* studies will help to design models for *in vivo* reconstitution, which is the ultimate goal of thymic "hormone" studies, in a more meaningful way. As mentioned earlier, convincing results with regard to *in vivo* restoration of thymus-related immune deficiencies by thymic factors are lacking.

1.4 OUTLINE OF THE PRESENT STUDY

To define more precisely the depressed lymphocyte proliferative capacity during ageing, as reported by many authors, mitogen responses of young and aged rats were compared under different conditions (Chapter II). Rats were initially chosen as the experimental model because of their greater availability and since survival studies and studies on the incidence of age-related lesions approached completion at that time (56).

In Chapter III, some aspects of the possible role of the thymus in age-related defects in T cell proliferative capacity are described. The sensitivity of intrathymic precursor T cells of young and old rats to the action of thymic factors will be compared, as well as T cell proliferative responses of rats exhibiting age-related thymus atrophy to a different extent.

In addition to the effects of ageing on the T cell proliferative capacity, the effects of tumour bearing on this parameter are also described (Chapter IV). Since reduced T cell proliferation in the tumour bearing spleen was found to be associated with an increase in the number of macrophages, we postulated that a quantitative rather than a qualitative change in the tumour-bearing splenic macrophages caused the suppression. The findings reported in Chapter V lend further support to this hypothesis, in addition to extending the previous data to other tumour models and lymphoid organs.

Chapter VI deals with the conditions required for macrophages to exert either suppressive or helper effects in T cell stimulation, a study which seemed warranted after the experiments reported in the previous Chapters had yielded conflicting data on the role of macrophages in T cell proliferation.

As outlined in the beginning of Chapter I, more detailed information on the nature and mode of action of thymic humoral factors is necessary before therapeutical application to correct age-related or other deficiencies in T cell immune competence can be envisaged. This requirement formed the basis for the experiments described in Chapters VII to IX, in which the effects of thymic epithelial culture supernatants (TES) on several T cell parameters and possible precursor T cells are reported.

Chapter VII contains a detailed description of the technique used to prepare TES and of its effects in the assay initially used for evaluation of the biological activity of TES preparations, i.e., mitogen-induced proliferation of thymocytes.

In the experiments described in Chapter VIII, the biological activity of TES is further evaluated by studying its effects on mixed lymphocyte reactivity, helper T cell function and intracellular cyclic AMP levels of thymocytes.

Chapter IX provides data on the effects of TES on two "markers" of thymocytes, i.e., Thy 1 antigen expression and resistance to cortisone, and on the effect of TES on another parameter for T cell function, i.e., killer T cell function in a CML assay.

A general discussion of the results is presented in Chapter X.

CHAPTER II

EFFECTS OF AGEING ON MITOGEN RESPONSES IN RATS

2.1 INTRODUCTION

Several authors have reported an age-related decline in mitogen-induced peripheral T lymphocyte proliferation in mice (123, 164, 239, 290). It cannot be judged from these reports whether this decrease in thymidine incorporation is, as the authors suggest, indeed due to a decrease in the number of stimulated cells. There could also be changes in the kinetics of the cell culture, e.g., a decreased survival of stimulated cells or a delay in the onset of proliferation of the same number of initially triggered cells, which could be overcome by prolonged culturing. Also differences in dose-response effects could occur. These would be reflected in differences in mitogen doses required for optimal stimulation. Up to now, however, age-related changes in mitogen stimulation have been studied at doses of mitogens and under culture conditions which were found to be optimal for lymphocytes from young animals. It was felt necessary, therefore, to study the mitogen response of lymphocytes from young and aged rats under various conditions, to construct mitogen dose-response curves and to determine viability and blast formation after culturing with mitogens.

The results presented in this Chapter provide baseline data concerning the age-related changes in Con A responses of spleen and blood lymphocytes and LPS responses of spleen cells from rats.

2.2 MATERIALS AND METHODS

2.2.1 Animals

(WAG x BN)F1 rats were used in all experiments. The parental strains were bred under specific pathogen free (SPF) conditions. The animals were fed standard AM mouse pellets (Hope Farms, Holland) with free access to tap water. Unless stated otherwise, female rats were used in all experiments. Culture and labelling conditions were established with rats of 12-14 weeks of age. Only data obtained with animals that showed no lesions on histological examination are included, except for the age group of 30 months in which all animals had

multiple lesions. Of the 20 rats of 30 months of age used, 18 had pituitary tumours, 3 had mammary tumours and various lesions were noted in other organs (49).

2.2.2 Mitogens, media, culture and labelling conditions

Lipopolysaccharide (LPS) from E. coli 0111:B4 was obtained from Difco Laboratories, Detroit, Mich.; Concanavalin A (Con A) was purchased from Calbiochem, San Diego, California. Fresh solutions of LPS and Con A (2 mg/ml medium and 1 mg/ml medium, respectively) were prepared for each experiment.

The culture media were either RPMI (RPMI 1640; Flow Laboratories, Irvine, Scotland) or HE (Dulbecco's modification of Eagle's medium on a Hanks salt solution base); both media were supplemented with penicillin (100 IU/ml), streptomycin (0.1 mg/ml) and 2 mM glutamine and buffered with bicarbonate.

As serum sources, either FCS (fetal calf serum, Flow Laboratories, Irvine, Scotland) or fresh RS (rat serum from Sprague Dawley rats) (360) were used. Blood and spleen cell cultures were placed in glass tubes (100 x 16 mm) and tightly sealed with rubber stoppers; they were kept in an incubator at 37°C without gassing.

Cultures were labelled with 0.15 µCi of ^{14}C-TdR by adding 50 µl of a solution containing 3 µCi ^{14}C-TdR (Radiochemical Centre, Amersham, England, specific activity (s.a.) 50 mCi/mmol), 194 mg glutamine (L-glutamine, Sigma, St. Louis, Mo.) and 0.01 mg thymidine (Sigma, St. Louis, Mo.) per 1 ml of HE (final s.a. 25 mCi/mmol). This solution will further be referred to as "labelling solution".

Radioactivity of the ^{14}C labelled compounds was measured by liquid scintillation counting (Nuclear Chicago Mark II counter). Labelled cells were harvested on glass fibre filters (type A-E, Gelman, Ann Arbor); the air-dried filters were placed in scintillation vials (Packard Instr., Zurich, Switzerland) and 5 ml of a toluene-based scintillation fluid (50 mg POPOP and 4 g PPO per litre toluene) was added.

2.2.3 Spleen cell cultures

The animals were anaesthetized with ether and killed by cervical dislocation; spleens were removed under sterile conditions and collected in HE. The spleens (pooled from 5 rats) were finely minced with a pair of scissors and filtered through a sieve of six layers of nylon gauze. Further manipulations

were carried out at 0°C. The cell suspensions were collected in Falcon tubes (2070), centrifuged at 200 g for 5 minutes, resuspended in a small volume (2 ml per spleen), and left in an ice bath for 15 to 30 min to allow dead cells to aggregate. Dead cell aggregates were removed by centrifugation at 50 g for 20 seconds (this sediment contained no living cells) and the supernatant was centrifuged at 200 g for 5 min. The procedure of dead cell aggregation and removal was repeated once. The final cell suspension was counted in a haemocytometer; cell viability as assessed by trypan blue staining was 85 to 90%. The cell suspensions were adjusted to 2 x 10^6 viable cells/ml. Triplicate cultures (1 ml of cell suspension in HE + 15% RS) were prepared for each experiment. In some experiments, 2 tubes were included for the determination of total cell number, cell viability and percentage of blast-like cells. The number of blasts was determined in May-Grünwald-stained smear preparations.

Mitogens were added in the amounts stated in the figures. After 24 h of culturing, the cultures were labelled with 0.15 µCi of ^{14}C-TdR.

Twenty-four hours later the tubes were put in an ice bath; the cells were harvested on glass fibre filters and washed with a solution of 0.5 g thymidine/l saline.

The filters were placed in scintillation vials, dried overnight at 37°C, and scintillation fluid was added. Results were expressed as the difference between the mean radioactivity incorporated into triplicate stimulated cultures and triplicate nonstimulated cultures; standard deviations within triplicate cultures were between 5 and 15%. Cells were usually cultured for 48 h; longer periods were used in some experiments (see Results), but the ^{14}C-TdR was added 24 h before harvesting, regardless of the length of the culture period.

2.2.4 Blood lymphocyte cultures

Animals were anaesthetized with ether; blood was taken by cardiac puncture and collected in sterile glass tubes coated with heparin (Venoject tubes, Jintan Terumo Co., Tokyo, Japan). Fifty ul of heparinized blood was pipetted into culture tubes and 2 ml of RPMI supplemented with 20% FCS and Con A were added. After 72 h of culturing, 0.15 µCi of ^{14}C-TdR was added in 50 µl of the labelling solution (see p. 48); the cultures were terminated 24 h later and processed as described for spleen lymphocyte cultures.

2.3 RESULTS

2.3.1 Effect of culture time on Con A stimulation in spleen cells from young and old rats

Three culture periods (48, 72 and 96 h) at 3 Con A concentrations (25, 50 and 75 µg per culture) were employed to investigate whether lymphocytes from young and old rats required different culture periods. No differences were found between incorporation at 48 and 72 h (Table 2.1); young lymphocytes always showed a two- to fourfold higher stimulation than did old lymphocytes (see also Figure 2.1). At 96 h, the response of spleen cells from both young and old rats had dropped to ~ 50% of the values obtained at 48 and 72 h, but the difference between old and young rats was still evident. Apparently, the lower incorporation values for old lymphocytes are not due to differences in the time-course of the reaction.

2.3.2 Effect of mitogen dosage on the Con A response of spleen and blood lymphocytes and the LPS response of spleen cells from rats of different ages

The response of blood and spleen lymphocytes from young and old rats to Con A was studied at multiple doses of mitogen to determine whether different doses would be required for maximal responses.

TABLE 2.1

COMPARISON OF Con A RESPONSIVENESS OF SPLEEN CELLS FROM YOUNG AND AGED RATS AFTER DIFFERENT CULTURE PERIODS

culture period	age	response in cpm x 10^{-3} at different Con A doses* 25 µg	50 µg	75 µg
48 h	3 months	124.3 ± 12.4	115.1 ± 9.6	99.6 ± 5.4
	28 months	41.1 ± 3.2	37.8 ± 1.8	42.2 ± 2.8
72 h	3 months	119.6 ± 8.8	120.7 ± 4.8	90.6 ± 6.2
	28 months	38.4 ± 1.9	43.9 ± 5.4	35.2 ± 2.1
96 h	3 months	61.2 ± 6.6	58.7 ± 2.7	41.6 ± 2.9
	28 months	20.3 ± 3.6	15.6 ± 1.2	7.5 ± 1.4

*Doses of Con A approximating the dosage giving a maximum response in young rats were selected (see Figure 2.2).

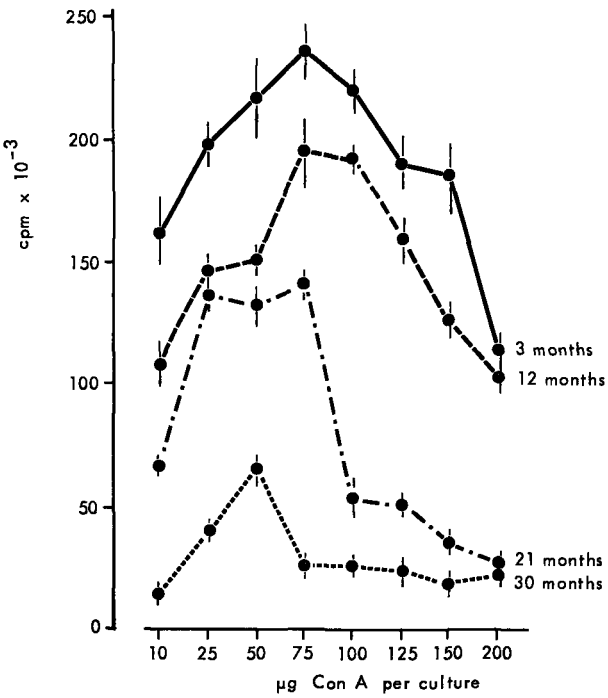

Figure 2.1

Lymphocyte proliferative response of whole blood from female rats of different ages as a function of Con A concentration. Each point represents the mean ± standard deviation of triplicate Con A-stimulated cultures. Data are representative for 4 separate experiments. Age groups: 3 months, 12 months, 21 months, and 30 months. For each age group, a pool of equal blood volumes of 5 rats was used. Background values in nonstimulated cultures were 1749, 1199, 1135 and 1122 cpm for animals of 3, 12, 21 and 30 months old, respectively.

Figure 2.1 presents data on the response of blood lymphocytes to Con A in a representative experiment. Cells from young rats always showed higher values than cells from old rats, whereas the dose-response curves showed similar patterns. Maximum responses were obtained with 75 µg of Con A, except for the oldest group in this experiment.

The Con A responses of cells from spleens of 3-month-, 12-month- and 21-month-old rats are compared in Fig. 2.2. At the age of 12 months, a decline in Con A response to 52-76% of the values for 3-month-old rats (range for all concentrations studied in 4 different experiments) were already observed; differences between 3 and 21 months are more striking: at the age of 21 months, TdR incorporation is only 20-35% of that in young lymphocytes. Just as for the Con A response in blood lymphocytes, no differences were found in dose-response effects for the different age-groups. Maximum responses were obtained at 25 or 50 µg of ConA.

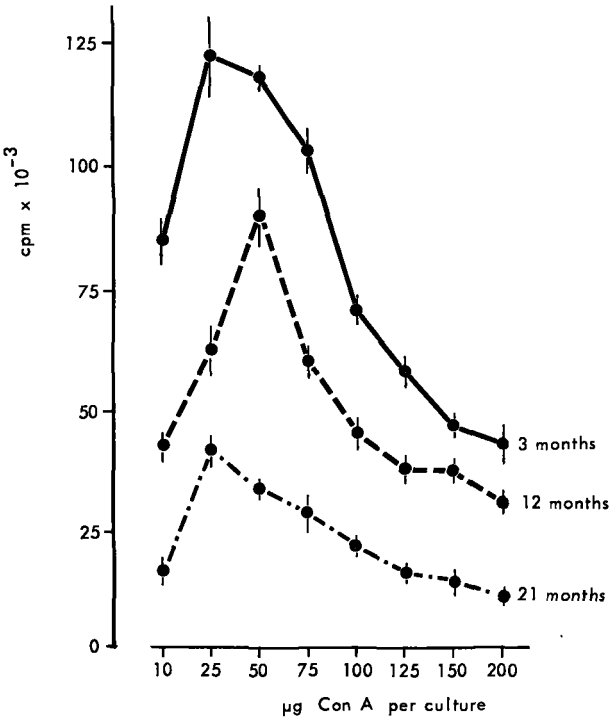

Figure 2.2

Lymphocyte proliferative response of 2 x 10^6 spleen cells from female rats of different ages as a function of Con A concentration. Each point represents the mean ± standard deviation of triplicate Con A-stimulated cultures. Data are representative for 4 separate experiments. Age groups: 3 months, 12 months and 21 months. For each age group, a pool of 5 spleens was used. Background values in nonstimulated cultures were 1305, 1968 cpm and 1661 cpm for animals of 3, 12 and 21 months old, respectively.

In 6 experiments (using animals of 3 and 21 months of age), the total number of cells left after 48 h of culture with 50 µg Con A and cell viability were determined (Table 2.2). There was no difference in the extent of cell death in spleen cell cultures from the 2 age-groups; cell recovery and viability are essentially comparable. There is a difference, however, in the number of blast-like cells (as determined in May-Grünwald Giemsa-stained smear preparations) in young and old lymphocyte cultures; the number of blasts in the cultures of old spleen cells is ~ 40% of that in young cultures, whereas the amount of ^{14}C-TdR incorporated into 21-month-old spleen lymphocytes varied from from 27 to 35% of that in young lymphocytes in these experiments. Thus, it is evident that the decreased ^{14}C-TdR incorporation values for old lymphocytes cannot be correlated with poor survival of the old spleen cells in vitro, but there seems to be a decrease in the number of blast-forming cells.

TABLE 2.2

CELL RECOVERY, SURVIVAL AND BLAST FORMATION IN SPLEEN CELL CULTURES FROM YOUNG AND OLD FEMALE RATS AFTER 48 h STIMULATION WITH 50 μg Con A

age	number of cells left in % of initial number	% viability	number of blasts in % of number of surviving cells	^{14}C-TdR incorporation in cpm x 10^{-3}
3 months	58 ± 6*	73 ± 5	47 ± 8	120.3 ± 10.5
21 months	59 ± 5	75 ± 4	20 ± 9	37.8 ± 4.9

*Figures represent the mean ± standard deviation of triplicate counts in 6 separate experiments; 200 cells were counted in each preparation.

The decline in LPS response (Fig. 2.3) is even more striking than that of the Con A response: at the age of 12 months, the LPS response has decreased to 18-35% (ranges scored at different doses) of the values for 3-month-old lymphocytes; this was further decreased to less than 15% at 21 months. No difference was found in the dose of LPS required for optimal stimulation; there was also no difference in cell survival and cell viability, but a ~ 70% decrease in the number of blast-like cells was observed at the age of 21 months.

Differential counts were performed on blood and spleen leucocytes from individual animals of 3 months and 21 months of age. Table 2.3 shows the mean values and the standard deviations for 6 animals per age-group. No significant difference in the relative number of spleen and blood lymphocytes was observed. The absolute number of leucocytes per volume of blood was the same for young and old animals.

TABLE 2.3

DIFFERENTIAL COUNTS ON SPLEEN AND BLOOD LEUCOCYTES FROM YOUNG AND OLD FEMALE RATS

cell types (per cent)	spleen		blood	
	3 months	21 months	3 months	21 months
monocytes	5 ± 2*	6 ± 1	7 ± 2	5 ± 2
lymphocytes	93 ± 4	92 ± 2	86 ± 3	88 ± 5
granulocytes	2 ± 1	3 ± 1	6 ± 3	8 ± 3
unidentified other cell types	< 1	2 ± 1	< 1	< 1

*Figures represent the mean values and standard deviations of counts on spleen and blood cells from 6 animals; 200 cells were counted.

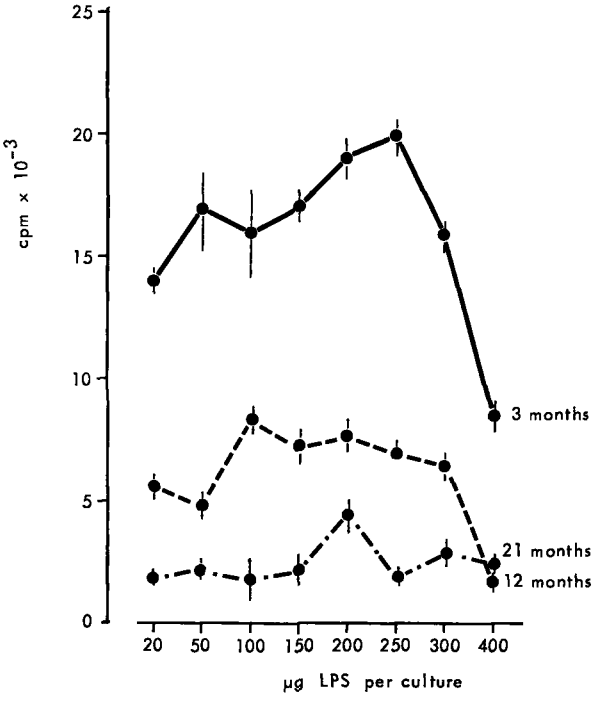

Figure 2.3

Lymphocyte proliferative response of 2 x 10^6 spleen cells from female rats of different ages as a function of LPS concentration. Each point represents the mean ± standard deviation of triplicate. LPS-stimulated cultures in an experiment representative for 3 separate experiments. Age groups: 3 months, 12 months and 21 months. For all age groups, a pool of 5 spleens was used. Background values in nonstimulated cultures were 1812, 2342 and 1408 cpm for animals of 3, 12 and 21 months old, respectively.

2.4 DISCUSSION

The present data show an age-related decrease in T and B cell mitogen responses in rats as measured by ^{14}C-TdR incorporation. It was suggested in the the Introduction that this phenomenon, which has also been described by others (123, 164, 239, 290) and attributed to a decreased capacity of the lymphocytes to proliferate, could also be due to other factors. Our experiments in rats, however, confirm the data reported by others for mice and suggest that the decrease in ^{14}C-TdR incorporation is indeed due to a decrease in the number of responsive cells, as indicated by the following results:

a) The lower ^{14}C-TdR incorporation by lymphocytes from old rats cannot be related to changes in cell recovery and viability in Con A or LPS stimulated cultures.

b) Prolonged culturing of lymphocytes from aged rats (72 and 96 h instead of 48 h) did not result in an increase in ^{14}C-TdR incorporation. Both aged and young lymphocytes showed lower incorporation values at 96 h than at 48 or 72 h.

c) When several doses of mitogen were used, an age-related decline in the spleen lymphocyte response to Con A and LPS was noted at all doses. The Con A response of lymphocytes in whole blood also showed a clear-cut decline at all doses, demonstrating that lymphocytes from old rats do not exhibit different requirements for mitogen dose.

d) The relative number of blast cells was higher in young than in old stimulated lymphocyte cultures.

These data on cell survival, proliferation time and mitogen dose suggest that the decreased ^{14}C-TdR incorporation of old lymphocytes is due to a decrease in the number of cells responsive to these mitogens. While this study was in progress, these data were confirmed by others (166) who showed that also the age-related decrease in PHA responsiveness of mouse spleen cells was due to a decrease in the number of responsive cells.

We do not share the view of others (58, 123, 146, 164) that thymic-independent mitogen responses appear to decline with age to a lesser degree than do the thymus-dependent responses: in the F1 hybrid rats used here, the age-related decline in LPS response was even more striking than that for the Con A response.

Although our results suggest that fewer cells are responsive to Con A and LPS in aged rats, it is still possible that also a decrease in thymidine uptake per cell occurs. This possibility was suggested by others (123, 164) who found that the age-related decrease in PHA responsiveness of mouse spleen cells was not correlated with a decrease in the relative number of Thy 1 positive cells in the spleen. However, since the T cell population can be subdivided into various subpopulations (for recent reviews, see ref. 14, 71), it is also possible that, although the number of Thy 1 positive cells remains the same, the ratio between responsive and nonresponsive cells changes. Moreover, a suppressive effect of spleen cells from long-lived mice on mixed lymphocyte reactivity (122) and on mitogen responsiveness (149) has been described; therefore, the possible role of suppressor cells has to be taken into account.

CHAPTER III

SOME ASPECTS OF THE ROLE OF THE THYMUS IN THE AGE-RELATED DECREASE IN THYMUS-DEPENDENT IMMUNE FUNCTIONS

3.1 INTRODUCTION

It recently (1, 166, 206) became apparent that the age-related decline in mitogen-induced proliferation of peripheral T lymphocytes from ageing rodents is due to a decrease in the number of reactive cells. It was found (see also Chapter II) that some other possibilities for the observed decrease in labelled thymidine incorporation into mitogen- or alloantigen-stimulated old lymphocytes (i.e., changes in kinetics and mitogen or allogeneic stimulator cell dose requirements) could be excluded (1, 166, 206) and that the actual number of stimulated cells had decreased (166, 206). Data in this Chapter suggest that the decrease in mitogen responsiveness of splenic T cells from ageing rats seems to be related to the extent of thymus atrophy: a more pronounced decrease is observed in animals exhibiting a more severe thymus atrophy.

The role of the thymus in the origin of the age-related immune deficiencies is still unknown. The small population of mature T cells in the thymus represents progeny of at least part of the major population of immature precursor T cells in the thymus (372, 374), but it is unclear whether these mature thymocytes migrate to the periphery to contribute to peripheral T cell function (335, 373). In addition, immature thymocytes seem to migrate to the periphery where they undergo further maturation (335, 373), possibly under the influence of thymic factors (13) which are considered to be secreted by thymic epithelial cells (13, 89, 335). Hence, depressed age-related T cell functions could be due to decreased thymus factor production (9, 19, 161) and/or a decrease in the number of intrathymic precursor T cells sensitive to the action of thymic factors. Experiments described in this Chapter were aimed at testing the latter hypothesis, employing thymocytes as a source of precursor cells. As a source of thymic factors, supernatants from cultured thymic epithelial cells (TES) were used. We recently demonstrated (209, 210, 212 and Chapters VII-IX) that such supernatants are capable of conferring upon thymocytes from young mice or rats various properties of mature T cells, such as a reduction in Thy 1-antigen density, increase in resistance to hydrocortisone, in intracellular cyclic AMP levels, mitogen responsiveness, mixed lymphocyte reactivity (MLR), killer T cell and helper T cell function. Data to be presented here indicate that thymocytes from old donors are no longer sensitive to the effects of TES, a putative analogue of thymic humoral function.

3.2 MATERIALS AND METHODS

3.2.1 Animals

Either male or female WAG/Rij rats were used in all experiments. The rats were bred and maintained as described in Chapter II. Spleen cells from 12-14-week-old (WAG x BN)F1 rats were used as allogeneic stimulator cells. The present determinations were performed on spleen cells from individual animals, in contrast to the experiments in the previous Chapter, which were performed with pooled spleen cells from 5 rats.

3.2.2 Mitogen stimulation and mixed lymphocyte reactivity (MLR) of thymus and spleen cells

In contrast to the macroculture system described in the previous Chapter, a microsystem was employed here. Animals were anaesthetized by ether and spleens or thymuses removed under sterile conditions and collected in RPMI 1640 buffered with 25 mM HEPES (Gibco Biocult Ltd. 240, Paisley, Scotland). Only individual animals were tested in the present experiments. The organs were minced with scissors and filtered through six layers of nylon gauze. Further steps were carried out at $0^{o}C$. The cell suspensions were collected in Falcon tubes (2070, Falcon Plastics, Los Angeles), centrifuged at 200 x g (spleen cells) or 130 x g (thymocytes) for 10 min and washed once in HEPES buffered RPMI. The final cell suspensions were counted in a haemocytometer and viability was assessed by trypan blue exclusion; cell viability was 85 to 90% for spleen cells and $>$ 95% for thymocytes. The cell suspensions were adjusted to 2×10^{6} viable cells/ml (for mitogen stimulation) or 10×10^{6} cells/ml (for MLR). The medium was bicarbonate buffered RPMI 1640 (2-0631; Flow Laboratories, Irvine, Scotland) supplemented with 15% heat-inactivated rat serum, penicillin (100 IU/ml), streptomycin (0.1 mg/ml), 5×10^{-5} M 2-mercaptoethanol (2-ME) and 2 mM glutamine.

For mitogen stimulation, cells were dispensed into flat-bottom wells of microtitre plates (M 220-29 ART; Greiner, Würtingen, FRG) (4×10^{5} cells/0.2 ml/well). Freeze-dried PHA (HA 15, lot K 1231; Wellcome, Beckenham, G.B.) was reconstituted according to the instructions of the supplier and dilution series were prepared in RPMI; from each dilution, 25 µl was added per well. For Con A (Pharmacia, Uppsala, Sweden), dilution series of a 1 mg/ml solution in RPMI were prepared and 25 µl of each dilution was added per culture. Cultures were kept at $37^{o}C$ in a humidified atmosphere of 95% air-5% CO_2.

MLR was performed in U-shaped wells of microtitre plates (M 220-24 ART; Greiner, Würtingen, FRG); 100 µl of the responder cell suspension (i.e., 1 x 10^6 cells) were cultured together with 0.5 x 10^6 stimulator spleen cells (which had been irradiated with 2500 rad) in a final volume of 0.2 ml per well. Control cultures consisted of responder cells alone, stimulator spleen cells alone and responder cells cultured with syngeneic spleen cells.

After 24 h (for mitogen stimulation) or 48 h (for MLR) cultures were labelled with 0.075 µCi of ^{14}C-TdR by adding 25 µl of the labelling solution described previously (Chapter II). Twenty-four h later, cultures were harvested on GF/A glass fibre filters (Whatman) with an automatic culture harvester (Cryoson, Midden Beemster, NL) and processed for liquid scintillation counting as described (Chapter II).

3.2.3 Thymic factors

Supernatants from thymic epithelial cultures were used as the source of thymic factors. Preparation of the cultures and collection and use of the supernatants were as described in detail elsewhere (210 and Chapter VII). Kidney or pancreas epithelial culture supernatants served as controls and were previously shown to exhibit no activity in the assays employed (210,212). Thymus epithelial culture supernatants will be referred to as TES and control supernatants as CS.

3.3 RESULTS

3.3.1 Effect of age on T cell proliferative responses to mitogens and allogeneic cells in the spleen and in the thymus

Figure 3.1 illustrates the effect of ageing on Con A and PHA responsiveness and MLR of female WAG/Rij rat spleen cells. These data have been obtained under conditions which were optimal for young rats. In Chapter II and ref. 206 , it was excluded that spleen cells from old rats might require different conditions than those from young rats for optimal responsiveness. The present findings indicate that also when spleen cells from individual animals are tested (in contrast to pooled spleen cells, as used in Chapter II), an age-related decrease in T cell proliferative responses is observed. In some of the experiments to be described later the same individuals used here were employed. Male and female BN/Bi rats (data not shown) as well as male and female (WAG x BN)F1 rats (Chap-

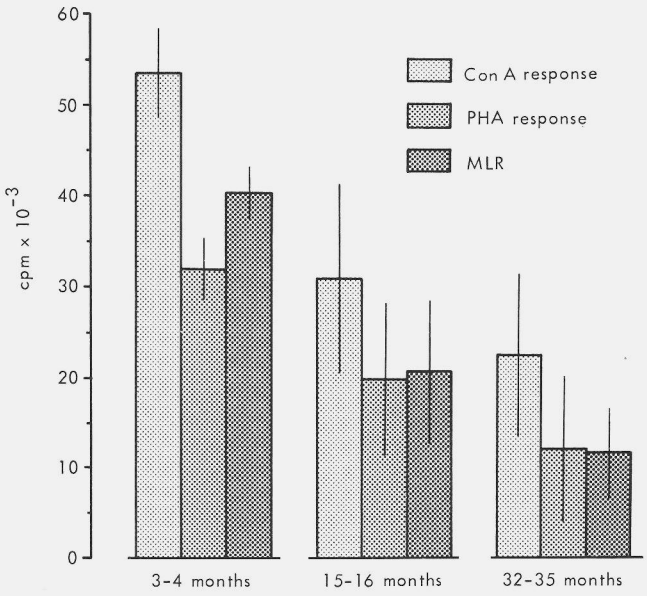

Figure 3.1

^{14}C-TdR incorporation into spleen cells from female WAG/Rij rats of different age groups stimulated with Con A, PHA or allogeneic cells. Figures represent the mean ± standard deviation of 10, 16 and 12 individual determinations in rats of the young, middle-aged and old groups, respectively.

ter II) exhibit a similar age-related change, i.e., a 40 to 50% reduction in the 15 to 16-month-old group and a 60 to 75% reduction in the 32 to 35-month-old group. With advancing age, a clear-cut increase in the standard deviation of the mean was observed (Figure 3.1), indicating that variations among individual animals also increased.

This increased variability is more strikingly observed in thymocyte responses to Con A and PHA and in MLR (Figure 3.2): thymocyte responses in the 15-16-month-old and 32-35-month-old group varied from 5 to 240% of the values obtained in young rats, indicating that in old rat thymuses wide variations in the ratio of responsive vs. nonresponsive cells occur.

3.3.2 Comparison between age-related changes in thymus morphology and T cell proliferative responses in the spleen of male and female WAG/Rij rats

Extensive pathological studies on the different rat strains of our Institute performed by Burek (56) revealed that: (a) male WAG/Rij rats die 6 to 9 months earlier than male and female rats of the BN strain or the (WAG x BN)F1

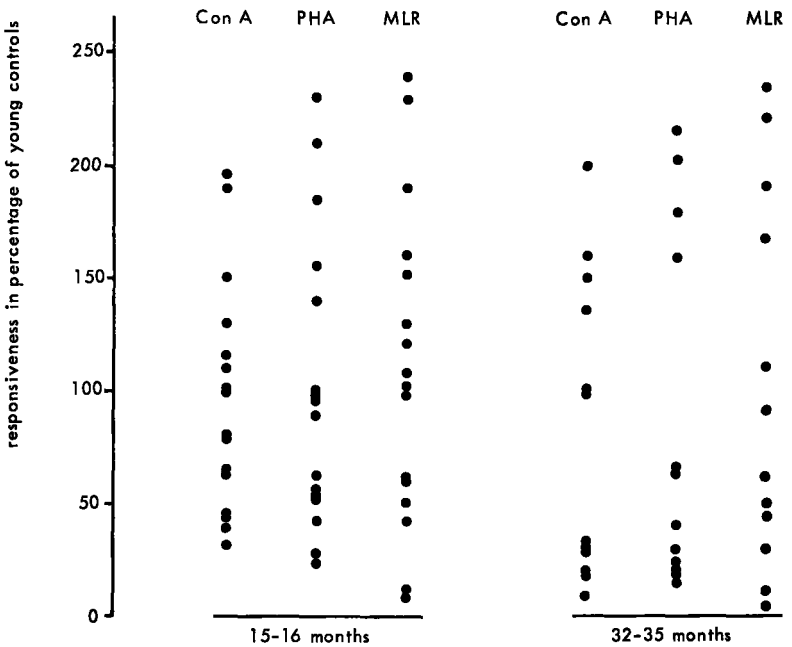

Figure 3.2

Relative responsiveness of thymocytes from female WAG/Rij rats of different age groups stimulated with Con A, PHA or allogeneic cells. Each point represents the response of an individual animal, expressed as a percentage of the response obtained in young (3-4-month-old) rats, which was estimated at 100%. The same animals as used for the determination of splenic T cell responses shown in Figure 3.1 were used. The mean response in cpm of thymocytes from young rats stimulated with Con A, PHA or allogeneic cells was 23837 ± 7586, 7133 ± 1718 and 17624 ± 1843, respectively (mean ± standard deviation of 10 individual animals).

hybrid or female WAG/Rij rats; (b) the onset of many age-associated lesions, such as, e.g., pituitary tumours, medullary thyroid carcinoma, ulceration of the forestomach and myocardial fibrosis, occurred 6 to 10 months earlier in the male WAG/Rij rats; (c) WAG/Rij male rats exhibited a more severe and earlier thymic involution than males and females of the other two strains. It was therefore investigated whether these differences in thymus morphology also reflected differences in proliferative capacity of peripheral T lymphocytes of the same animals.

The main age-associated changes in the thymuses from 20 female and 17 male 18-month-old WAG/Rij rats are given in Table 3.1. In the males, a pronounced cortical atrophy was present in all animals, agglomerations of epithelial cells were recognizable in many animals, but a few thymuses only contained specialized epithelial structures such as tubules and cords. No cysts were found. In

TABLE 3.1

COMPARISON OF THYMUS MORPHOLOGY
OF 18-MONTH-OLD FEMALE AND MALE WAG/Rij RATS

Features	Female rats*	Male rats*
Cortical atrophy	8/20	17/17
Epithelial cell clusters	19/20	13/17
Distinct epithelial cords and/or tubules	16/20	7/17
Epithelial cysts	13/20	0/17

*The animals used for this comparison were also used for experiments 1 and 2 shown in Table 3.2.

TABLE 3.2

COMPARISON OF ^{14}C-TdR INCORPORATION INTO PHA AND Con A STIMULATED SPLEEN CELLS FROM 18-MONTH-OLD FEMALE AND MALE WAG/Rij RATS

Exp.	Sex	1.5 µl PHA	2.5 µl PHA	7.5 µg Con A	10 µg Con A
1	female	5762 ± 1471*	5342 ± 1145	6311 ± 1785	6778 ± 2143
	male	2698 ± 955	3006 ± 1543	2849 ± 1469	3222 ± 1881
2	female	15437 ± 5942 (48%)**	16727 ± 6975 (50%)	30868 ± 11650 (55%)	29599 ± 13205 (55%)
	male	4506 ± 1384 (14%)	4237 ± 2227 (13%)	8827 ± 4815 (16%)	9327 ± 7483 (17%)

* Figures represent the mean cpm of the mean values of triplicate cultures from 10 individual animals ± standard deviation of the mean; 7 instead of 10 male rats were investigated in experiment 2.

**In the same experiment, 5 male and 5 female 4-month-old rats were also tested. The mean response of this group to PHA was 32212 ± 3263 cpm and to Con A 53899 ± 5003 cpm. The values in parentheses represent the response of the 18-month-old animals, expressed as a percentage of the response obtained in young rats which was estimated as 100%.

the female group of the same age, thymic cortical atrophy was encountered in less than half of the animals and extensive epithelial structures were found in the thymuses of all animals except one. These epithelial structures often included cords, tubules and large cysts. Subsequently, PHA and Con A responsiveness of spleen cells from these rats are compared. At the ages of 1, 4 and 8 months, no differences in responsiveness to either mitogen are observed between male and female rats (data not shown). However, at the age of 18 months, the mitogen response of spleen cells from female rats was 2 to 4 times higher than the response in male rats (Table 3.2). In experiment 2, young rats were also tested; in comparison, the responses of 18-month-old females were reduced by 45 to 50%, whereas those of males of the same age were reduced 83 to 87%. Thus, the extent of the decline in T cell proliferative capacity in the spleen might be related to the degree of thymus atrophy.

Thymocyte responses to mitogens and alloantigens of male and female WAG/Rij rats could not be compared since cell recovery of male thymuses was too low for that purpose.

3.3.3 Effects of thymic epithelial culture supernatant (TES) on T cell proliferative responses of thymocytes from female WAG/Rij rats of different ages

T cell mitogen responsiveness and MLR of thymocytes from young rats can be strongly enhanced by the addition of TES (Figure 3.3). In addition, the data shown in Figure 3.3 indicate that, with advancing age, thymocytes are no longer sensitive to the effects of TES. In the oldest age group, TES completely fails to affect PHA, Con A and MLR responses, whereas the effect of TES in the middle-aged group is lower than in young rats. No correlation was found between baseline T cell proliferative responses (in cultures without TES; shown for the same animals used here in Figure 3.2) and the effect of TES, i.e., irrespective of whether the 32-35-month-old rats exhibited enhanced or depressed thymocyte responses (as compared to young rats), no effect of TES was observed.

3.4 DISCUSSION

A more pronounced defect in peripheral T cell proliferative capacity was found in ageing rats exhibiting a more severe thymus atrophy. By what mechanism the ageing thymus influences the peripheral thymus-dependent immune functions is not yet fully understood. Hirokawa and co-workers (156-158) demonstrated

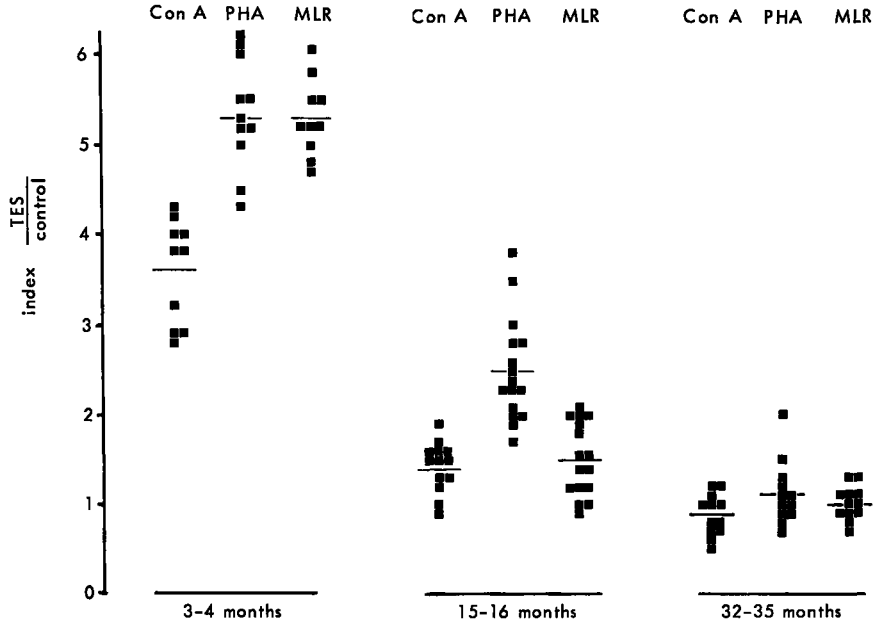

Figure 3.3

Effect of thymic epithelial culture supernatant (TES) on thymocytes from female WAG/Rij rats of different age groups stimulated with Con A,PHA or allogeneic cells. Each point represents the factor by which the response of an individual animal is increased by TES (as compared to cultures containing control supernatant). The same animals as used for the experiments shown in Figures 3.1 and 3.2 were used.

that intrinsic age-related thymic changes must occur, since old thymuses transplanted into young thymectomized recipients fail to reconstitute several T cell functions. Since total thymuses were used in these experiments (156-158), both a decline in thymic humoral function or dysfunction of thymus microenvironment and a decrease in intrathymic precursor T cell content could be responsible for the observed defect. The experiments discussed in this Chapter indicate that mitogen responses and MLR of thymocytes from old rats can no longer be enhanced by the addition of factors present in TES. If the effects of TES on young thymocytes reflect a T cell maturation process, these findings might be explained by a loss of intrathymic precursor T cells with ageing.

Intrathymic precursor T cells originate mainly from the bone marrow in the adult animal (33, 98). Hence, an age-related decrease in intrathymic precursor T cells could be due to defects in the bone marrow thymocyte progenitors. This hypothesis is supported by the experiments of Tyan (356, 357), who showed that thymic regeneration of young whole body irradiated mice was significantly impaired when the recipients received marrow cells from donors 100 weeks of age or older.

However, a decrease in marrow thymocyte progenitors cannot be the only explanation for the observed decrease in precursor cells in the thymus of old animals. This is based on the following observations: (a) old thymuses transplanted into young thymectomized recipients are not effective in T cell function reconstitution (156-158); (b) young bone marrow transplanted into old irradiated recipients fails to restore immune functions (159, 246). Thus, apparently young bone marrow cannot cooperate efficiently with the old thymus to build up the thymus-dependent immune system. It seems likely that the aged thymus no longer provides the proper microenvironment for further differentiation of stem cells into intrathymic precursor T cells.

Thymic humoral function is considered to be impaired during ageing by several investigators who observed a decline in thymic factor levels in the serum of aged mice and man (9, 19, 161). The physiological role of serum thymic factors in T cell maturation is unknown, but it has been suggested that they might play a role in the maturation of T cells in the periphery (335, 373). Whether only depressed thymic humoral function is responsible for the intrinsic defect of the old thymus discussed above remains unknown. Young thymus grafts in combination with young bone marrow grafts reconstitute several T cell functions in irradiated old recipients (159), but these experiments were performed with total thymuses. The data presented in this Chapter suggest that lack of thymic factor production cannot be the only cause of deficient thymus function, but depressed production of thymic factors and decreased precursor cell content of the thymus might be related events.

CHAPTER IV

EFFECT OF TUMOUR BEARING ON T CELL-MITOGEN RESPONSES IN RATS

4.1 INTRODUCTION

It is well-known that the frequency of tumours increases in mice (319), rats (56) and man (235) with advancing age. Thus, in the framework of our ageing studies, it was considered worthwhile to investigate to what extent tumours might affect thymus-dependent immune functions. The objectives of the experiments described in this Chapter were to collect baseline data on the effect of tumour bearing on T cell-mitogen responses and to attempt to elucidate the mechanism(s) by which tumours might affect this T cell parameter. Obviously, aged rats cannot be used for such a study, due to the great variations in T cell proliferative responses observed in old age (see Chapter III). Therefore, young rats transplanted with fragments of an originally spontaneously occurring tumour (rather than chemically or virally induced tumours, which have been used so far in this type of study; see, e.g., ref. 2, 129, 145, 186, 189, 197) were employed as a model. A strong decline in the Con A response of spleen cells from tumour bearing rats was observed and this decline was associated with an increase in the number of phagocytic cells. Therefore, the effect of macrophages on normal rat spleen cells was also investigated. From the evidence presented here, it seems likely that the defective Con A response of spleen cells from tumour bearers is caused by an increase in the number of macrophages. Results which suggest that the decline in Con A response is not due to soluble suppressive factors released by macrophages are also presented.

4.2 MATERIALS AND METHODS

4.2.1 Animals, mitogens, media, spleen cell cultures

All of these experimental conditions were as described Chapter II.

4.2.2 Tumour and experimental plan

The tumour used is a carcinoma of the bladder which originally occurred spontaneously in a 30-month-old BN/Bi male rat (50). It has been serially passaged for two years in (WAG x BN)F1 rats by subcutaneous (sc) transplantation;

it is a progressively growing tumour leading to death of the host within 3-4 months after sc transplantation of fragments of 15-20 mg wet tumour tissue.

Animals referred to as "tumour-bearing" (TB) were 12-14 weeks old female (WAG x BN)F1 and had received sc transplants of 15-20 mg wet tumour tissue on both flanks 18 days before the mitogen responses were assessed. By that time, tumours had reached a diameter of ~ 2 cm and had a weight of ~ 5 g (the total tumour mass represented ~ 5% of the body weight). Control animals (N) had been sham-operated. Mitogenic response of spleen lymphocytes to 2 different doses of Con A (approximating the dose giving maximum response) was determined by measuring ^{14}C-thymidine incorporation. Separate experiments (data not shown) showed no shifts in the dose-response curves in TB rats.

The proliferative response of Con A stimulated spleen cells was estimated by counting the number of lymphoblasts in May-Grünwald Giemsa stained smear preparations. Spleen cells were also examined before culturing, in both May-Grünwald Giemsa stained smear preparations and after the addition of 0.07% neutral red (NR) solution which stains the phagocytic cells.

In the initial experiments designed to determine the effect of tumour-bearing on mitogen responses, each N or TB animal was tested individually. Once these baseline data were established, further experiments were performed with cell pools from 5 animals.

4.2.3 Macrophage-depletion of spleen cells

Treatment of spleen cells by the carbonyl iron/magnet technique was performed as follows: 10^8 spleen cells were suspended in 10 ml HE (Dulbecco's modification of Hanks Eagle's medium) + 15% rat serum (RS) in a Falcon tube (2070); 0.5 gram carbonyl iron powder was added and the tube was incubated at 37°C for 30 min. The tube was then placed in ice and the iron powder removed by by placing a magnetic bar in the suspension; after cleaning the magnet with sterile cloth, this procedure was repeated several times until iron particles were no longer visible on the magnet. The cells were centrifuged, counted and adjusted to 2 x 10^6/ml HE + 15% RS. About 70-80% of the original cell population was recovered after treatment, with a viability of 95%. Control cells were treated in the same way, except that no carbonyl iron was added.

Glass-adherent cells were removed by incubating 40 x 10^6 spleen cells in 10 ml HE + 15% RS in glass tissue culture flasks (surface 200 cm^2) for 60 min at 37°C; after removal of the nonadherent cells, the adherent layer was washed and the nonadherent cells, together with the washing fluid, were transferred to a fresh tissue culture flask and allowed to adhere at 37°C for another 60 min.

The nonadherent cells were then centrifuged, counted and readjusted to a concentration of 2 x 10^6/ml HE + 15% RS. The recovery of nonadherent cells was ~ 70-80% of the original cell suspension with a viability of 95%. Control suspensions were preincubated in Falcon tubes (2070) for the same period (recovery > 90%).

Both treatments resulted in a selective decrease in phagocytic cells; in normal rat spleen cell suspensions, the number of cells which took up neutral red decreased from ~6% to < 2%, and differential counts showed a decrease in the number of monocytes from 6% to < 2%.

4.2.4 Preparation of macrophages

Since the number of phagocytic cells in TB-spleen suspensions was increased (from ~ 6% to ~ 14%), the effect of excess numbers of macrophages on the response of normal spleen cells was investigated. For this purpose, peritoneal macrophages from thioglycollate-treated rats were used unless stated otherwise.

F1 rats of 14-18 weeks of age were injected intraperitoneally with 8 ml of 3% thioglycollate medium (Difco, Detroit, Mich.). Four days later, the peritoneal cavity was rinsed with 25 ml cold HE (containing 1000 IU heparin). The collected peritoneal exudate cells (PEC) were centrifuged at 4°C for 10 min at 200 g, washed once with cold HE + 15% RS, counted and adjusted to the desired concentration in HE + 15% RS. All manipulations were carried out at 4°C. The final yield was usually 30 to 40 x 10^6 cells/animal.

Cytological analysis on May-Grünwald Giemsa stained smear preparations revealed that the PEC were composed of 62% monocytes, 30% granulocytes and 8% lymphocytes (mean of 10 individual animals); ~ 90% of these cells took up neutral red.

In some experiments, PEC collected from untreated rats were also used; in those cases, the final yield was 4 times lower than that of thioglycollate-injected rats and the composition of the cell suspension was different: 45-55% lymphocytes; the remainder appeared on neutral red staining to be phagocytic cells.

To determine whether the non-phagocytic cells in PEC (i.e., ~ 10% and ~ 50% of the cell population in thyoglycollate treated and normal rats, respectively) contributed to the effects described later, macrophage-depleted PEC were used in some experiments. Macrophage-depletion was performed as described for spleen cells (section 4.2.3). The remaining number of cells taking up neutral red in either carbonyl iron/ magnet treated or nonadherent PEC was < 2%.

4.2.5 Testing for possible macrophage-derived inhibitory factors

The possibility that soluble macrophage-derived factors interfered with TdR incorporation (263, 264) was investigated by employing the following procedures:

a) Except that the tubes were kept horizontally, 10^7 N or TB spleen cells were cultured in 1 ml under the same conditions as those described for mitogen stimulation (Chapter II). In addition, cultures of 8.5×10^6 spleen cells to which 1.5×10^6 PEC had been added were prepared. The supernatants were harvested after 48 h, centrifuged at 500 g for 20 min, passed through a Millipore filter (0.45 µm) and tested for inhibition of TdR incorporation into Con A stimulated spleen cell cultures. Culture conditions were as described previously (Chapter II); supernatants were tested in dilutions of 1:2.5 and 1:5. At the latter dilution, the concentration of the inhibitory factor should be about the same as in normal cultures, since 5 times more cells were used for the preparation.

b) Three labelling procedures were compared: (1) normal labelling (used in all other experiments, i.e., labelling during the last 24 h of a 48 h culture period); (2) pulse labelling (labelled during the last $3^1/2$ hours of the 48 h culture period); (3) pulse labelling during the last $3^1/2$ h *after* the cells had been washed 3 times with 5 ml HE containing 15% RS and readjusted to 1 ml. Using the latter procedure, soluble factors which could possibly affect TdR uptake are removed.

4.3 RESULTS

4.3.1 Some characteristics of spleen cell suspensions from TB rats

A cytological analysis of pooled spleen cell suspensions from 5 animals is given in Table 4.1. The absolute number of spleen cells from TB animals was increased by a factor of 1.5 $\pm$ 0.2. The relative number of lymphocytes had decreased, with a concomitant increase in the numbers of monocytes and granulocytes. The number of cells taking up neutral red was also increased. After excision of the tumour, the composition of the spleen cell suspensions returned to normal values (Table 4.1).

TABLE 4.1

PERCENTAGE OF CELL TYPES IN SPLEEN CELL SUSPENSIONS FROM NORMAL AND TUMOUR-BEARING RATS

cell type	normal	tumour bearing	13 days* after excision	tumour bearing after mø depletion**
lymphocytes	93 ± 2***	80 ± 3	89 ± 2	91 ± 3
monocytes	6 ± 1	13 ± 2	7 ± 2	5 ± 1
granulocytes	2 ± 1	6 ± 3	3 ± 1	2 ± 1
neutral red staining cells	6 ± 2	14 ± 2	8 ± 2	4 ± 2

* For effects of excision on Con A responses, see Table 4.2
** mø-depletion was performed by carbonyl iron/magnet treatment; for effects of mø-depletion on Con A responses, see Figure 4.3
***Figures represent the mean ± S.E. of triplicate counts on pooled spleen cells from 5 rats in 3 separate experiments; 200 cells were counted.

4.3.2 Con A response of spleen cells from TB rats

The results obtained for the Con A response of splenic lymphocytes in 3 experiments are shown in Figure 4.1. Five normal and 5 tumour bearing animals were tested in each experiment and the individual as well as the mean values are plotted. In a total of 15 experiments, a 3-10-fold decrease of TdR incorporation in lymphocytes from TB animals was found for both doses of Con A (approximating the dosage giving a maximum response; see Chapter II, Figure 2.2).

The decrease in Con A responsiveness was reversed after tumour excision. A representative experiment is shown in Table 4.2: 6 days after excision, the Con A response is significantly higher than in the TB animals; it has further increased to almost normal values one week later.

To exclude the possibility that the observed effects were restricted to female F1 rats, some experiments were performed in male F1 rats and in female and male BN/Bi rats (the strain in which the tumour originated). Comparable effects of tumour bearing were observed in both strains and sexes (data not shown).

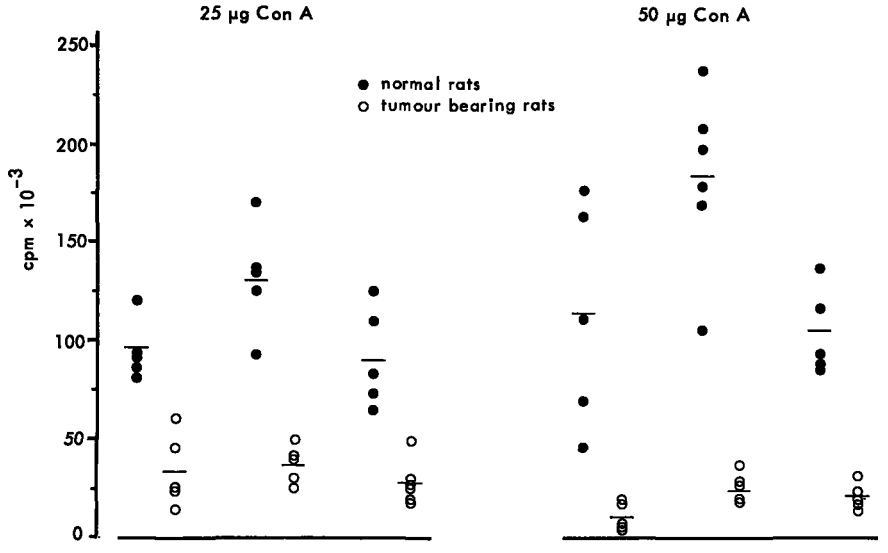

Figure 4.1

^{14}C-TdR incorporation into 2 x 10^6 spleen cells from normal and tumour bearing rats after stimulation with Con A.
Each point represents the difference between the mean of triplicate Con A-stimulated cultures and the mean of triplicate nonstimulated cultures from an individual animal. The means of each group are indicated by horizontal lines. TdR incorporation into cultures without mitogen varied from 1000 to 1400 cpm and was not affected by tumour bearing. The significance of the difference between normal and tumour bearing rats was determined by Student's t-test and p values < 0.001 were observed in all experiments.

TABLE 4.2

^{14}C-TdR INCORPORATION IN CPM x 10^{-3} INTO 2 x 10^6 Con A STIMULATED SPLEEN CELLS FROM NORMAL, TUMOUR BEARING AND POST-TUMOUR-BEARING RATS

Con A dosage	normal	tumour bearing	p-value	6 days after excision	13 days after excision
25 µg	9.1 ± 4.0*	1.1 ± 0.6	< 0.005	3.7 ± 2.7**	6.9 ± 3.3***
50 µg	11.4 ± 5.6	1.2 ± 0.6	< 0.005	2.5 ± 2.0	9.8 ± 5.0

* Mean cpm of the mean values of triplicate cultures from 5 individual animals ± standard deviation of the mean. P-values were determined by Student's t-test.
** Significant difference with TB-group ($p < 0.05$).
***Significant difference with TB-group ($p < 0.005$); difference with N-group not significant.

4.3.3 Effect of macrophages on TdR incorporation into Con A stimulated spleen cells of normal rats

The effect of macrophage-depletion or addition was studied with spleen cells from normal rats. In spleen cell cultures to which 5% PEC were added, TdR incorporation into Con A stimulated spleen cells was reduced to ± 50% of the values observed in control cultures (Figure 4.2); at 10% PEC, a further decrease to ± 30% of the normal values was observed. In separate experiments (207), it was demonstrated that PEC treated with carbonyl iron/magnet did not exhibit inhibitory effects, suggesting that the small percentage of nonphago-

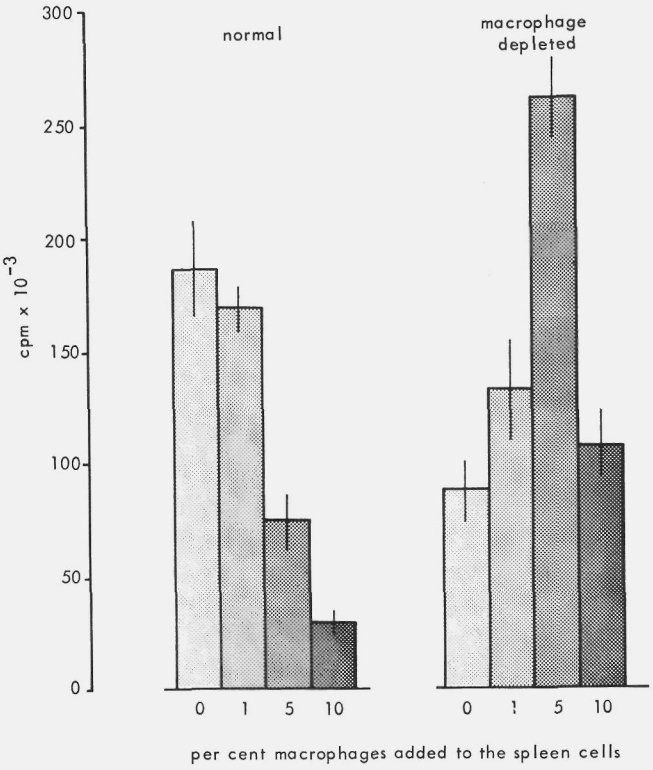

Figure 4.2

The effect of macrophages on TdR incorporation into Con A stimulated cultures from normal spleen cells and macrophage-depleted spleen cells.
The data are representative of five experiments using different cell preparations. Mø-depletion was accomplished by carbonyl iron/magnet treatment. Various numbers of PEC were added to 2 x 10^6 spleen cells which were stimulated with 50 µg Con A. The results represent the arithmetic mean ± standard deviation of triplicate cultures. PEC were unresponsive to Con A, when cultured alone. TdR incorporation into control cultures (without mitogen) was not affected by PEC and varied from 1200 to 1500 cpm.

cytic cells among PEC did not contribute to the observed effects. Also nonadherent PEC (data not shown) do not inhibit TdR-incorporation, further supporting the notion that the effects exerted by PEC were due to macrophages.

Similar results (211) were obtained with PEC from untreated rats, except that 20-25% PEC had to be added in order to observe similar levels of inhibition as with PEC from thioglycollate treated rats. Since PEC from untreated rats contain lower numbers of phagocytic cells (see section 4.2.4) and the phagocytic cells seem to be responsible for the observed inhibition (see above), these findings suggest that the lower efficiency of PEC from untreated rats is due to their lower phagocytic cell content.

Macrophage depletion (accomplished by glass adherence or the carbonyl iron/magnet technique) results in a decrease in the Con A response of spleen cells (Figure 4.2). This effect is most noticeable at Con A concentrations of 50 µg or higher; at 10 or 25 µg of Con A, no effect of macrophage-depletion is often observed (208). PEC-macrophages enhanced the response of such macrophage-depleted cell suspensions if added in amounts of 1 and 5% (complete reconstitution is usually observed at 5%), demonstrating that the decrease was not merely due to removal of a highly responsive subpopulation of T cells. Larger numbers of PEC-macrophages (10% and higher) resulted in inhibition of the response. Thus, it seems that the final macrophages to lymphocytes ratio in the cultures (irrespective of the origin of these macrophages, i.e., splenic or peritoneal) determines whether TdR incorporation will be enhanced or inhibited: if spleen cell cultures stimulated with 50 µg Con A contain $> 5\%$ phagocytic cells, a reduction in TdR incorporation after Con A stimulation will be observed.

4.3.4 Effect of macrophage-depletion on TdR incorporation into Con A stimulated TB rat spleen cells

Because addition of PEC-macrophages to normal spleen cell cultures (containing $\sim 6\%$ phagocytic cells) resulted in a marked decrease in TdR incorporation after Con A stimulation, the effect of macrophage-removal from TB spleen cell suspensions (containing $\sim 14\%$ of phagocytic cells) was investigated.

Figure 4.3 shows a representative experiment testing the effect of carbonyl iron/magnet treatment on TdR incorporation into Con A stimulated cultures of N and TB spleen cells. With N spleen cells, little or no effect of macrophage-removal was seen at the low Con A dose, whereas a partial decrease in TdR incorporation was observed at the higher dose. The decreased Con A response of TB spleen cells could be completely restored, however, by carbonyl iron/magnet

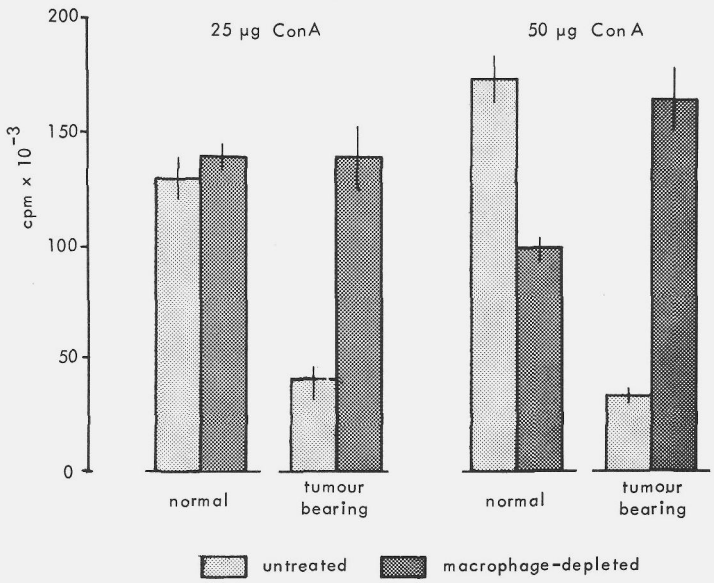

Figure 4.3

The effect of carbonyl iron/magnet treatment on TdR incorporation into Con A stimulated cultures from N and TB spleen cells.
Figures are representative of five experiments (see Table 4.5); 2 x 10^6 spleen cells were cultured per tube. The results represent the arithmetic mean ± standard deviation of triplicate cultures. TdR incorporation into cultures without mitogen varied from 1100 to 1500 cpm and was not affected by tumour bearing or macrophage depletion.

treatment: at both doses of Con A, the TdR incorporation after macrophage removal was no longer significantly different from that found in N spleen cell cultures. The increase in TdR incorporation was accompanied by a decrease in the number of NR-positive cells from ~14% (before treatment) to ~4% (after treatment) (Table 4.1). The number of cells left after carbonyl iron/magnet treatment was similar in N and TB spleen cells (70-80% recovery). When macrophages were removed from TB spleens by means of glass adherence, an increase in TdR incorporation comparable to that observed after carbonyl iron/magnet treatment, as well as a similar decrease in the number of NR-positive cells was seen (data not shown).

4.3.5 Possible inhibitory factors in TB spleen cell supernatants

It has been suggested that the decrease in TdR incorporation in mitogen stimulated spleen cell cultures containing 10% or more PEC (109, 263,

264) is due to macrophage-derived cold thymidine resulting from ingestion and degradation of DNA released from dying cells. Similarly, also the macrophage-induced suppression of TdR incorporation into spleen cells stimulated with allogeneic cells from animals bearing MSV-tumours was due to dilution of labelled thymidine (110, 183). If such inhibitory factors are responsible for the decrease in ^{14}C-TdR uptake observed in our system, supernatants from TB spleen cell cultures should transfer the effect. Alternatively, extensive washing of the cells before addition of ^{14}C-TdR, with consequent removal of the inhibitory factors from the supernatant, should reconstitute the response of TB spleen cells. Both possibilities were investigated.

Table 4.3 demonstrates that, irrespective of the labelling procedure used, ^{14}C-TdR uptake into TB spleen cell cultures was reduced to ~ 40% and ~ 20% of the values obtained in normal spleen cells for 25 to 50 µg Con A, respectively. Thus, removal of the TB spleen cell supernatant prior to the addition of the isotope did not lead to enhancement of the response. It seems unlikely that sufficient cold thymidine is produced during the 3 1/2 h labelling period to cause inhibition of TdR incorporation.

TABLE 4.3

EFFECT OF VARIOUS LABELLING PROCEDURES ON ^{14}C-TdR INCORPORATION INTO Con A STIMULATED SPLEEN CELLS FROM TUMOUR BEARING RATS

labelling procedure	25 µg Con A	50 µg Con A
normal labelled	39 (35 - 42)	21 (17 - 24)
pulse labelled	40 (37 - 50)	19 (14 - 25)
pulse labelled after washing	38 (30 - 47)	22 (20 - 29)

For each labelling procedure, the response in 2 x 10^6 normal control spleen cells is estimated at 100%, whereas the response of tumour bearing spleen cells is given as a percentage of the control. Figures represent the mean values and the range of 3 separate experiments in which pooled spleen cells from 5 animals were used. Absolute values of cpm in normal spleen cells varied from 15 to 20 x 10^3 cpm when the cultures were labelled in the normal way and from 4.7 to 6.2 x 10^3 cpm in the pulse-labelled cultures (irrespective of whether the cultures were washed prior to the addition of the isotope).

Table 4.4 illustrates that, although all supernatants tested caused inhibition of ^{14}C-TdR uptake, differences between N and TB supernatants were not observed. Similarly, supernatants from normal spleen cell cultures containing 15% PEC had an inhibitory effect comparable to that of N supernatants. Also, in TB supernatants from cultures containing 2 x 10^6 cells per ml which were tested undiluted, extra inhibitory effects, as compared to that of N supernatants, could not be demonstrated. Other variables (cell concentration, concentration of supernatant, incubation period) also did not reveal inhibitory factors.

In addition, it was investigated whether the proliferative response of TB spleen cells, as determined by counting the number of blast-like cells, was inhibited. In 3 separate experiments, the Con A response of TB spleen cells was found to be depressed by the criteria of both ^{14}C-TdR incorporation and lymphoblast counts (Table 4.5). Although the number of lymphoblasts was much less depressed than ^{14}C-TdR uptake, it can be concluded that soluble inhibitory factfactors are not the only cause of the observed decline in TdR incorporation.

4.4 *DISCUSSION*

Reduced responses to Con A were observed in spleen cell cultures from rats bearing subcutaneous transplants of an in vivo passaged line of a spontaneous bladder carcinoma. This is in agreement with the results of other investigators

TABLE 4.4

EFFECT OF VARIOUS CULTURE SUPERNATANTS ON ^{14}C-TdR INCORPORATION INTO Con A STIMULATED NORMAL SPLEEN CELLS

source of supernatant	concentration of supernatant 20%	40%
normal spleen cells	54 (50 - 68)	55 (51 - 58)
tumour-bearing spleen cells	56 (42 - 69)	45 (40 - 49)
normal spleen cells + 15% PEC	57 (51 - 60)	57 (50 - 50)

2 x 10^6 normal control spleen cells were stimulated with 50 µg Con A. The response in cultures to which the various supernatants were added is given as a percentage of the response in cultures without added supernatant. Figures represent the mean values and the range of 4 separate experiments in which pooled cells from 5 animals were used.

TABLE 4.5

^{14}C-TdR INCORPORATION AND LYMPHOBLAST COUNTS IN Con A STIMULATED SPLEEN CELLS FROM NORMAL AND TUMOUR-BEARING RATS

	normal control	tumour bearing	tumour bearing in per cent of control
percentage of lymphoblasts	80 (78 - 82)	60 (54 - 64)	75
^{14}C-TdR uptake in cpm x 10^{-3}	26.1 (24.3 - 30.2)	4.9 (3.3 - 6.5)	18.8

2 x 10^6 spleen cells were cultured with 50 µg Con A. The number of lymphoblasts is given as a percentage of the number of surviving cells at the end of the culture period (mean of duplicate counts on 200 cells). For ^{14}C-TdR uptake, mean values of triplicate cultures are given. Figures represent the mean values and range of 3 separate experiments using pooled spleen cells from 5 animals.

who demonstrated that Moloney-sarcoma-virus-induced tumours (145, 186) and chemically induced tumours (2, 376) lead to a state of PHA nonresponsiveness in the spleens of tumour-bearing mice.

Data shown in this Chapter indicate that the impairment in T-cell mitogen responsiveness was accompanied by an increase in the relative number of phagocytic cells in the spleens of TB animals and a concomitant decrease in the relative number of lymphocytes (from ~ 93% to ~ 80%). This decline is not sufficient to account for the observed reduction in TdR incorporation; therefore, other explanations have to be sought. It was demonstrated by others that, in cultures containing 10% or more peritoneal macrophages, TdR incorporation into mitogen stimulated cells is strongly inhibited (179, 263). In our culture system, the addition of peritoneal macrophages to normal spleen cell cultures also reduced TdR incorporation. In contrast, addition of 5 or 10% of macrophages to macrophage-depleted cell suspensions resulted in an increase in TdR incorporation, demonstrating that the reduction observed in normal spleen cells was not due to inhibitory properties of peritoneal macrophages, larger numbers of peritoneal macrophages (>10%), however, caused inhibition of TdR incorporation in such macro phage-depleted spleen cell suspensions. Consequently, the effect of macrophage removal on the decreased Con A response of TB spleen cells was studied. Treatment of TB spleen cells with the carbonyl iron/magnet technique or glass-adherence led to a complete restoration of the Con A response (on a per cell basis); this was accompanied by a decrease in the number of phagocytic cells from ~ 14% (before treatment) to ~ 4% (after treatment).

Thus, it becomes apparent that Con A responsive T cells are present in TB spleen, although the response of untreated (not macrophage-depleted) cells is depressed. This rules out the possibility that the tumour-induced impairment in T-cell-mitogen responsiveness is due to intrinsic defects in the thymus-dependent lymphocytes.

There are several explanations for the reconstituting effect of macrophage depletion on the defective Con A response in TB spleen cell suspensions. First, the spleens of TB animals may contain increased numbers of nonreactive cells (as suggested in 2, 129, 197) which dilute out the Con A responsive population and are removed by these techniques. This seems unlikely, since only 20-30% of the cells is lost after macrophage-depletion, whereas the response is increased four- to sixfold. Kirchner and colleagues (110, 186, 189) proposed that, in MSV tumour-bearing mice, suppressor cells with macrophage properties which inhibited the proliferative response of T lymphocytes were present. The data obtained in the present study suggest a third possibility, namely, that the inhibition is attributable to a quantitative rather than a qualitative change in the spleen macrophage population. However, we cannot exclude the possibility that a qualitatively distinct suppressor cell with macrophage-like properties has been removed by these techniques, but a simple removal of excess numbers of macrophages also seems a likely explanation.

The question may be raised whether increased numbers of macrophages indeed interfere with T cell proliferation or only inhibit ^{14}C-TdR incorporation by releasing cold thymidine into the culture supernatants, as was suggested by others (109, 110, 183, 264; see also Chapter II).

In the present study, no evidence for soluble inhibitory factors in the supernatants of TB spleen cell cultures was obtained; N and TB spleen cell supernatants (irrespective of the way in which they were prepared or diluted) had similar effects on TdR uptake into mitogen stimulated normal spleen cell cultures. Washing of TB spleen cells prior to pulse labelling did not change the results. In our culture system, however, the chance that competing cold thymidine interferes with ^{14}C-TdR uptake is low, since ^{14}C-TdR of low specific activity is used (0,03 Ci/mmol, in contrast to others (109, 110, 183, 264), who used ^{3}H-TdR of 2.2 Ci/mmol or 6 Ci/mmol). The suppression of TdR uptake we have observed in TB spleen cells was also reflected in a decrease in the number of lymphoblasts. However, inhibition of TdR incorporation was more marked than the decrease in the number of lymphoblasts. It is difficult to explain an 80% reduction in TdR uptake by a 25% reduction in blast formation, but selective survival of stimulated cells could contribute to this discrepancy.

CHAPTER V

TUMOUR-INDUCED CHANGES IN T CELL MITOGEN RESPONSES IN RATS: SUPPRESSION OF SPLEEN AND BLOOD LYMPHOCYTE RESPONSES AND ENHANCEMENT OF THYMOCYTE RESPONSES

5.1 INTRODUCTION

In the previous Chapter, the strong reduction in the Con A responsiveness of spleen cells from rats bearing transplants of a syngeneic tumour was shown to be associated with an increase in the number of phagocytic cells (macrophages) from 6% (normal) to 14% (tumour-bearing, TB). The decreased Con A response of TB spleen cells was completely restored to normal values by depletion of adherent cells or phagocytic cells. Further identification of these splenic "suppressor" cells is required. Do they represent a distinct type of suppressor cells or do they occur also in normal rats and in other organs from TB rats? Experiments described in this Chapter demonstrate the capacity of PEC from non-tumour bearing rats to negate the restorative effect of macrophage depletion of TB spleen cells, supporting the notion that the suppression of both PHA and Con A responsiveness in the TB spleen is not due to the presence of a unique suppressor cell type, but to the small increase in the number of macrophages. Furthermore, it is shown that macrophages with inhibitory effects may occur in the spleen of rats grafted with either one of 4 different types of tumours, all of spontaneous nonviral origin and nonimmunogenic, indicating that the phenomenon of "suppressor" macrophages is not restricted to virally or chemically induced tumours.

In addition, the effect of TB on T-cell-mitogen responses of peripheral blood lymphocytes was investigated and found to be comparable to its effect on spleen cell responsiveness, i.e., strong reduction in both PHA and Con A responsiveness and restoration by depletion of phagocytic cells.

An additional observation in this study was that the thymus of TB animals showed a marked involution which was mostly confined to the cortical area, a phenomenon which also occurs in mice treated with corticosteroids (5, 44, 47, 173). Therefore, the effect of TB and corticosteroid treatment on the T-cell-mitogen responsiveness of rat thymocytes was compared. The present data indicate that TB leads to a decrease in mitogen nonresponsive, corticosteroid-sensitive thymocytes.

5.2 MATERIALS AND METHODS

5.2.1 Animals, tumours and experimental plan

All experimental conditions have been earlier described (Chapter IV). Twelve- to 14-week-old female (WAG x BN)F1 rats were used for transplantation. The tumour used in most experiments is a carcinoma of the bladder originally occurring spontaneously in a 30-month-old BN/Bi male rat (50). In some experiments, rats grafted sc with similar fragments of tumour tissue from an in vivo transplantation line of a ureter carcinoma (50), a fibrosarcoma or a rhabdomyosarcoma were used; all tumours were of spontaneous origin and originated in BN/Bi rats.

5.2.2 Mitogen stimulation of spleen and thymus lymphocytes

For these experiments, a microculture system was used, in contrast to the *macrosystem* used for the studies in TB rats described in Chapter IV. The experimental conditions have been described previously (Chapter III).

5.2.3 Blood leucocyte cultures

Animals were anaesthetized with ether and blood was withdrawn by cardiac puncture. Per 10 ml of blood, 0.1 ml heparin (Tromboliquine, Organon, Oss, The Netherlands, 5000 IU per ml) was added. Ten ml of blood were mixed with 3 ml of a 5% dextran solution in saline (M = 200,000, Batek 860, Porret Products, Amsterdam, The Netherlands) in a Falcon 3033 tube and incubated at 37°C for 1 h at an angle of 45°. The upper lymphocyte-rich layer was removed and washed twice in RPMI (centrifugation at 220 g for 15 min). The final cell suspension (cell viability > 95%) was adjusted to 1×10^6 cells per ml RPMI supplemented with serum, antibiotics, 2-ME and glutamine as described above (Chapter III) and dispensed into the wells of flatt bottom microtitre plates (2×10^5 cells/0.2 ml per well). Mitogen stimulation, labelling and harvesting were performed as described for spleen cells (Chapter III), except that cultures were labelled after 48 h and harvested after a total culture period of 72 h.

5.2.4. Macrophage-depletion of spleen cells and peripheral blood leucocytes and preparation of macrophages.

These techniques were performed as described in Chapter IV. In the present experiments, only carbonyl iron/magnet treatment was used to remove macrophages, since it was previously shown (Chapter IV) to have similar effects as glass-adherence.

5.2.5 Treatment of animals with corticosteroids

F1 rats of 16-18 weeks of age were injected intraperitoneally with various doses of hydrocortisone acetate (HC, Hydro-Andreson; Organon, Oss, The Netherlands; 25 mg/ml). Three days later, thymuses were removed and the number of cells remaining as well as the response of these cells to PHA and Con A were determined. Control animals were injected with comparable volumes of saline.

5.2.6 Histological studies of the thymus

Histological studies were made on thymuses of 16 tumour bearing (TB) rats with varying tumour loads which had received transplants of the bladder tumour 20-30 days before sacrifice. In addition, thymuses from rats treated with 2.5, 5, 7.5, 10 and 15 mg HC were investigated (6 rats per group), as were thymuses from 20 age-matched control rats. The thymuses were fixed in 4% buffered formalin. After routine processing, 5 μ slides were stained with Hematoxylin Phloxine Saffron.

5.3 RESULTS

5.3.1 Effect of tumour bearing (TB) on PHA and Con A responsiveness of spleen cells

At all Con A doses, ^{14}C-TdR incorporation into TB spleen cells is less than in spleen cells from normal rats (Figure 5.1). ^{14}C-TdR incorporation into PHA stimulated cultures was always less affected by tumour-bearing than that into Con A stimulated cultures, but also decreased. The decreased response to both mitogens could be restored to normal values by treatment with carbonyl iron/

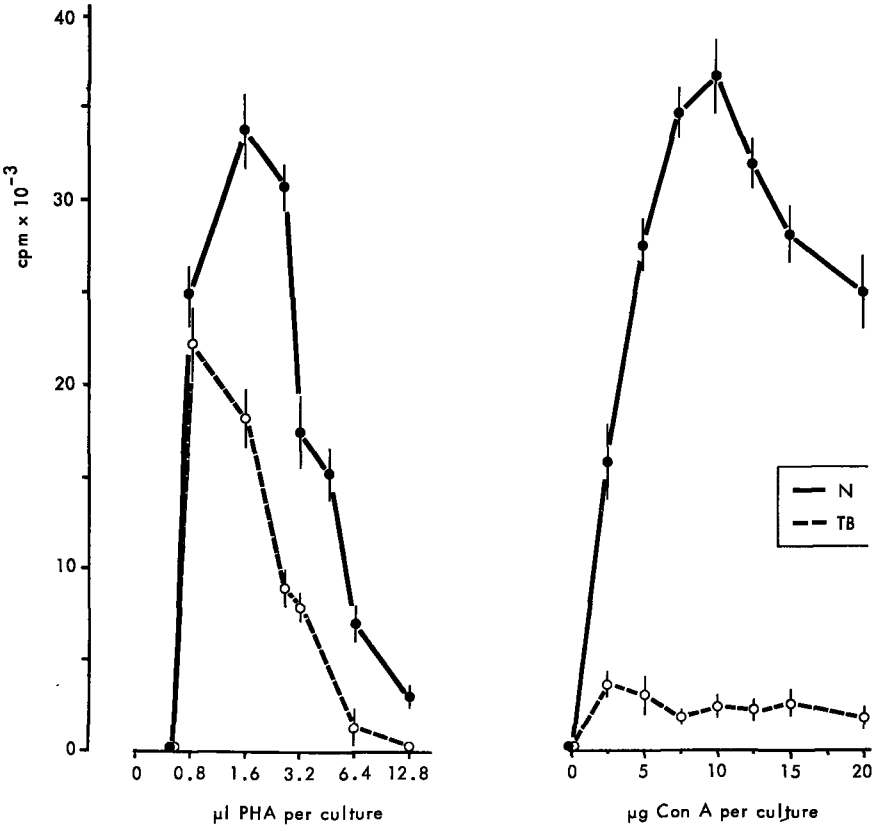

Figure 5.1

^{14}C-TdR incorporation into 4 x 10^5 spleen cells from N and TB rats after stimulation with various doses of PHA or Con A. The results represent the arithmetic mean ± standard deviation of triplicate cultures. The data are representative of 5 experiments in which pooled spleen cells from 5 N or 5 TB (grafted with bladder tumour) animals were used. TdR incorporation into control cultures (without mitogen) varied from 40 to 170 cpm and was not significantly affected by TB.

magnet (Figure 5.2), which is associated with a decrease in the number of phagocytic cells to normal values (Chapter IV, Table 4.1).

When 10% peritoneal exudate macrophages (PEC) from thioglycollate-stimulated rats were added to macrophage-depleted TB spleen cell suspensions (Figure 5.2), ^{14}C-TdR incorporation into mitogen stimulated TB spleen cells decreased to the values observed before macrophage depletion, indicating that the state of TB can be mimicked by the addition of exogenous macrophages. ^{14}C-TdR incorporation was found to be also inhibited in spleen cells from rats grafted with 3 other types of tumours (Table 5.1) and the response could be restored by

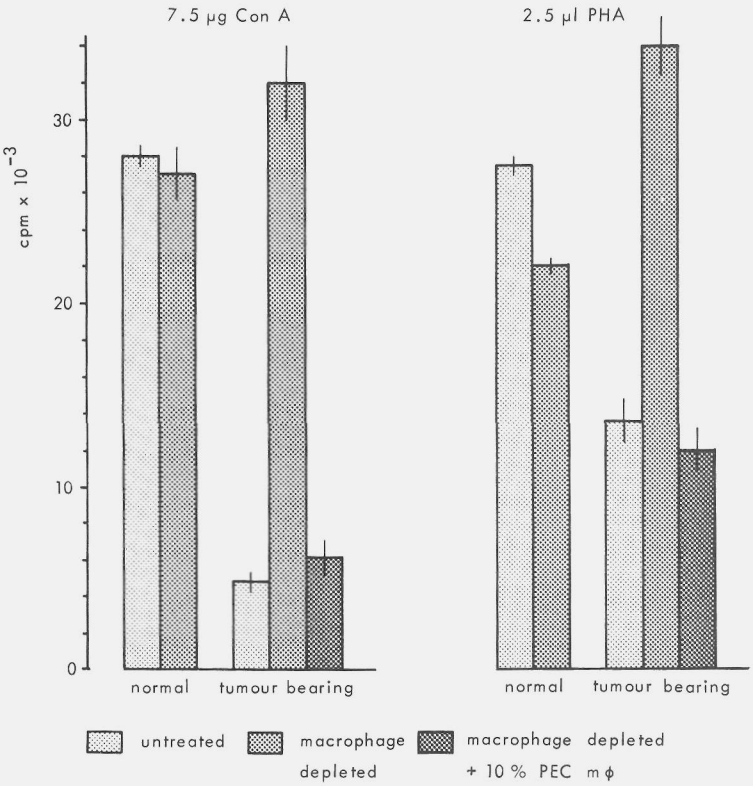

Figure 5.2

The effect of carbonyl iron/magnet treatment and addition of peritoneal macrophages on TdR incorporation into Con A or PHA stimulated 4 x 10^5 spleen cells from N and TB rats. A representative experiment in which pooled spleen cells from 5 N or 5 TB (grafted with bladder tumour) animals were used is shown. The results represent the arithmetic mean ± standard deviation of triplicate cultures. Mitogen doses giving optimal responses were used. TdR incorporation into cultures without mitogen varied from 30 to 175 cpm and was not significantly affected by either TB, macrophage depletion or the addition of PEC.

macrophage-depletion. This restorative effect could again be negated by addition of PEC from either normal (data not shown) or thioglycollate-treated rats (Table 5.1).

5.3.2 Effect of tumour bearing on PHA and Con A responsiveness of peripheral blood leucocytes

^{14}C-TdR incorporation into PHA and Con A stimulated blood leucocyte cultures was also strongly affected by tumour bearing (Figure 5.3). Differential

TABLE 5.1

EFFECT OF CARBONYL IRON MAGNET TREATMENT ON ^{14}C-TdR INCORPORATION INTO Con A AND PHA-STIMULATED SPLEEN CELLS FROM TB RATS

type of tumour*	mitogen**	untreated TB spleen cells	mø-depleted TB spleen cells	mø-depleted TB spleen cells + 10% PEC cells
ureter carcinoma	Con A	18.1 ± 6.2***	114.2 ± 8.3	17.0 ± 11.6
	PHA	48.3 ± 9.2	121.5 ± 18.3	35.1 ± 8.3
rabdomyo-sarcoma	Con A	7.7 ± 2.5	102.6 ± 13.0	14.3 ± 4.4
	PHA	34.0 ± 5.6	108.6 ± 10.1	43.1 ± 12.1
fibro-sarcoma	Con A	11.3 ± 6.5	110.1 ± 8.9	21.3 ± 6.4
	PHA	53.6 ± 5.0	106.1 ± 11.2	49.5 ± 4.8

* In all cases, the total tumour mass represented 5-10% of the body weight.

** Only the values for mitogen doses giving optimal responses are presented.

***The response in cultures of TB spleen cells is given as a percentage of that obtained with normal spleen cells, which is estimated at 100 in each experiment. Numbers represent the mean values ± standard deviation of 3 separate experiments in which pooled spleen cells from 5 rats were used. For absolute values of ^{14}C-TdR incorporation, see Figures 5.1 and 5.2. The response of unstimulated cultures was not affected by TB, mø-depletion and/or addition of PEC.

TABLE 5.2

EFFECT OF CARBONYL IRON/MAGNET TREATMENT ON ^{14}C-TdR INCORPORATION INTO Con A AND PHA STIMULATED BLOOD LYMPHOCYTES FROM TB RATS

mitogen*	untreated N blood	untreated TB blood	mø-depleted TB blood	mø-depleted TB blood + 10% PEC cells
Con A	12214 ± 4220** (100%)	4560 ± 2703 (37%)	16856 ± 7283 (138%)	5007 ± 1508 (41%)
PHA	35225 ± 8020 (100%)	21530 ± 4860 (61%)	42048 ± 9832 (119%)	19373 ± 2895 (55%)

* Only the values for mitogen responses giving optimal responses are presented.

**Figures represent the mean cpm of triplicate cultures in 3 separate experiments ± standard deviations; in each experiment, pooled blood from 5 rats was used. The response given in parentheses is the percentage of the response obtained in N blood. ^{14}C-TdR incorporation into unstimulated cultures was not affected by TB, mø depletion and/or addition of PEC. Rats grafted with the bladder tumour were used.

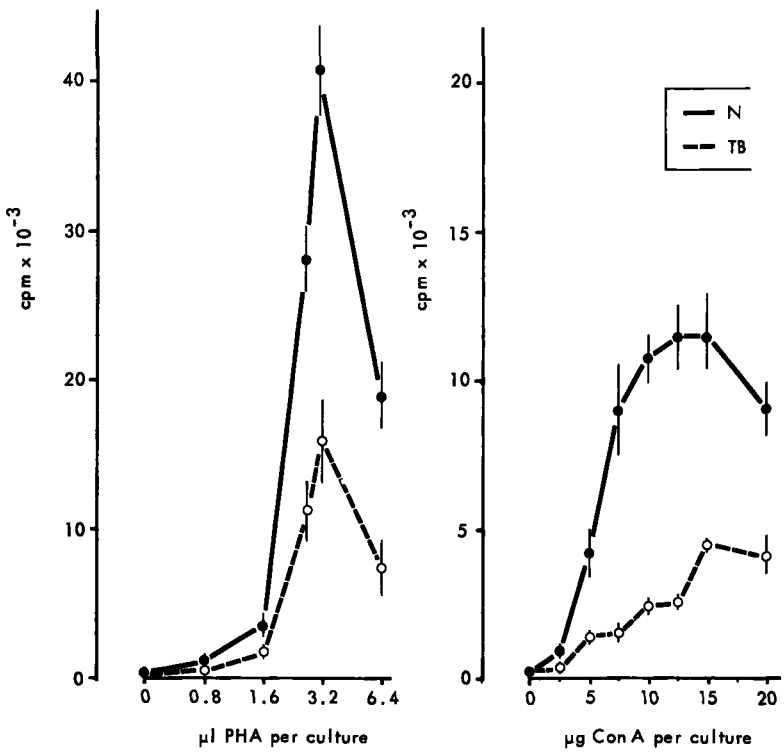

Figure 5.3

^{14}C-TdR incorporation into 2 x 10^5 peripheral blood lymphocytes from N and TB rats after stimulation with various doses of PHA or Con A. The results represent the arithmetic mean ± standard deviation of triplicate cultures. The data are representative of 5 experiments in which pooled peripheral blood from 5 N or TB (grafted with bladder tumour) animals was used. TdR incorporation into unstimulated cultures varied from 100 to 200 cpm and was not affected by TB.

counts on peripheral blood samples (after Dextran treatment) from TB rats revealed that the relative number of granulocytes and monocytes had increased to 20-25%, in normal rats, granulocytes and monocytes represent 4-14% of the total number of leucocytes. Carbonyl iron powder treatment of TB blood led to a complete restoration of the response (Table 5.2), accompanied by an increase in the relative number of lymphocytes to normal values (~90%), while the number of granulocytes and monocytes decreased to normal values. Addition of PEC abolished the restorative effect of macrophage depletion (Table 5.2), suggesting that the depressed response of TB blood lymphocytes is also caused by an increase in the number of monocytes and/or granulocytes.

5.3.3 The effect of tumour-bearing (TB) or hydrocortisone-acetate treatment on thymus morphology and cell yield

On histological examination, the effect of TB on thymic tissue appeared variable, but more severe changes were observed with increasing tumour load. In the most severe cases, the thymus was reduced to a small strand of fibroblastic and histiocytic cells with no recognizable distinction between the cortical and medullary areas. Only sporadic epithelial cells and some dispersed lymphocytes were observed. In other cases with intermediate atrophy, the thymus was decreased in size mainly because of lymphocyte depletion and shrinkage of the cortical area.

The morphological changes in the thymus caused by cortisone treatment were comparable to those induced by TB. A dose-dependent decrease in thymus volume was observed, mainly due to lymphocyte depletion and shrinkage of the cortex. Some differences between TB and HC thymuses, however, were observed. Even in the rats treated with the highest dose of HC, a clear distinction between cortical and medullary areas could be recognized; an atrophy as severe as that observed in some TB rats was not evident in the HC rats. Furthermore, macrophages were relatively prominent in those TB thymuses which exhibited severe atrophy.

TABLE 5.3

EFFECTS OF TB ON CELL NUMBER AND T CELL MITOGEN RESPONSIVENESS IN THYMUS FROM N, TB AND CORTISONE-TREATED RATS

rats		number of cells left (% of control)	2.5 µg PHA	7.5 µg Con A	number of animals
control		100%	1	1	30
TB	5-10 g	28.0 ± 3.5*	2.9 ± 0.6**	2.9 ± 0.65	10
	10-20 g	8.2 ± 2.4	12.4 ± 3.1	9.5 ± 1.9	14
	20-30 g	3.8 ± 0.8	2.3 ± 0.5	2.1 ± 0.4	10
HC	5 mg	8.0 ± 1.7	13.5 ± 1.15	8.2 ± 0.4	30
	10 mg	5.6 ± 0.5	19.3 ± 1.3	10.4 ± 0.8	30
	15 mg	3.0 ± 0.3	18.8 ± 1.4	9.7 ± 0.9	30

* Each value represents the mean ± standard error of the mean (SE) of the indicated number of animals. The absolute number of thymocytes in normal animals was $381.9 \pm 14 \times 10^6$ (mean ± SE of 30 rats). Rats grafted with the bladder tumour were used in these experiments.

**Numbers represent the factor by which the response is increased as compared to the normal control. The mean values ± SE of the indicated number of animals is given. For absolute values of ^{14}C-TdR incorporation see Figure 5.4.

Both TB and cortisone treatment of rats results in a strong decrease in the number of thymocytes recovered from each thymus (Table 5.3). The reduction in cell yield caused by a tumour mass of 10-20 grams was comparable to treatment with 5 mg of HC. In animals carrying a tumour mass larger than 20 grams, the reduction was comparable to that found after treatment with 15 mg HC.

5.3.4 Effect of tumour bearing or hydrocortisone acetate treatment on PHA and Con A responsiveness of thymocytes

The cells remaining in the thymuses of TB rats exhibit increased responses to PHA and Con A (Figure 5.4). The dose-response profile to both mitogens in TB thymocytes was similar to that observed in normal thymocytes. A summary of the results obtained in individual animals with mitogen doses giving maximum responses is given in Table 5.3. The increase in T cell mitogen responsiveness is dependent on the size of the tumours: with increasing tumour mass, the atrophy of the thymus was more severe, the number of thymocytes was decreased and the response to T cell mitogens was increased. However, this does not apply to animals carrying very large tumours (total tumour mass $>$ 20 grams), in which the increase was much less pronounced.

The cells present in the HC-treated thymus also showed an increase in responsiveness to both PHA and Con A (Table 5.3). PHA responses were more increased than Con A responses, as has been reported for HC-treated mice (325). The increase in both PHA and Con A responsiveness and reduction in cell number observed in rats treated with 5 mg HC was similar to that observed in animals carrying a tumour mass of 10-20 grams. Surprisingly, the increase in mitogen responsiveness in animals carrying a tumour mass larger than 20 grams was much lower than that observed in groups treated with high doses of HC, although the number of cells remaining was comparable.

5.4 DISCUSSION

The experiments reported above demonstrate a marked decrease in the PHA and Con A responsiveness of spleen cell cultures from rats bearing subcutaneous transplants of a bladder tumour (Figure 5.1). Mitogen responses were restored by treatment of the spleen cell suspensions with techniques leading to macrophage depletion. Similar results were obtained with spleen cells from rats bearing subcutaneous transplants of either one of 3 other types of tumours (Table 5.1), thus demonstrating that the observations were not restricted to

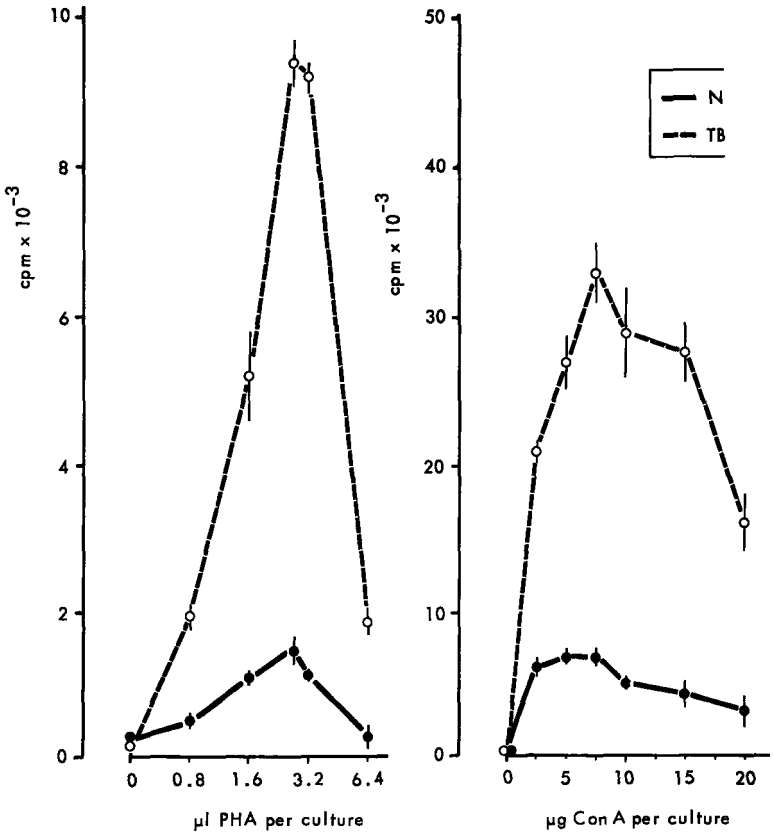

Figure 5.4

^{14}C-TdR incorporation into 4 x 10^5 thymocytes from N and TB rats after stimulation with various doses of PHA or Con A. A representative experiment in which pooled thymus lymphocytes from 5 N and 5 TB (grafted with bladder tumour) rats (carrying a total tumour mass of 10-15 g) were used. The results represent the arithmetic mean ± standard deviation of triplicate cultures. TdR incorporation into unstimulated cultures was not affected by TB and varied from 50 to 125 cpm.

one type of tumour. An increase in mitogen or allogeneic cell responses after macrophage depletion has also been reported for spleen cells from mice and rats bearing transplants of virus induced tumours (110, 131, 183, 185-187, 189, 261, 361) and was interpreted as being due to removal of a qualitatively distinct type of suppressor macrophage. However, the data obtained in our system suggest that the suppression phenomenon can also be explained in a quantitative way. It was shown in the previous Chapter that the number of macrophages in TB spleen cell suspensions was increased from the normal value of ~ 6% to ~ 14%. It was also shown that ^{14}C-TdR incorporation into mitogen stimulated normal spleen

cell cultures to which 5% or more peritoneal macrophages were added was reduced to the levels observed in TB spleen cultures (Chapter IV, ref. 207, 208), a phenomenon observed by several other investigators (27, 109, 179, 234, 262, 315, 337). Together with the fact that the restorative effect of macrophage-depletion of TB spleen cells could be negated by the addition of 10% PEC-macrophages, these data suggest that the inhibition phenomenon in TB spleen cell cultures is attributable to an increase in the number of macrophages rather than to the presence of qualitatively distinct suppressor cells. The final macrophage to lymphocyte ratio in the cultures (irrespective of the origin of these macrophages) determines whether ^{14}C-TdR incorporation will be inhibited. However, the possibility that both a quantitative and a qualitative effect are responsible for the final suppressive effects in TB spleen cannot be excluded. It has been shown (27, 179, 377) that, on a per cell basis, macrophages display varying levels of suppressive effects on lymphocyte proliferation, dependent upon their degree of "activation". Therefore, besides the actual macrophage to lymphocyte ratio, the magnitude of macrophage "activation" also determines whether lymphocyte proliferation will be inhibited. Only cell separation studies might disclose whether, as was suggested by others (110, 131, 183, 185-187, 189, 261, 361), a qualitatively distinct type of macrophage is present in the spleen of TB mice or rats, i.e., whether tumour bearing also leads to "activation" of splenic macrophages. However, this could be dependent upon the type of tumour.

With regard to the types of tumours used in the present study the following has to be emphasized: it has been suggested that suppressor macrophages develop in the spleen as a consequence of stimulation by tumour - and/or viral antigens (188, 361) or alloantigens (361) and that this phenomenon represents a feedback mechanism in order to prevent excessive autonomous proliferation of lymphocytes upon strong antigenic stimulation (188). The present data indicate that similar phenomena also occur using four different types of tumours (originally occurring spontaneously) which appeared to be nonimmunogenic by conventional in vivo immunization-challenge procedures (irrespective of the cell dosage used or the time lapse between immunization and challenge) (R.L.H. Bolhuis, J.C. Klein and A.M. Kruisbeek, unpublished results). Therefore, it seems unlikely that appearance of "suppressor" macrophages is only a result of stimulation by immunogenic tumours.

The present experiments also show that the occurrence of "suppressor" macrophages is not restricted to TB spleen: the decreased mitogen responsiveness of peripheral blood leucocytes from TB animals (Figure 5.3) could be returned to normal values by treatment with carbonyl iron powder (Table 5.2), while the relative number of lymphocytes and phagocytic cells reverted to nor-

mal values after this treatment. These results indicate that great care has to be taken in interpreting a decrease in T cell mitogen responsiveness in blood from cancer patients (as reported, e.g., in 77, 289, 313) as a reflection of an impairment in thymus-dependent immunity due to intrinsic defects in the T cells. In the present study, the decreased responses were a result of variations in the macrophage population rather than in the lymphocytes themselves.

A relation between the cell number in the thymus and the weight of the tumour was found: histological studies revealed that both the cortical and the medullary areas had decreased in size, but the most impressive lymphocyte depletion was found in the cortex. ^{14}C-TdR incorporation into both PHA and Con A stimulated thymocytes was markedly increased (Figure 5.4, Table 5.3) in TB rats, dependent upon the size of the tumour. This suggests the disappearance of nonresponsive cortical thymocytes with consequent enrichment in mitogen-responsive medullary thymocytes. However, in rats carrying the heavy tumours, the increase in response was smaller, despite the strong decrease in cell number. One possible explanation could be an increase in macrophage content up to inhibitory levels, since macrophages were more prominent in the atrophic thymuses associated with a heavy tumour burden. Separate work (208) revealed that addition of PEC-macrophages to mitogen stimulated thymocyte cultures inhibited ^{14}C-TdR incorporation at high concentrations of macrophages (5% and more).

The possibility that corticosteroid-mediated effects contributed to the observed effects of TB on the thymus was considered. Corticosteroids exert a variety of effects on most lymphoid organs (recently reviewed in ref. 12), of which thymus shrinkage is one of the most prominent. In the rat strain used here, the thymocyte population remaining after HC treatment showed increased responsiveness to both PHA and Con A, as has been reported for mice (5, 47, 173, 325). The increase observed in rats treated with 5 mg HC was comparable to that observed in animals carrying a tumour mass of 10-20 grams and the reduction in the number of thymocytes was also comparable. The close similarity between the effects of cortisone treatment and TB is suggestive for a role of corticosteroids in the thymic involution observed in TB hosts, a suggestion advanced by others (86, 103, 314) on the basis of histological studies. The fact that a reduced sensitivity to anti-Thy 1 antiserum plus complement has been observed in cortisone-resistant thymocytes (199, 284, 325) as well as in TB mouse thymocytes (232) is also suggestive of a corticosteroid-mediated effect in the thymic involution in TB rats. Preliminary data indicate that serum corticosterone levels are enhanced in TB rats (unpublished observations). However, Simu et al. (314) found that removal of the adrenals attenuated thymus involution in tumour bearing rats but did not prevent it, suggesting that adrenal secretion is not exclusively responsible for this phenomenon. The mechanism re-

sponsible for thymic involution in tumour bearing mice was ascribed by others to fewer T lymphocyte precursors in the bone marrow (79), to enhanced migration of thymocytes from the cortex to the peripheral lymphoid tissue, where they can exercise a killer cell function (108), or to tumour-derived factors inducing thymus atrophy (118, 180).

In conclusion, our data with spleen and blood lymphocyte cultures indicate that poor mitogen reactivity of lymphocytes from cancer patients or TB animals should be interpreted with great caution: it is possible that altered reactivity is mediated by other cell types rather than being due to intrinsic defects in the lymphocytes. Accordingly, this type of study should always be accompanied by cytological analysis of the cell suspensions. Small changes in the macrophage to lymphocyte ratio can cause large changes in mitogen-induced lymphocyte stimulation. However, the severe changes observed in the thymus of TB rats suggest that the presence of the tumour might eventually (dependent upon time) lead to deficiency in functional thymus-dependent lymphocytes in the periphery, since it has been suggested (335, 373) that the cortical thymocytes represent the direct precursor cells of mature peripheral T cells.

CHAPTER VI

HELPER AND INHIBITORY EFFECTS OF MACROPHAGES IN ACTIVATION OF T CELLS

6.1 INTRODUCTION

The experiments described in this Chapter were designed in part to provide an explanation for the following discrepancies between our data on alloantigen and mitogen induced rat T lymphocyte proliferation and those from the literature:

a) We consistently obtained good mixed lymphocyte reactivity (MLR) in rat spleen cell cultures, in contrast to others (38, 233, 262), who observed that rat spleen cells responded to allogeneic cells only after macrophages had been removed from the responding cells. Analogous to these findings, it was also reported that splenic macrophages suppressed in vitro generation of cytotoxic effector cells in a rat MLC (38, 262, 371).

b) T cell mitogen responsiveness of rat spleen cells either decreased or remained the same after macrophage depletion (207, 208; Chapters IV and V) in our studies, which is in agreement with some authors (361) but in contrast to other reports showing that, after carbonyl iron treatment (131) or glass adherence (111, 112, 115), rat spleen cell responses to PHA and Con A were increased.

Thus, it was postulated that the normal rat spleen contains adherent "suppressor" cells which are either T cells (111-115) or macrophages (38, 131, 233, 262, 371), whereas our data indicated that rat splenic macrophages may act as accessory or helper cells (207, 208) rather than as suppressor cells. Since T lymphocyte proliferative capacity is used as a parameter for determining T cell reactivity in many of the studies reported in this thesis, it seemed necessary to search for explanations for these discrepancies. Similar discrepancies can be found in the literature on mouse T lymphocyte proliferation induced by mitogens or alloantigens: both a suppressor function of splenic macrophages (27, 28) or adherent T cells (369) and a helper function of splenic macrophages (240, 377) have been reported. Also studies on the effect of the addition of peritoneal exudate macrophages to lymphocyte cultures have so far given conflicting results: strong suppression of lymphocyte proliferative responses have been reported for both mice (27, 28, 109, 377) and rats (179, 208, 211, 262), but enhancement has also been observed in mice (377) and rats (179).

In view of these conflicting data on splenic and peritoneal macrophages, it is difficult to ascertain the nature of the regulatory role which macrophages have in lymphocyte proliferation under normal conditions. The experiments described in this Chapter were intended to devise a model which explains these so far conflicting data from the literature.

It should be emphasized that these experiments were by no means aimed at criticizing data indicating a helper role of macrophages. Studies on both antigen- (reviewed in 259) and mitogen- (reviewed in 271) -induced T cell proliferation have provided a firm basis for considering Ia-antigen-positive macrophages as essential helper cells. It was the puzzling finding that many other investigators failed to observe helper effects of macrophages (27, 28, 38, 131, 233, 262, 371), but in contrast obtained evidence for suppressive effects, which needed further clarification.

6.2 MATERIALS AND METHODS

6.2.1 Animals, tumours

In all experiments, cell pools from five 12-14 week-old WAG/Rij rats were used. Rats were reared under SPF and maintained under clean conventional conditions (160). When indicated, germ-free (GF) rats were used. For determination of MLR, spleen cells from (WAG x BN)F1 rats were used as stimulator cells.

6.2.2 Mitogen stimulation, mixed lymphocyte reactivity, macrophage depletion techniques and collection of peritoneal macrophages

Cell suspensions from blood, thymus and spleen were prepared as previously described (Chapters III and V). The medium routinely used was bicarbonate buffered RPMI 1640 (2-0631; Flow Laboratories, Irvine, Scotland) supplemented with 15% rat serum, penicillin (100 IU/ml), streptomycin (0.1 mg/ml), 5×10^{-5} M 2-mercaptoethanol (2-ME) and 2 mM glutamine. In some experiments, 2-ME was left out (see Results). Both mitogen stimulation and mixed lymphocyte reactivity were performed as described (Chapter III). Macrophage depletion and preparation of peritoneal macrophages (PEC) were performed as described in Chapters IV and V. The number of remaining phagocytic cells in all experiments was $< 2\%$, except in spleen cells from GF rats which contained no phagocytic cells after carbonyl/iron magnet treatment. Macrophage depletion was always performed on the re-

sponder cell population. PEC were obtained from the same strain of rats used as responder cell donors in MLR (i.e., the WAG/Rij). For the composition of PEC suspensions, see Chapter IV.

6.3 RESULTS

6.3.1 Effects of macrophage depletion and addition of macrophages on T cell mitogen responses

When rat spleen cells were cultured without 2-ME, Con A- and PHA-responses decreased after carbonyl iron/magnet treatment (Table 6.1) or glass adherence (data not shown; see ref. 208). This low level of responsiveness can be fully restored to normal by the addition of appropriate numbers of PEC, which, however, suppress the response if added in higher numbers. As shown in Chapter IV, carbonyl iron/magnet treated PEC showed no reconstituting or suppressive effects, suggesting that the phagocytic cells in the PEC population were responsible for the above findings. On the other hand, when spleen cells were

TABLE 6.1

EFFECT OF MACROPHAGE DEPLETION AND ADDITION OF MACROPHAGES ON ^{14}C-TdR INCORPORATION INTO RAT SPLEEN CELLS STIMULATED WITH Con A or PHA (IN THE ABSENCE OF 2-ME)*

percentage of macrophages added	response untreated spleen cells		response macrophage depleted spleen cells	
	Con A	PHA	Con A	PHA
0	100%	100%	40.7 ± 10.8**	70.5 ± 8.8
1	96.3 ± 8.5	95.8 ± 7.6	64.6 ± 8.8	95.2 ± 5.5
5	50.5 ± 10.1	75.4 ± 8.3	109.3 ± 11.2	101.2 ± 6.6
10	29.6 ± 9.6	51.2 ± 6.5	85.5 ± 16.3	90.2 ± 6.3
15	15.5 ± 3.1	35.6 ± 5.7	36.3 ± 7.4	61.4 ± 8.1

* In cultures without 2-ME, ^{14}C-TdR incorporation values were 6 to 8 times lower than in cultures with 2-ME; the latter values are shown in Fig. 6.1A and B.

**Responses are given as a percentage of the control (100%). Figures represent the mean values ± standard deviation of 4 separate experiments.

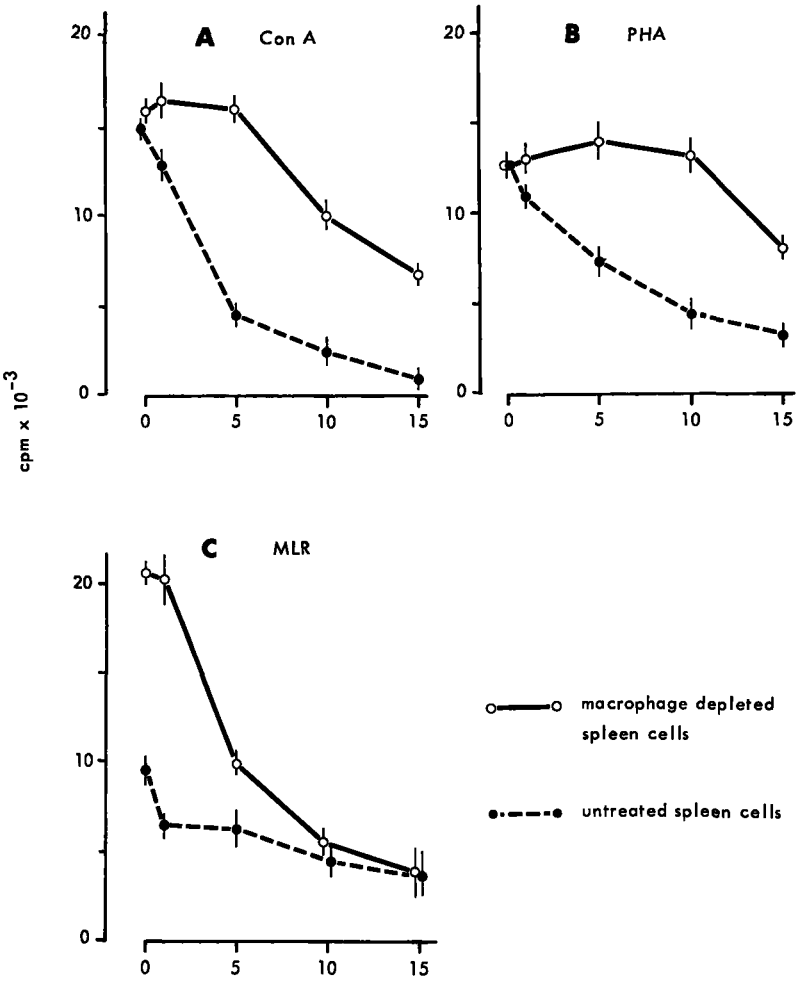

Figure 6.1

^{14}C-TdR incorporation into untreated and macrophage depleted rat spleen cells stimulated with Con A (A), PHA (B) or allogeneic cells (C). Figures represent the mean ± standard deviation of triplicate cultures and are representative for 7 separate experiments. Thymidine incorporation into control cultures (without mitogen or with syngeneic cells) varied between 260 and 800 cpm and was not influenced by macrophage depletion or addition (indicating that macrophages do not contribute to the measured DNA synthesis).

TABLE 6.2

EFFECT OF MACROPHAGE DEPLETION AND ADDITION OF MACROPHAGES ON ^{14}C-TdR INCORPORATION INTO RAT SPLEEN CELLS STIMULATED WITH Con A OR PHA (IN THE PRESENCE OF 2-ME)

percentage of macrophages added	response untreated spleen cells		response macrophage depleted spleen cells	
	Con A	PHA	Con A	PHA
0	100%	100%	103.5 ± 22.8*	100.5 ± 21.5
1	88.7 ± 5.1	91.5 ± 7.5	115.1 ± 8.2	112.6 ± 10.1
5	39.3 ± 6.6	59.8 ± 8.5	106.4 ± 7.6	104.3 ± 6.6
10	15.3 ± 3.5	42.6 ± 7.2	75.2 ± 4.7	102.7 ± 9.2
15	6.4 ± 4.5	26.0 ± 5.3	50.8 ± 6.5	75.1 ± 6.8

*Responses are given as a percentage of the control (100%). Figures represent the mean values ± standard deviation of 7-11 separate experiments. For absolute values of ^{14}C-TdR incorporation, see Fig. 6.1A and B.

cultured in the presence of 2-ME, no change in PHA and Con A responsiveness was observed after macrophage depletion (Table 6.2, Fig. 6.1 AB). On addition of increasing numbers of PEC, a gradual suppression of the response was observed. Fewer PEC were required to obtain suppression in untreated spleen cells than in macrophage-depleted spleen cells. When 5% PEC (consisting of >90% of phagocytic cells) were added to macrophage-depleted spleen cells (containing less than 2% macrophages), TdR incorporation values remained at the levels observed in untreated spleens (containing ~ 5% macrophages). PHA responsiveness was always less suppressed than the Con A response. For the following experiments, only data obtained in culture systems with 2-ME will be discussed.

When the effects of macrophage depletion and addition on PHA- and Con A responses of lymphocytes from 3 sources are compared, the following points can be noted (Table 6.3): (a) blood and thymocyte responses decrease after macrophage depletion, whereas spleen cell responses remain the same; (b) the decreased responses of blood and thymic lymphocytes can be restored by the addition of PEC; (c) higher numbers of PEC suppress the response in all types of lymphocytes, but this suppression is most pronounced in thymocytes.

These data also illustrate that the presence of 2-ME does not replace the requirement for macrophages (i.e., spleen cell responses remained the same after macrophage depletion, but blood and thymocyte responses decreased, despite the presence of 2-ME), a finding also reported by others (294). There is the possibility that, after macrophage "depletion", the number of remaining macro-

TABLE 6.3

EFFECT OF MACROPHAGE DEPLETION AND SUBSEQUENT ADDITION OF MACROPHAGES ON ^{14}C-TdR INCORPORATION INTO Con A or PHA STIMULATED RAT SPLEEN CELLS, BLOOD LYMPHOCYTES AND THYMOCYTES (IN THE PRESENCE OF 2-ME)

response macrophage depleted	percentage of macrophages added	Con A	PHA
spleen cells	0	103.5 ± 22.8*	100.5 ± 21.5
	1	115.1 ± 8.2	112.6 ± 10.1
	5	106.4 ± 7.6	104.3 ± 6.6
	10	75.2 ± 4.7	102.7 ± 9.2
	15	50.8 ± 6.5	75.1 ± 6.8
blood lymphocytes	0	51.3 ± 7.6	63.0 ± 7.5
	1	n.d.	n.d.
	5	98.3 ± 6.5	102.8 ± 7.3
	10	n.d.	n.d.
	15	45.2 ± 6.3	65.8 ± 7.3
thymocytes	0	38.6 ± 7.5	44.8 ± 12.9
	1	85.3 ± 8.2	97.2 ± 12.1
	5	35.2 ± 6.8	105.0 ± 17.4
	10	12.7 ± 9.6	48.3 ± 16.3
	15	6.5 ± 5.3	35.3 ± 8.5

*Responses given as a percentage of the values obtained in untreated cell suspensions, which were estimated at 100%.
Figures represent the mean ± standard deviation of 3 separate experiments. Absolute values of ^{14}C-TdR incorporation in untreated cell suspensions ranged from 8198 to 12104 cpm (Con A-blood lymphocytes), 28096 to 39125 cpm (PHA-blood lymphocytes); 3470 to 5362 cpm (Con A-thymus lymphocytes) and 947 to 1254 cpm (PHA-thymus lymphocytes); for spleen cells, see Fig. 6.1A and B.

phages in the spleen, in the presence of 2-ME, is still sufficient for optimal responsiveness, whereas it is not in blood and thymus. The effect of 2-ME in lymphocyte cultures will not be further discussed, because both the above findings and literature data indicate that, in the absence of 2-ME, splenic macrophages can display either an inhibitory (27, 28) or a helper effect (240, 377).

All of the above data were obtained with mitogen doses giving optimal responses. At supraoptimal doses, high numbers of macrophages exert less inhibitory effects (208), as observed by some investigators (27, 179) but in contrast to others (131). Apparently, inhibitory effects of macrophages are also a function of mitogen concentration; however, only data obtained with optimal mitogen doses will be discussed, since insufficient literature data are available on this subject to make a satisfactory comparison.

6.3.2 Effect of macrophage depletion and addition of macrophages on mixed lymphocyte reactivity

The data illustrated in Fig. 6.1C indicate that a good MLR in rat spleen cells is obtained. However, on macrophage depletion, a strong enhancement of MLR was observed. This could be abolished by the addition of peritoneal exudate macrophages (Fig. 6.1C, Table 6.4). When 5% PEC (consisting of more than 90% phagocytic cells) were added to macrophage-depleted spleen cells (containing less than 2% macrophages), ^{14}C-TdR incorporation values decreased to the values observed in untreated spleen cells (containing $\sim$ 5% macrophages). This indicates that, on a per cell basis, PEC exhibited similar suppressive effects as did splenic macrophages. When PEC from unstimulated rats were employed (these contain 45-50% phagocytic cells), two times more PEC had to be added in order to obtain similar suppressive effects as with 5% PEC from thioglycollate treated rats (data not shown). These data demonstrate that also in our system splenic macrophages suppress MLR, though not to the same extent as reported by others (38, 233, 262).

This difference might be due to a difference in the number of macrophages, since (a) it was reported by others (131, 262) that the spleen cell suspensions of their rats contained 8-10% macrophages, which is higher than the number we

TABLE 6.4

EFFECT OF MACROPHAGE DEPLETION AND ADDITION OF MACROPHAGES ON ^{14}C-TdR INCORPORATION INTO RAT SPLEEN CELLS STIMULATED WITH ALLOGENEIC CELLS (IN THE PRESENCE OF 2-ME)

percentage of macrophages added	response untreated spleen cells	response macrophage depleted spleen cells
0	100%	259.2 ± 76.3
1	77.4 ± 10.1*	187.3 ± 61.5
5	55.6 ± 6.8	95.0 ± 14.1
10	37.8 ± 5.8	55.3 ± 8.9
15	27.4 ± 6.7	44.8 ± 5.9

*Responses given as a percentage of the control values (100%) obtained in untreated cell suspensions. Figures represent the mean ± standard deviation of 5 separate experiments. For absolute values of ^{14}C-TdR incorporation, see Fig. 6.1C.

find (~5%) and (b) we observed that, with increasing numbers of exogenous macrophages (PEC), there was a gradual decrease in MLR. This hypothesis was tested by studying the behaviour of spleen cells from germ-free (GF) rats, which contain lower numbers of phagocytic cells (< 3%) than rats kept under clean conventional conditions (160). A comparison of the effect of macrophage depletion on MLR of normal and GF rat spleen cells is given in Fig. 6.2. The results indicate that opposite effects of macrophage depletion are obtained, normal rats again exhibiting an increase, whereas a strong decrease is observed in GF rats. On addition of 5% PEC, the response of GF rats is restored to normal values, whereas that of normal rats decreases to previously observed low values. This indicates that PEC can exert either a helper or an inhibitory effect. As with the suppressive effects of PEC (see above), two times more PEC from unstimulated rats had to be used in order to obtain similar reconstitution of macrophage-depleted GF spleen cells as with PEC from unstimulated rats (data not shown).

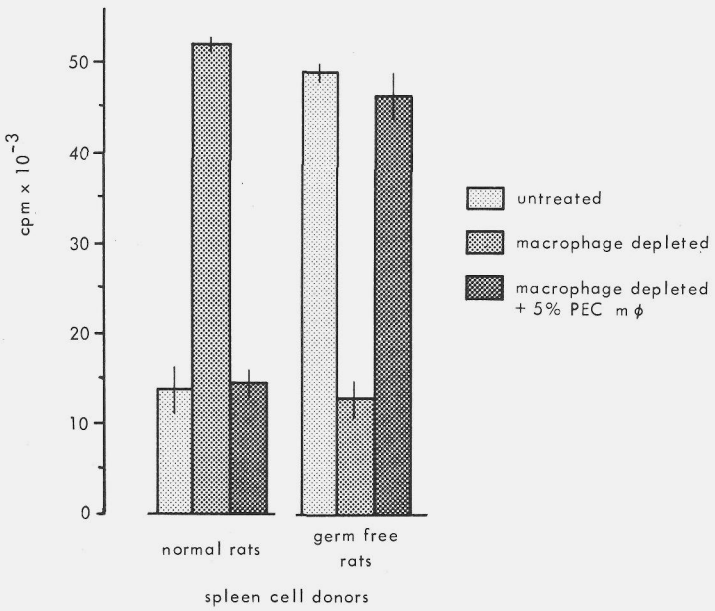

Figure 6.2

Comparative effect of macrophage depletion and addition on mixed lymphocyte reactivity of spleen cells from normal rats and from germ-free rats.
Figures represent the mean ± standard deviation of triplicate cultures and are representative for 3 separate experiments. Thymidine incorporation into syngeneic control cultures varied from 244 to 561 cpm and was not statistically significantly different among the various groups.

6.4 DISCUSSION

This discussion will be mostly limited to data on mouse and rat lymphocyte proliferation, since both inhibitory and helper functions of phagocytic cells in mitogen or alloantigen stimulation have been extensively investigated in these species. It is realized that there is profound functional and morphological heterogeneity among macrophages; however, for the purpose of this discussion, we will refer to all cells that are mononuclear and phagocytic as "macrophages".

On the basis of the above reported results, we postulate a model for the role of macrophages in mitogen- or alloantigen induced lymphocyte proliferation which is presented in Fig. 6.3. It needs no further discussion that, irrespective of the type of lymphocyte or stimulant used, increasing the number of macrophages in the culture always eventually led to suppression. If it is assumed that there is a specific optimal macrophage: lymphocyte ratio for each type of lymphocyte and stimulatory agent, we are faced with the following 3 possible situations:

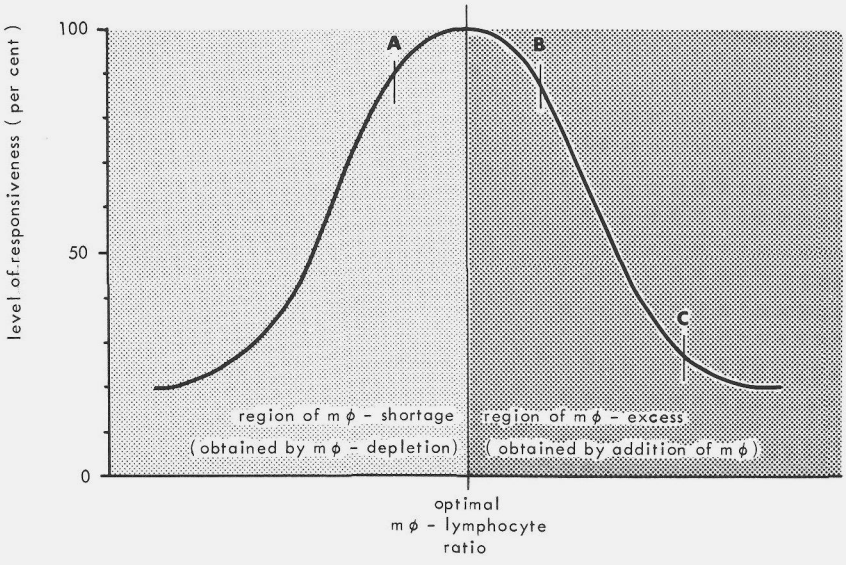

Figure 6.3

Theoretical model for the relationship between lymphocyte responsiveness and macrophage to lymphocyte ratio in the culture.

A) Macrophage depletion leads to a decrease in mitogen responses of blood and thymic lymphocytes or spleen cells (the latter only in the absence of 2-ME) and MLR of spleen cells from GF rats which can be restored by the addition of exogenous macrophages.

B) Macrophage depletion has no effect on mitogen responses of spleen cells but there is an eventual decrease when macrophage depletion is complete.

C) Macrophage depletion leads to enhancement of mitogen responses of tumour-bearing spleen cells (see Chapters IV and V) and MLR of normal spleen cells, which is counteracted by the addition of peritoneal macrophages.

Obviously, the macrophage:lymphocyte ratio is expressed here on the basis of the total number of lymphocytes, thereby neglecting the fact that different activating signals stimulate different T cell subpopulations, which may vary in their macrophage dependency. However, there are no techniques available to study the effect of macrophages on each of these T cell subpopulations separately, so we have to work with this simplified model.

The difference between the effect of macrophage depletion of spleen cells on mitogen reactivity and MLR (i.e., mitogen reactivity remaining the same and MLR exhibiting an increase) could be due to the fact that, relative to the number of responsive cells, a certain number of macrophages is in the region of excess for MLR and in the optimal region for mitogen responsiveness. It may be relevant in this respect to realize that mitogen responses involve a higher number of responsive cells than MLR.

The phenomenon of "naturally" occurring "suppressor" macrophages in rat and mouse spleen as reported by various authors (27, 28, 38, 131, 233, 262, 371) could be explained on the basis of this model by assuming that the number of macrophages in the spleens of the animals used was in the region of macrophage excess (point C in Fig. 6.3), in which macrophage depletion leads to increased responses. This hypothesis is supported by the fact that the number of macrophages was reported to be higher (8-10%) in some of these reports (131, 262) than that we find ($\sim$5%) and also by the observation that the enhancement can be abolished by addition of peritoneal macrophages (27, 262; present data). In contrast, authors reporting helper functions of splenic macrophages (207, 208, 240, 361, 377; present data with GF rats) may have been using cell suspensions under conditions of situation A of Fig. 6.3. In this situation, it can be expected that the response of macrophage depleted cells can be restored by addition of peritoneal macrophages. This phenomenon indeed occurs (240, 377, this Chapter), as does suppression when too high numbers are added (this Chapter).

Apart from the fact that the initial number of macrophages determines the consequences of macrophage depletion, the efficiency of the technique used for that purpose is, of course, also decisive in this respect. Thus, absolute macrophage dependency of T cell mitogenesis could be detected by many authors only after rigorous purification of human peripheral blood lymphocytes (224, 227, 254, 277, 305), guinea pig lymph node cells (105, 225, 294, 295) and mouse lymph node (148) and thymus (251) cells. The failure of others to demonstrate a macrophage dependency of lectin- or alloantigen-induced proliferation might be due to incomplete macrophage depletion. This difficulty can be overcome by using spleen cells from GF rats (this Chapter), which contain a lower number of phagocytic cells to start with.

Variations in the "naturally" occurring number of macrophages in the spleen may be of genetic origin (283) but may also be due to changes in the health status of laboratory animals. In a period in which the rats in our Institute suffered from a respiratory disease (57), their spleen cell responses to PHA, Con A and alloantigens were severely depressed, while the number of macrophages in the spleen increased to 10-12% (unpublished observations). On macrophage depletion, complete restoration of the responses, associated with a decrease in the number of macrophages to the levels observed previously ($\sim$ 5%), was observed. Thus, infections might also influence the outcome of macrophage depletion and seriously affect the interpretation of the results. In contrast, the data obtained with GF rats (this Chapter) indicate that, under conditions where less antigenic stimulation occurs, the number of macrophages is depressed. This leads to effects opposite of those of macrophage depletion.

Splenic "suppressor" macrophages have been reported by many authors under a variety of "abnormal" conditions. Depressed splenic lymphocyte proliferative responses of mice (110, 132, 183, 185-189) and rats (131, 261, 361) bearing viral-induced tumours, of rats bearing spontaneous tumours (207, 211, Chapters IV and V), of mice undergoing a GvH reaction (272, 306) and of mice treated with C. parvum (182, 187, 306, 307) or pyran (26, 27) were shown to be mediated by macrophages (27, 110, 132, 182-189, 207, 211, 261, 306). It has been postulated that, in tumour bearing animals, macrophages in the spleen are activated due to stimulation by tumour- and/or viral associated antigens (188, 361) or alloantigens (361) and that such activated macrophages can exert a suppressive effect. Based on the observations that (a) the number of macrophages is increased in hosts bearing viral-induced (186) or nonimmunogenic spontaneous tumours (207, 211, Chapter IV); (b) the depressed responses of tumour bearers are restored after macrophage depletion (110, 132, 183, 185-189, 207, 211, 261; Chapters IV and V); (c) this restoration can be counteracted by readdition of normal macrophages (211, Chapter V); and (d) normal macrophages have been

implicated as the suppressive elements in numerous supposedly normal conditions (27, 28, 38, 109, 179, 187, 211, 233, 262, 371), we propose that the suppressor phenomenon observed in tumour-bearer spleens is due merely to an increase in the number of phagocytic cells. Thus, spleen cells from tumour bearers would be in situation C of the model (Fig. 6.3), at least with the tumour models we employed (207, 211, Chapters IV and V), since on a per cell basis tumour bearer spleen macrophages had a similar suppressive effect as peritoneal macrophages (211, Chapter V). However, we cannot exclude that qualitative effects also play a role in the extent of suppression under other conditions, as was discussed in Chapter V. Cell separation studies will have to reveal whether a qualitatively distinct type of macrophage is present in the spleen of animals bearing virus-induced tumours. So far, a quantitative change in the splenic macrophage population suffices to explain the observed suppression.

The present discussion has so far neglected the extensive studies of Waksman and his co-workers (111-115), who suggested that the suppressor cells in rat spleens are adherent thymus-dependent cells, which are distinct from macrophages. A similar adherent T cell with suppressor properties was more recently reported to also occur in the mouse spleen (369). This conclusion was based on the observations that: (a) thymectomy (113, 114) or anti-Thy 1 treatment (369) removed splenic suppressor cells; and (b) addition of purified peritoneal or spleen macrophages could not suppress the response of nonadherent spleen cells (111, 115). A partial explanation for the discrepancies between their findings and all of the studies discussed here (demonstrating that macrophages are the suppressor elements) could be that only 2% of macrophages were used (111, 115), a number probably too low to obtain suppression. Oehler et al., (262) provided another explanation for these disparate findings, i.e., they postulated that in vitro splenic macrophages may require thymus influence in order to acquire suppressor activity. In thymus-deficient rats, splenic macrophages might become less activated or migrate from the spleen.

In this relatively long discussion, a summary of present literature reports on macrophage-lymphocyte interaction in which splenic or peritoneal macrophages were employed is given. By combining those data with our own, a model which explains why some authors report helper and others mainly inhibitory effects of macrophages was constructed. The model is, however, descriptive, i.e., it does not provide explanations for the mechanism by which macrophages inhibit or enhance T cell proliferation. It illustrates that, when T cell proliferative capacity is used as a parameter for the determination of T cell reactivity, it should be accompanied by a determination of the composition of the cell suspension, since small changes in the macrophage : lymphocyte ratio can cause great changes in reactivity.

CHAPTER VII

EFFECT OF THYMIC EPITHELIAL CULTURE SUPERNATANT ON T CELL MITOGEN RESPONSIVENESS OF THYMOCYTES

7.1 INTRODUCTION

As stated in Chapter I, more basic information concerning the role of thymic humoral function in T cell differentiation is required before therapeutic application of thymic factors in age-related or other presumably thymus-dependent immune deficiencies can be envisaged. In the following Chapters, the effects of factors present in thymic epithelial culture supernatant (TES) on several T cell parameters and target cells will be reported. This Chapter describes in detail the conditions required to obtain biologically active supernatants from cultured epithelial cells. These conditions were defined by using enhancement of T cell mitogen responsiveness in thymocytes as an indication for activity. Admittedly, effects on this parameter must be interpreted with caution, but since they were shown to be accompanied by effects in more functional assays for T cell reactivity (see next Chapters) and because of its reproducibility, this assay is valuable as a routine method for testing the activity of supernatants. Because of their greater availability, rats were used in the present experiments, but supernatants with similar activity can be obtained from human and mouse thymus epithelial cultures (data not shown). We present evidence for the in vitro release of humoral factor(s) by rat thymic epithelial cultures which increase the responsiveness of rat thymocytes to both PHA and Con A by the criteria of ^{14}C-thymidine incorporation and blast formation. Furthermore, the effect of TES on lymphocytes from spleen and lymph nodes and on cortisone-resistant thymocytes was studied.

7.2 MATERIALS AND METHODS

7.2.1 Animals

All experiments were performed with female (WAG x BN)F1 rats of 12-15 weeks of age; WAG/Rij or BN/Bi rats were also used in some experiments. The animals were maintained as described earlier (Chapter II). Rats injected intraperitoneally with a single dose of 15 mg hydrocortisone acetate (HC, Hydro-Andreson; Organon, Oss, NL; 25 mg/ml) were used in some experiments. The effects of this

dosage of hydrocortisone on thymic histology, cell yield and mitogen responses were reported in Chapter V. Thymuses were removed three days after injection and used for mitogen stimulation. Control animals were injected with a comparable volume of saline.

7.2.2 Mitogen stimulation of thymus, spleen and lymph node lymphocytes

All experiments were performed as described in Chapter III. For each experiment, thymuses, spleens or lymph nodes from 3-5 rats were pooled, except when cortisone-treated rats were used, in which case only individual animals were tested. Thymocytes and spleen cells were cultured for 48 h, whereas lymph node cells were cultured for 72 h.

In some cases, separate tests were performed for the determination of the number of lymphoblasts in stimulated thymocyte cultures. Smears of cells cultured for 48 h were stained with May-Grünwald Giemsa. Results were expressed as the mean of counts on 200 cells of triplicate cultures. In addition, separate samples were prepared for the determination of cell survival by trypan blue exclusion.

7.2.3 Thymic epithelial and control cultures

Thymuses from 5-week-old female WAG/Rij or (WAG x BN)F1 rats were aseptically removed and cut into small fragments ($\sim$1-2 mm^3) after removal of capsular tissue. The tissue fragments were washed 4 times with RPMI 1640 buffered with 25 mM HEPES (Gibco) in order to remove the bulk of lymphocytes; centrifugation at 35 x g for 20 sec was performed between the washings. Tissue fragments from 2 thymuses were suspended in a small volume of medium sufficient to cover just the surface (75 cm^2) of plastic tissue culture flasks (Costar 3075, Cambridge, Mass.). Cultures were kept at 37°C in closed flasks without extra CO_2. The culture medium consisted of HEPES buffered RPMI supplemented with 2 mM glutamine, penicillin (100 IU/ml), streptomycin (0.1 mg/ml) and 10% heat-inactivated rat serum. After 24 h, extra medium was added (final culture volume 10 ml). The first supernatant was collected on day 4 and the medium was subsequently collected and renewed twice a week (10 ml/flask). Cultures were usually lymphocyte-free after the second medium change (as observed by inverted microscope). As a control, supernatants from thymic lymphocytes were prepared by culturing a cell suspension from the thymus which was prepared in the same way as described for mitogen stimulation of thymocytes (Chapter III); 2 thymuses

were used per culture flask. In addition, control cultures from kidney and pancreas were prepared in the same way as described for thymic epithelial cultures.

7.2.4 Testing of the supernatants

Media collected from the cultures were centrifuged at 700 x g for 10 min at 4°C; the supernatants were filtered through Millipore filters (0.45 µm) and stored in small samples at -20°C. Supernatants were never thawed and frozen more than 3 times. They were added to the cell suspensions, keeping the final cell concentration constant.

In some experiments (see Section 7.3.5), the effect of preincubation of thymocytes with supernatant (instead of addition together with the mitogens) was investigated. Cell suspensions (2×10^6 cells/ml) were incubated at 37°C for various periods in Falcon 2057 tubes in culture medium containing supernatant (dilution 1:20) and washed 3 times with 10 ml of culture medium. Centrifugation at 130 x g for 10 min was performed between the washings; after washing, the cells were reconstituted to the original volume.

7.2.5 Electron microscopy

Thymus cultures were fixed *in situ* with a phosphate buffered 2% glutaraldehyde solution (pH 7.3) after various culture periods. The cells were harvested with a "policeman" and centrifuged at 1000 x g for 10 min. After postosmication the cell pellet was dehydrated in graded ethanol series and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and viewed under a Philips EM 300 electron microscope.

7.3 RESULTS

7.3.1 Nature of the thymic epithelial and control cultures

Outgrowths were visible around the thymus explants from day 2. On day 4, they were 3-6 mm in diameter and consisted of a mixture of large rounded granular cells (about 90%) with a few extended flat cells in between (about 10%). After the second medium change most explants had been removed. At days 7-11,

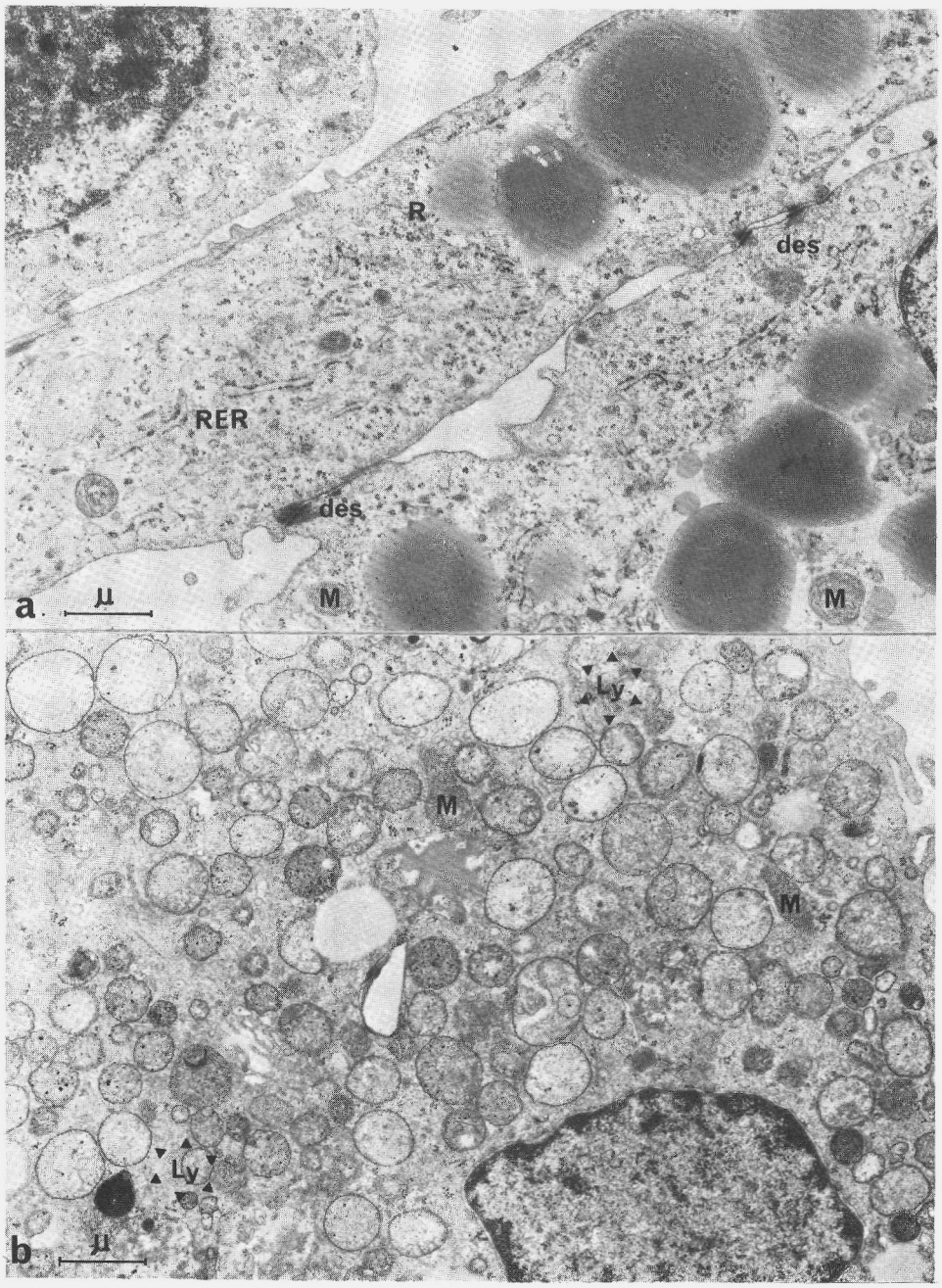

Figure 7.1

Cytoplasmic details of a flat extended epithelial cell in a 15-day-old thymic culture (a) and of a large round cell in a 6-day-old thymic culture (b). Ly = lysosomes (arrows); M = mitochondrion; DES = desmosome with attached tonofilaments; RER = rough endoplasmatic reticulum; R = ribosomes.

more flattened cells with long extensions gradually appeared, while the number of round cell colonies decreased, never reaching diameters of more than 10 mm. The cells remaining in culture after day 11 formed large sheets which reached sizes of 30-40 mm in diameter; these were mainly composed of polygonal cells of varying sizes in a mosaic-like arrangement, resembling epithelial colonies and usually representing 95% or more of the total population at day 14. In addition, an occasional isolated large round cell was observed between epithelial colonies.

Electron microscopy of thymic cultures showed that both the few flat cells in the early cultures and the polygonal cells in the sheets in later cultures (between day 14 and 28) displayed prominent epithelial characteristics (desmosomes, tonofilaments, see Fig. 7.1a) and signs of active protein synthesis, i.e., many polyribosomes in the flat cells from early cultures and, in addition, well-developed rough endoplasmatic reticulum containing a homogeneous product in the polygonal cells from later cultures. The characteristics of active protein synthesis gradually disappeared in cultures older than $\sim$ 4 weeks, after which the cultures stopped growing. The abundantly present large rounded granular cells in early cultures displayed microfilaments and signs of active phagocytosis (many lysosomes, Golgi zones); there were no signs of desmosomes or signs of protein synthesis (Fig. 7.1b).

Sheets of polygonal cells resembling epithelial colonies were also observed in kidney and pancreas cultures; in addition, numerous fibroblasts were present ($\sim$50% contamination). No fibroblasts were observed in rat thymic epithelial cultures.

7.3.2 Effect of various culture supernatants on ^{14}C-TdR incorporation into PHA and Con A stimulated thymocytes

At a dilution of 1:20, supernatants from thymic epithelial cultures (TES) were capable of increasing PHA as well as Con A responses (Table 7.1), while supernatants from nonthymic cultures had no such effect. There was also no enhancing activity in control tissue supernatants collected at culture periods other than day 11 (supernatants were collected twice a week between days 4 and 32). A difference between the effect of TES on PHA and Con A responses was consistently observed: in approximately 200 tests with approximately 100 different supernatants from 7 to 32-day-old cultures, the PHA response was always more increased (by a factor of 2.5 to 6) than was the Con A response (by a factor of 1.5 to 3.5). The enhancing activity of the supernatants was dependent on the culture time of the thymic epithelial cells: the peak activity was usually

TABLE 7.1

EFFECT OF VARIOUS CULTURE SUPERNATANTS ON PHA AND Con A RESPONSES OF RAT THYMOCYTES (DETERMINED BY ^{14}C-TdR INCORPORATION)

source of supernatant*	PHA response	Con A response
thymus, batch 1	1.6 ± 0.2**	1.4 ± 0.2
thymus, batch 2	3.5 ± 0.6	2.1 ± 0.3
pancreas	1.1 ± 0.2	1.0 ± 0.2
kidney	1.0 ± 0.1	1.0 ± 0.2
thymocytes	0.4 ± 0.1	0.5 ± 0.1

* Supernatants were collected from 11-day-old cultures and tested at a dilution of 1:20; batch 1 from thymus was collected from a 4-day-old culture.

**Factor by which the response is increased as compared to cultures to which no supernatant was added (mean ± standard deviation of the mean of 15 separate experiments with the same batch). In this table and all following tables, mitogen doses giving maximal responses were used. ^{14}C-TdR incorporation into control cultures (without mitogen) was not affected. For absolute values of ^{14}C-TdR incorporation, see Fig. 7.2 and Table 7.2.

reached at day 11 or 14, when the cultures consisted mainly of epithelial cells. Supernatants of cultures older than 28 days were usually less active. The TES used in all further experiments was a pool of supernatants from 11, 14 and 18-day-old cultures. Other experiments demonstrated that TES from either WAG/Rij or F1 rats enhanced mitogen responses of thymocytes from male and female BN/Bi, WAG/Rij or F1 rats to the same extent (data not shown), indicating that the effect of TES is not strain or sex-dependent.

7.3.3 Dose-effect curves of TES and mitogens

Dose-effect curves for TES and control supernatants are shown in Fig. 7.2. Kidney supernatant was inactive at all dilutions. Similar experiments also failed to reveal activity of other control supernatants. Thymocyte supernatants always inhibited the response. Such an inhibition could be due simply to the presence of competing pools of cold thymidine, as a result of lymphocyte death (263, 323).

Subsequently, the effect of a certain dilution of TES on the response to various doses of PHA and Con A was investigated. It was found that the mitogen dose-response profile of thymocytes cultured in medium containing TES was simi-

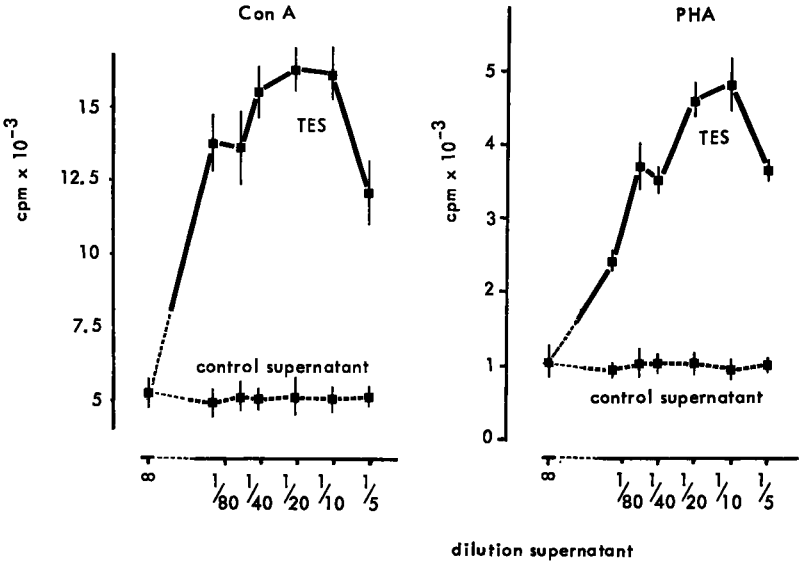

Figure 7.2

^{14}C-TdR incorporation into PHA and Con A-stimulated thymocytes cultured with various dilutions of TES or kidney supernatant. Mitogen doses giving maximal responses were used. Figures represent the mean ± standard deviation of triplicate cultures and are representative for 5 separate experiments. TdR incorporation into control cultures (without mitogen) was not affected by the addition of supernatant and varied from 40 to 100 cpm.

lar to that observed in thymocytes cultured in control medium and that TES enhanced the response of both PHA and Con A at all mitogen doses (data not shown).

7.3.4 Effect of TES on blast formation in PHA and Con A-stimulated thymocyte cultures

Using the number of blast-like cells in mitogen-stimulated cultures as another parameter for cellular proliferation, it was found that TES not only enhanced TdR incorporation into PHA-stimulated cultures but also induced an increase in the number of lymphoblasts (Table 7.2). However, the increase in TdR incorporation into Con A-stimulated cultures was hardly reflected in the number of blast-like cells: a 2-fold increase in cpm was accompanied by a 10% increase in lymphoblasts. Alternatively, such a discrepancy could also be a result of selective survival of stimulated cells, but significant differences in cell survival between TES and control supernatant cultures were not observed.

TABLE 7.2

EFFECT OF TES ON ^{14}C-TdR INCORPORATION AND BLAST FORMATION IN PHA AND Con A-STIMULATED RAT THYMOCYTES

supernatant*	PHA response		Con A response	
	^{14}C-TdR (cpm)	blasts (per cent)	^{14}C-TdR (cpm)	blasts (per cent)
control (kidney)	647 ± 179** (320-1140)	45.1 ± 9.9** (30-56)	4637 ± 745 (3670-6238)	76.3 ± 5.6 (68-80)
TES	2612 ± 998 (1150-4370)	78.3 ± 5.1 (72-86)	8267 ± 2907 (4800-13602)	87.2 ± 4.7 (80-94)
ratio TES: control***	4.04	1.74	1.99	1.14

* Supernatants were added in a dilution of 1:20

** Mean cpm or mean % of blast like cells of the mean values of triplicate cultures in 15 separate experiments ± standard deviation of the mean and the range of these values. The number of lymphoblasts is given as a percentage of the number of surviving cells in the culture.

***Factor by which the response is increased (TES/control).

TABLE 7.3

SUMMARY EFFECTS OF THE ADDITION OF TES OR PREINCUBATION WITH TES ON PHA AND Con A RESPONSES OF RAT THYMOCYTES

preincubation period (h)	PHA response ratio TES:control	Con A response ratio TES:control
0*	4.0 ± 0.5**	2.3 ± 0.3
1	0.9 ± 0.2	1.0 ± 0.1
6	1.3 ± 0.2	1.3 ± 0.2
24	3.1 ± 0.4	1.9 ± 0.2

* Thymocytes were incubated for the indicated period of time with TES containg medium (TES dilution: 1:20), washed 3 times and reconstituted to the original volume; thereafter, mitogen responsiveness was determined. 0 h = TES added together with the mitogens.

**Factor by which the response is increased as compared to cultures containing control supernatant (mean ± standard deviation of 6 separate experiments). In cultures without supernatant, ^{14}C-TdR incorporation was not affected by the washing procedure.

7.3.5 Time-dependency of the effect of TES on thymocytes

Table 7.3 illustrates that cells preincubated for 24 h in TES containing medium and subsequently washed 3 times prior to the addition of the mitogens also exhibit an increase in PHA and Con A responsiveness. In contrast, thymocytes preincubated for 1 h with TES and washed thereafter demonstrated no elevated PHA and Con A responsiveness; 6 h preincubation followed by washing resulted in a slight increase in the response, but much less than that observed after 24 h of preincubation. Thus, it seems that although the addition of TES together with the mitogens provides the best conditions for enhancement of mitogen responsiveness, also incubation with TES alone leads to increased responses. A representative experiment shown in Table 7.4 illustrates that a preincubation of 24 h and, to a lesser extent, of 6 h, leads to a strong decline of the response.

7.3.6 Possible target cell for the effect of TES within the thymus

Treatment of rats with 15 mg hydrocortisone acetate (HC) resulted in most instances in a decrease in the number of thymocytes to ~ 5% of the cell number in a normal thymus. The remaining cells exhibited a 20 to 40-fold increase in PHA and a 10 to 20-fold increase in Con A responsiveness as compared to normal

TABLE 7.4

EFFECTS OF THE ADDITION OF TES OR PREINCUBATION WITH TES ON PHA AND Con A RESPONSE OF RAT THYMOCYTES

	PHA response		Con A response	
preincubation period (h)	control supernatant	TES	control supernatant	TES
0*	1447 ± 58**	4368 ± 906	6835 ± 1314	14719 ± 535
1	1183 ± 18	1122 ± 61	5208 ± 119	5236 ± 265
6	1066 ± 29	1352 ± 13	3916 ± 176	5077 ± 122
24	860 ± 54	2001 ± 157	3512 ± 703	5981 ± 64

* Thymocytes were incubated for the indicated period of time with TES containing medium (TES dilution: 1:20), washed 3 times and reconstituted to the original volume; thereafter, mitogen responsiveness was determined. 0 h = TES added together with the mitogens

**Figures represent the mean cpm ± standard deviation of triplicate cultures from a representative experiment.

thymocytes. Because of the large variation in the effect of HC-treatment on mitogen responsiveness among individual animals, TES was tested only on thymocytes from individual animals. A summary of the effect of TES on mitogen responses of thymocytes from 35 normal and 43 HC-treated rats is given in Figure 7.3. In most of the HC-treated rats, mitogen responses were not enhanced due to the addition of TES. However, TES did enhance mitogen responses in some HC-treated rats, although not to the same extent as in untreated ones. Incomplete removal of cortisone-sensitive thymocytes could be the reason for this phenomenon. This possibility is supported by the observation that in those HC-treated animals in which TES did have an effect, the number of remaining thymocytes was higher (10-15% of a normal thymus), while the responsiveness to PHA and Con A was lower.

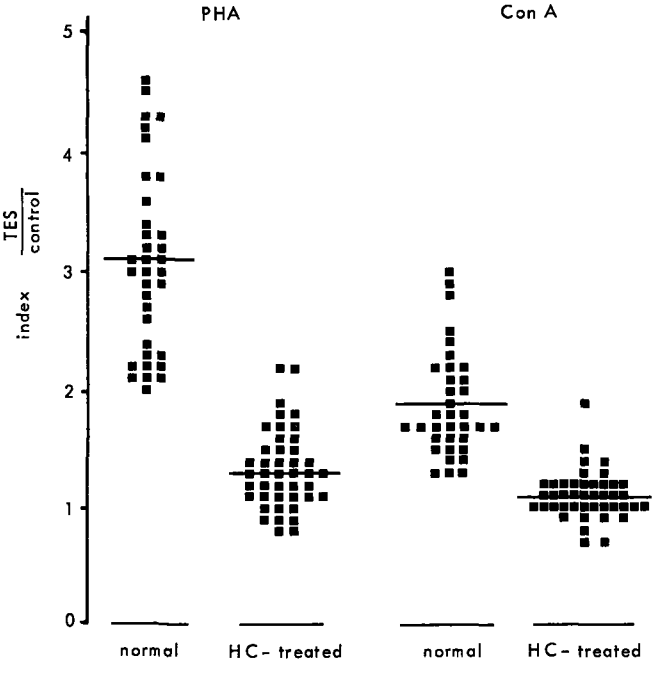

Figure 7.3

Effect of TES on PHA and Con A responsiveness of thymocytes from normal and corticosteroid-treated rats. Mitogen doses giving maximal responses were used. Each point represents the factor by which the response of an individual animal is increased by TES (as compared to cultures containing control supernatant from kidney cultures). The mean increase in PHA and Con A response was 3.1 ± 0.8 and 1.9 ± 0.4, respectively for normal rats and 1.3 ± 0.3 and 1.1 ± 0.2 in corticosteroid-treated rats (mean ± standard deviation of the mean).

7.3.7 Comparison of effects of TES on PHA and Con A responsiveness of thymus, spleen and lymph node cells.

In the previous paragraph, it was suggested that TES did not influence the mitogen responsiveness of the small mature population of cells in the thymus which are resistant to cortisone treatment. Subsequently, the capacity of TES to affect PHA and Con A responsiveness of lymphocytes from lymphoid organs which are considered to contain relatively more immunocompetent T cells was investigated. Table 7.5 gives a summary of experiments in which the effect of TES was tested simultaneously on thymic, splenic and lymph node lymphocytes. Only thymocytes cultured with TES displayed significantly increased responsiveness to PHA and Con A, whereas relatively little effect of the addition of TES could be detected in cell suspensions from other lymphoid organs, though a slight increase in PHA response was sometimes observed. If TES was added in dilutions other than 1:20 (it was tested in a range from 1:5 to 1:50), there was also no

TABLE 7.5

EFFECT OF TES ON ^{14}C-TdR INCORPORATION
INTO PHA AND Con A STIMULATED RAT LYMPHOCYTES

mitogen	lymphocyte source	control supernatant	TES*	ratio TES: control
PHA	thymus	1144 ± 639 (560 - 2246)	3944 ± 1138** (2625 - 5765)	3.5 ± 0.9***
	spleen	34005 ± 3254 (30685 - 37527)	42754 ± 7602 (34598 - 50185)	1.25 ± 0.1
	lymph node	20247 ± 4905 (13557 - 23933)	25000 ± 9252 (12843 - 34213)	1.2 ± 0.2
Con A	thymus	4362 ± 2494 (1686 - 8158)	10470 ± 3490 (6217 - 15096)	2.4 ± 0.9
	spleen	38866 ± 8509 (31293 - 48255)	43186 ± 11337 (31797 - 54506)	1.1 ± 0.1
	lymph node	13848 ± 1305 (13071 - 15355)	15246 ± 1021 (14392 - 16377)	1.1 ± 0.05

* Supernatants were added in a dilution of 1:20.
** Mean cpm of the mean values of triplicate cultures from 4 separate experiments ± standard deviation of the mean and the range of these values.
***Factor by which the response is increased as compared to cultures containing control supernatants.

effect on spleen and lymph node cell mitogen responsiveness. It should be noted (Table 7.5) that in absolute cpm often an increase in the response of spleen and lymph node cells was found. Due to the high baseline responses (i.e., in cultures without TES), this effect was not significant.

7.4 DISCUSSION

The data presented here indicate that TES contains one or more factors which increase TdR incorporation into PHA and Con A stimulated thymocytes (Table 7.1; Figure 7.2). For PHA responsiveness, it could be demonstrated that this increase in TdR incorporation is partly due to an increase in the number of mitogen-responsive cells (Table 7.2), but for the Con A response the increase in the percentage of blast-like cells is not significant. Furthermore, it was evident that TES had to be present preferentially during the entire culture period in order to exert its maximal effect (Table 7.3). As regards the target cell specificity, it seems that, relative to the number of responsive cells the thymus contains most of the target cells for TES activity (Table 7.5) and that these cells are probably found in the cortisone-sensitive, mitogen-nonresponsive subpopulation (Fig. 7.3).

The notion that the stimulating factor(s) in TES stem(s) from the epithelial cells in the cultures and not from other contaminating cell types is based on the following observations: (a) fibroblasts were not observed in rat thymic epithelial cultures, in contrast to the observations in mouse (257) and human (282) thymic epithelial cultures; moreover, kidney and pancreas cultures are heavily contaminated with fibroblasts, but their supernatants failed to exhibit enhancing activity; (b) supernatants from cultures containing lymphocytes, i.e., early cultures from thymuses which had not been washed prior to culturing, were less active; (c) supernatants from thymocyte cultures inhibited mitogen responses; (d) the most "active" supernatants are obtained from cultures which consist mainly of epithelial cells with secretory characteristics as determined by electron microscopy.

Thus, our data suggest that cultured epithelial cells of thymic origin may be the source of one or more factors which exhibit *in vitro* T cell function-enhancing activity, such as has been reported for thymic extracts. In our system, cellular interaction between the epithelial cells and the thymocytes is not necessary, in contrast to the suggestions proposed by others for mouse thymocytes (257) or spleen cells from nude mice (366, 375). This does not rule out the possibility that, for a certain subpopulation of thymocytes, contact with soluble thymic epithelial factors alone is not sufficient for induction of

mitogen responsiveness. Preliminary data (A.M. Kruisbeek and W.Boersma, unpublished observation) with rat thymocytes separated on continuous BSA gradients indicate that only a small subpopulation of nonresponsive cells of intermediate density is induced to PHA and Con A responsiveness by TES, whereas a larger number of nonresponsive, cortisone-sensitive, high-density cells remain unaffected by the presence of TES. Perhaps this latter subpopulation requires means other than contact with thymic humoral factors (e.g., interaction with thymic epithelial cells) to be induced to mitogen responsiveness or cannot be induced at al.

The relationship between TES and thymic humoral factors obtained from thymus extracts (thymosin, THF, thymopoietin) or from serum (TF) remains obscure. Addition of THF to mouse spleen cell cultures stimulated with T cell mitogens (297) or allogeneic cells (358) leads to inhibition of TdR incorporation, whereas pre-incubation of the cells with THF for 24 h (for mitogenic stimuli) or for 1 h (for allogeneic stimulation) leads to enhancement of the response. In rat spleen cell cultures containing TES (Table 7.5) or preincubated in TES (data not shown), neither inhibition nor significant enhancement was observed, suggesting that TES acts in a way different from THF. Alternatively, this could be due to species differences, but incubation of normal mouse spleen cells with thymopoietin (32) or with thymic epithelial monolayers (366, 375) also failed to enhance mitogen responses. In the latter reports (366, 375) incubation on thymic epithelial cells did enhance Con A responsiveness and graft-versus-host reactivity of spleen cells from nude mice or "B" mice.

It is possible that the number of target cells in a spleen from an intact animal, relative to the number of competent T cells, is low, so that only modest increases in TdR incorporation can be expected. Unless cell separation studies are performed to remove the already responsive cells, it is difficult to determine whether other lymphoid organs also contain target cells for the action of TES or other thymic factors. As mentioned in Chapter 1.3, *in vivo* experiments indicate that the spleen contains a population of postthymic precursor T cells which, under the influence of thymic humoral function, can differentiate into immunocompetent T cells (reviewed in ref. 335). Thus, if TES represents an *in vitro* analogue of thymic humoral function, it should theoretically also affect spleen cells. The only conclusion from the present experiments is that, relative to the number of responsive cells, the number of target cells for the enhancing effect of TES is greatest in the normal thymus.

Similar reasoning also leads to a more careful interpretation of the results obtained with cortisone-resistant thymocytes. The most likely explanation for the observed findings is that the target cell for TES activity resides among the cortisone-sensitive cells in the thymus, which have disappeared after

cortisone treatment. This view is supported by the observation that TES only enhanced the response in thymocytes from cortisone-treated rats when the number of remaining thymocytes was higher in these animals, while the responsiveness to PHA and Con A was lower. The latter findings might be explained by incomplete removal of cortisone-sensitive cells. Another possible explanation could be that the target cell is a PHA-nonresponsive but cortisone-resistant cell which is induced to respond to PHA by TES; however, the high baseline PHA responsiveness (due to enrichment of responsive cells) may not allow detection of a (relatively) small enhancement.

In conclusion, supernatants from thymus epithelial cultures seem to offer an alternative approach to the study of humoral thymus function. So far, such supernatants have been shown to induce surface markers characteristic for T lymphocytes in prethymic precursor cells (270, 282). The present findings suggest an enhancing effect of TES on mitogen responses of intrathymic precursor cells. However, responsiveness to T cell mitogens alone is not sufficient for determination of T cell function and insight into the biological activity of TES in terms of induction of T cell maturation can only be obtained by examining its activity in other assays for T cell function as well. This will be the subject of further experiments reported in the following Chapters.

It should be emphasized that it remains to be investigated whether the above findings represent a true differentiation of immature, nonresponsive cells to responsive cells. Also multiplication of preexisting responsive cells or selective survival of responsive cells could have resulted in increased mitogen responsiveness.

CHAPTER VIII

EFFECT OF THYMIC EPITHELIAL CULTURE SUPERNATANT ON MIXED LYMPHOCYTE REACTIVITY, HELPER T CELL FUNCTION AND INTRACELLULAR CYCLIC AMP LEVELS OF THYMOCYTES AND ON ANTIBODY PRODUCTION TO SRBC OF NUDE MOUSE SPLEEN CELLS

8.1 INTRODUCTION

In the preceding Chapter, data concerning the effect of TES on T cell mitogen responsiveness were presented. The need for more insight into the biological activity of TES prompted us to study its in vitro effect on several other T cell parameters. The present Chapter describes the activity of TES on:

1) In vitro response of rat thymocytes to allogeneic cells. The mixed lymphocyte reaction (MLR) represents a more functional assay for T cell reactivity than does responsiveness to T cell mitogens, since it involves the proliferation of antigen-specific clones of thymus-dependent lymphocytes. Cocultivation of nude mouse spleen cells (300) or normal mouse thymocytes (257) on thymic epithelial monolayers increased the responder cell capacity of these cells. However, it was not reported (257, 300) whether the enhancing effects on MLR could also be obtained with thymic epithelial supernatants.

2) In vitro antibody production of mouse spleen cells to SRBC. TES will be shown to be capable of enhancing the anti-SRBC response of nu/nu mouse spleen cells in vitro, as was reported for thymosin and THF (6, 43). Any increased antibody production could, of course, reflect an effect on T cells (or precursor T cells), B cells or both. In order to evaluate whether the activity of TES was expressed on the function of precursors of functional T cells, its effect on the anti-SRBC response of spleen cells from "B" mice and of such spleen cells in the presence of thymocytes was also tested.

3) Intracellular cAMP level of mouse thymocytes. It has been shown that thymosin, thymopoietin, pig serum thymic factor (TF), adenosine-3',5'-cyclic monophosphate (cAMP) and products increasing its cellular level (such as β-adrenergic activators) induce the appearance of the Thy 1 antigen on prethymic precursor cells (25, 30, 301, 302), suggesting that thymic fac-

tors might act through stimulation of cAMP synthesis. Some suggestions for induction of T cell function (i.e., T helper cell activity) together with the induction of Thy 1 by thymosin was also reported (302). A direct effect of thymic factors on intracellular cAMP levels of prethymic precursor cells was only described for thymopoietin (301). With regard to intrathymic precursor cells, only the thymic extract THF (201) and the human serum thymic factor SF (7) were capable of increasing the level of cAMP in mouse thymocytes *in vitro*, whereas thymosin exerted no such effect (8, 258). It was also shown that cAMP could mimic the enhancing effect of THF in the *in vitro* GvH assay (201). These findings were interpreted (25, 30, 201, 301, 302) as suggesting that intracellular cAMP production may play an essential role in the differentiation of precursor T cells, either prethymic or intrathymic. Thus, it was of interest to establish whether TES, apart from its effect on T cell functions, could also enhance intracellular cAMP levels.

8.2 MATERIALS AND METHODS

8.2.1 Animals

Mixed lymphocyte reactivity and mitogen responsiveness were routinely evaluated with thymocytes from 6- to 10-week-old female WAG/Rij rats as responder cells, but similar effects can be obtained with mouse thymocytes (see Chapter IX). In some experiments, spleen cells from 10-week-old female WAG/Rij rats were used as responder cells. Spleen cells from 12-14-week-old female (WAG x BN)F1 rats were used as allogeneic stimulator cells and spleen cells from WAG/Rij rats served as syngeneic control cells.

The *in vitro* anti-SRBC response was determined with spleen cells from 2- to 3-month-old homozygous athymic nude mice which had been partially back-crossed to CBA/Rij mice. In some experiments, spleen cells from female CBA/Rij mice were used which had been thymectomized at the age of 6 weeks, lethally irradiated with a dosage of 850 rad of X-rays (300 kV, 60 rad/min) when they were 8 weeks of age and reconstituted with 5×10^6 syngeneic bone marrow cells by intravenous injection; these animals were used 4 weeks later and will be referred to as "B mice".

The cAMP assay was performed with thymocytes from C57BL/6J mice 6-8 weeks of age. Other lymphoid organs of these animals were tested in some experiments.

8.2.2 Thymic epithelial and control cultures

Thymic epithelial cultures and supernatants were prepared as described in the preceding Chapter. All experiments reported here were performed with a pool of supernatants from cultures of 10 days and older, consisting mainly of epithelial cells, as determined by electron microscopy. Unless otherwise stated, supernatants were present during the entire culture period in each assay. Three or more batches of supernatants from both kidney and pancreas epithelial cultures (Chapter VII) and from thymic lymphocyte cultures were used as control supernatants in all assays and always tested over the same range of concentrations as TES. None of these three control supernatants had any effect; therefore, only the data obtained with kidney epithelial control supernatant are shown in the figures and tables.

In some experiments, rat responder thymocytes at a cell concentration of 4×10^6/ml were incubated alone for various time periods with TES (TES dilution: 1/15). They were then washed 2 times, reconstituted to the desired concentration and tested for their responder capacity in the one-way MLR. After washing and resuspension, the final TES concentration was 1/750, a concentration which was shown to have no effect when present during the entire culture period (see Figure 7.1).

8.2.3 Mitogen stimulation and mixed lymphocyte reactivity of rat thymocytes

These assays were performed as described in Chapter III, except that in some MLR experiments, the number of responder cells or stimulator cells was varied from 0.25 to 2×10^6 cells per well.

8.2.4 In vitro response of nu/nu mouse spleen cells to SRBC

Mice were killed by ether anaesthesia and spleens were removed under sterile conditions and collected in culture medium which consisted of Ham's F-12 (IF-083; Flow Laboratories, Irvine, Scotland), supplemented with 10% heat inactivated foetal calf serum (Flow Laboratories), penicillin (100 IU/ml), streptomycin (0.1 mg/ml), 0.2% (w/v) sodium bicarbonate, 20 mM HEPES (HEPES buffer, pH 7.3; Gibco, Glasgow, Scotland), and 2 mM L-glutamine. For each experiment, spleens from three or four mice were pooled. Cell suspensions were prepared as described (Chapter III) and the cells were dispensed into the wells of flat-bottom Microtest II plates (3040; Falcon Plastics) (1×10^6 spleen cells per

well). TES or control supernatants were added and 1 x 10^6 SRBC were finally added per well (final culture volume 0.2 ml). Four replicate cultures were made for each experiment; controls consisted of spleen cells cultured without SRBC. Cultures were kept for 4 days at 37°C in a humidified atmosphere of 5% CO_2, 7% O_2 and 88% N_2. At the end of the culture period, the culture supernatant was removed by inverting the plates and gently shaking. The cells were resuspended by adding 0.07 ml balanced salt solution (252) supplemented with 5% foetal calf serum and shaking the plates on a microshaker (AM69; Cooke microtitre system). After the addition of 0.07 ml of a 4% SRBC suspension and 0.06 ml guinea pig serum (diluted 1/3, unabsorbed; G87-2161B, Flow Laboratories) as a source of complement, the cell mixture was used for a plaque forming cell (PFC) assay as described by Cunningham and Szenberg (87). Results were expressed as the mean number of PFC from four replicate cultures; standard deviation within these replicate cultures never exceeded 10%.

Spleen cells from "B cell mice" either alone or supplemented with varying numbers of thymocytes from 6-week-old female CBA/Rij mice were used as responder cells in some experiments; further culture conditions were kept the same. Spleen cells from these mice will be referred to as "T cell deprived" spleen cells. Thymocyte suspensions were prepared in the same way as described for spleen cell suspensions.

8.2.5 Determination of intracellular cAMP levels of mouse thymocytes

Mice were sacrificed by cervical dislocation. The thymuses were aseptically collected in petri dishes (3003, Falcon Plastics, Oxnard, Cal.) containing Earle's solution (01-39110, Central Lab. Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) and gently pressed through a fine stainless steel sieve. The cell suspension was passed through a 110 x 7 mm glass column containing 25 mg Leuko-Pak nylon wool (Fenwal Laboratories, Div. of Travenol Laboratories Inc., Morton Grobe, Ill., U.S.A.) at 20°C in order to remove aggregates. The column was washed 3 times with Earle's solution; the eluted cells were washed twice in the same solution and counted with a Coulter counter Model ZF. Substances to be tested were diluted to the desired concentration in Earle's solution containing target cells at a final concentration of 2 x 10^6 cells/ml. Each assay tube contained 5 ml of suspension. After 5 min of incubation at 37°C the tubes were centrifuged at 4°C for 5 min at 600 g. Cell viability before and after incubation was >95%. The supernatant was discarded in order to measure intracellular cAMP levels only and the pellet was resuspended in 0.5 ml of 50 mM TrisHCl, 4 mM EDTA buffer, pH 7.5, by means of a

vortex mixer. Tubes were placed in liquid nitrogen for 1 min and subsequently in a boiling water bath for 2-3 min. The coagulated proteins were spun down at 4°C for 5 min at 1500 g. The supernatant was directly assayed for cAMP or further purified by means of Bio-Rad AG1-X8 chromatography. Cyclic AMP was eluted with 2 N formic acid according to the method of Kuehl et al. (213). Cyclic AMP was measured by Gilman's competition binding procedure (130) utilizing a binding protein isolated from bovine skeletal muscle. The assay was performed with cAMP kit TKR (The Radiochemical Centre, Amersham, England). Each determination was performed in duplicate; the within-assay coefficient of variation was less than 9% and the between assay coefficient of variation was lower than 12%. Results were expressed in picomoles (pmol) of cAMP per 10^7 cells. Net values were obtained by subtracting the background cellular levels of cAMP (i.e., the level of cAMP in thymocytes incubated without any addition). In addition to TES and control supernatants, we also studied the effect of nonspecific stimulators of cAMP, i.e., D-L-Isoproterenol (Sigma Chemical Co., St. Louis, Mo. U.S.A.) and prostaglandin E_1 (PGE_1) (a kind gift of Unilever Research, Vlaardingen, The Netherlands) and of the antagonist of isoproterenol, D-L-Propranolol (Sigma Chemical Co.). In some experiments, mouse spleen and lymph node lymphocytes (prepared in the same way as described for thymocytes) and rat peripheral blood lymphocytes (prepared by Ficoll-Hypaque separation) were also used as target cells.

8.3 RESULTS

8.3.1 Effect of various supernatants on ^{14}C-TdR incorporation into WAG/Rij thymocytes stimulated by semiallogeneic spleen cells

^{14}C-TdR incorporation into WAG/Rij thymocytes stimulated with irradiated (WAG x BN)F1 spleen cells was found to be markedly enhanced by the addition of thymic epithelial culture supernatant (TES), (Figure 8.1). Control supernatants from kidney epithelial cultures (Figure 8.1), pancreas epithelial cultures or thymus lymphocyte cultures (data not shown) have no such effect. TES does not enhance the syngeneic control MLR nor does it influence ^{14}C-TdR incorporation into responder or stimulator cells when cultured alone. The peak of activity of TES is usually observed at a dilution of 1:20 or 1:10; in subsequent experiments, a dilution of 1:15 was routinely employed. Separate experiments indicated that TES does not influence the kinetics of the one-way MLR (data not shown).

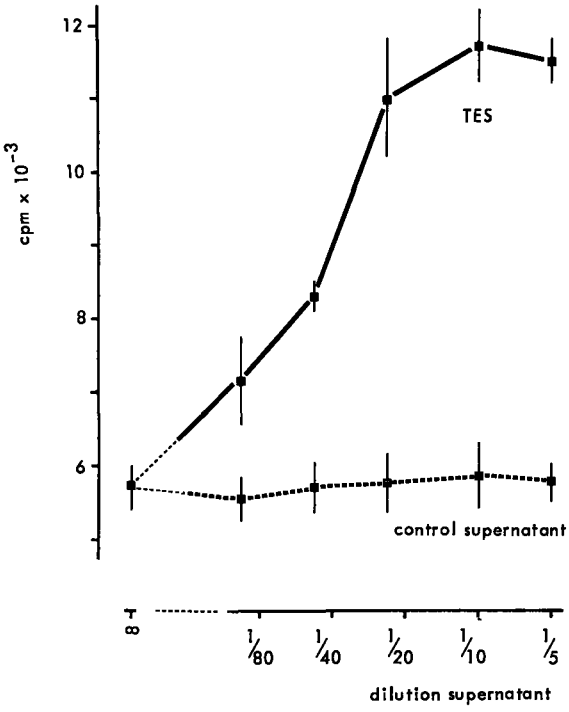

Figure 8.1

Effect of various dilutions of TES and kidney epithelial control supernatant on ^{14}C-TdR incorporation into rat thymocytes stimulated with irradiated semiallogeneic (F1) spleen cells. Figures represent the mean ± standard deviation of triplicate cultures containing 1 x 10^6 responder cell and 0.5 x 10^6 stimulator cells. Data are representative for four separate experiments. TdR incorporation into control cultures (syngeneic MLR, responder cells alone, stimulator cells alone) varied from 200 to 500 cpm and was not affected by the addition of TES.

The above findings were obtained with a responder cell number giving rise to optimal MLR reactivity. TES was subsequently tested over a range of responder cell numbers, keeping the stimulator cell number constant at 0.5 x 10^6. Figure 8.2 (left) shows that in cultures containing TES with 0.25, 0.5 and 1.0 x 10^6 responder cells, the response was comparable to that obtained in control (TES-free) cultures with 0.5, 1.0 and 2.0 x 10^6 responder cells, respectively. These findings indicate that, when TES is present, only half of the number of responder cells is required in order to obtain responses as observed in TES-free cultures.

When the number of stimulator spleen cells was varied (Figure 8.2, right) while the number of responder cells was kept at 1 x 10^6, it appeared that the optimum effect of TES was observed at stimulator cell numbers giving rise to

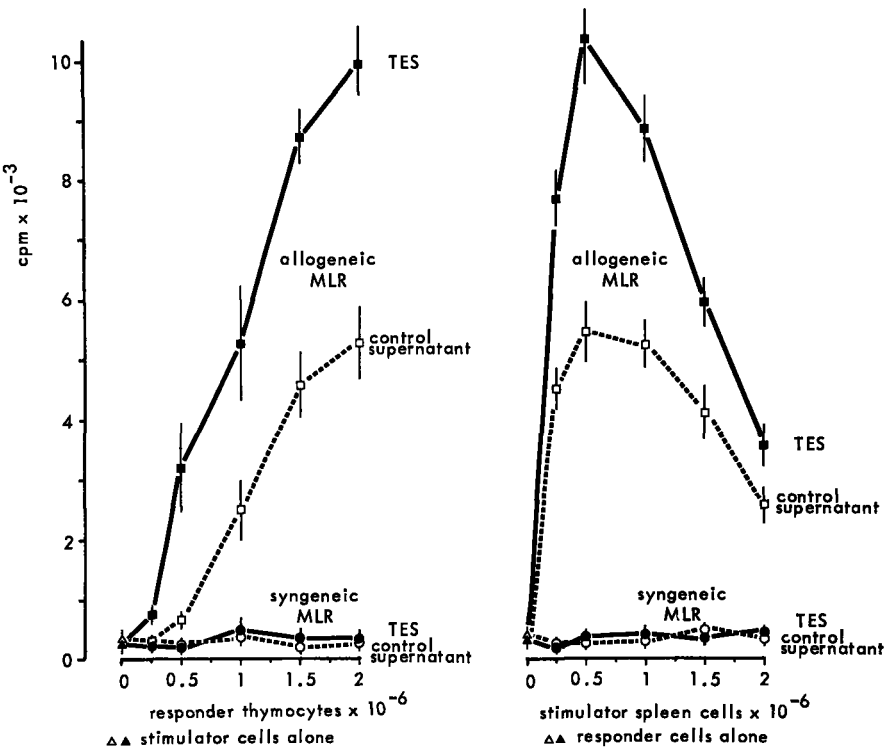

Figure 8.2

Effect of TES and kidney epithelial control supernatant on ^{14}C-TdR incorporation in one-way MLR of rat thymocytes at varying numbers of responder cells (left) or stimulator spleen cells (right). TES was added in a dilution of 1:15. Figures represent the mean ± standard deviation of triplicate cultures and are representative for four separate experiments. TdR incorporation into control cultures (see legends to Figure 8.1) varied from 150 to 500 cpm and was not affected by the addition of TES at any dosage of responder or stimulator cells.

optimum MLR, while at supraoptimal stimulator cell numbers less effect of TES was observed.

TES exerted relatively greatest activity at a low responder cell number, when no significant MLR could be detected in thymocytes cultured without TES (Figure 8.3). Under these conditions, spleen cells do respond to semiallogeneic cells, but this response was not enhanced by TES (Figure 8.3).

Since the above conditions did not permit identification of the cell type influenced by TES (i.e., the responder cell, the stimulator cell or both), we tested the effect of preincubation of thymocytes alone in medium containing TES on their subsequent (after removal of TES) capacity to act as responder cells

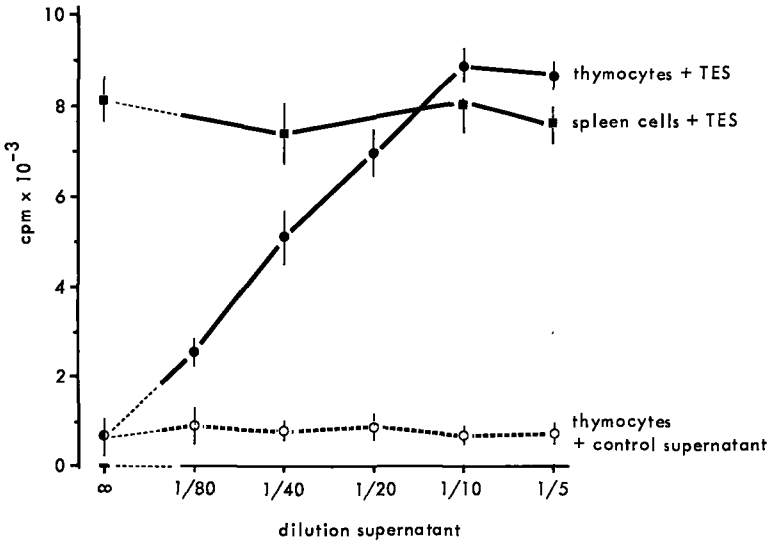

Figure 8.3

Effect of TES and kidney epithelial control supernatant on ^{14}C-TdR incorporation in one-way MLR. Figures represent the mean ± standard deviation of triplicate cultures containing 0.1 x 10^6 responder cells and 0.25 x 10^6 stimulator cells. For responder spleen cells, only the data obtained with TES are presented since these were not significantly different from those obtained with CS.

in a one-way MLR. Table 8.1 shows that preincubation of responder cells in TES for 24 h also resulted in an enhancement of ^{14}C-TdR incorporation, while 1 h preincubation had no such effect. For comparison, the T cell mitogen reactivity of these preincubated cells is also included in the table. Together with the fact that preincubation of stimulator cells with TES was found not to result in increased MLR (data not shown), these data suggest that the TES-induced increase in MLR is due to an effect of TES on the responder thymocytes. TES had virtually no effect on the responsiveness of spleen cells to alloantigens or mitogens (Table 8.1).

8.3.2 Effect of various supernatants on the *in vitro* production of antibody forming cells to SRBC

Spleen cells from nude mice develop only low numbers of plaque forming cells (PFC) *in vitro* when cultured together with SRBC. When TES is added at the beginning of the culture period, a significant increase in the antibody re-

TABLE 8.1

EFFECT OF THE ADDITION OF TES OR PREINCUBATION WITH TES ON ALLOANTIGEN, PHA AND Con A RESPONSES OF RAT THYMOCYTES AND SPLEEN CELLS

lymphocyte source	TES treatment*	MLR	PHA response	Con A response
thymus	continuous presence	2.1 ± 0.3**	4.0 ± 0.4	2.4 ± 0.6
	1 h preincubation	0.9 ± 0.1	0.9 ± 0.2	1.0 ± 0.1
	24 h preincubation	2.2 ± 0.2	3.6 ± 0.5	1.9 ± 0.3
spleen	continuous presence	1.1 ± 0.1	1.2 ± 0.1	1.1 ± 0.1
	1 h preincubation	1.2 ± 0.1	1.2 ± 0.2	0.8 ± 0.1
	24 h preincubation	1.1 ± 0.1	1.1 ± 0.2	1.1 ± 0.3

* For determination of MLR, responder cell numbers were readjusted to 1.5×10^6/0.1 ml and stimulated with 0.5×10^6/0.1 ml irradiated F_1 spleen cells. For mitogen responsiveness, cell numbers were readjusted to 4×10^5/0.2 ml and stimulated with mitogen doses giving optimum responses.

**Factor by which the response is increased as compared to cultures incubated in the same way with kidney epithelial control supernatant (mean ± standard deviation of six separate experiments). For absolute values of TdR incorporation, see Figs. 8.1 and 8.2 (MLR-thymocytes), and Chapter VII (PHA/Con A thymocytes and spleen cells). ^{14}C-TdR incorporation into one-way MLR of spleen cells varied from 4300 to 7800 cpm, with control values (in syngeneic MLR) from 200 to 500 cpm.

sponse is observed (Figure 8.4A), whereas addition of kidney epithelial culture supernatant (Fig. 8.4A) or supernatant from thymocytes or pancreas epithelial cultures (data not shown) had no such effect. The peak of activity was usually observed at a dilution of 1:10 or 1:20. Separate experiments indicated that TES did not change the peak day of the response (data not shown).

The capacity of nude mouse spleen cells to exhibit enhanced primary *in vitro* anti-SRBC responses in the presence of TES could reflect a direct influence of TES on B lymphocyte precursors of antibody-forming cells, on T lymphocyte precursors of helper cells or both. In order to discriminate among these possibilities, TES was also tested in cultures of spleen cells from "B mice".

It can be seen from Figure 8.4B that addition of TES to such cultures had no enhancing effect, indicating that TES does not directly facilitate triggering of B cells by SRBC in the absence of T cell participation. Addition of 0.5 to 2 x 10^6 thymocytes without TES to spleen cells from "B mice" did not result in a significant increase in the number of PFC, illustrating the poor helper cell capacity of thymocytes (Figure 8.4B). However, when TES was added together with thymocytes to spleen cells from "B" mice cultured with SRBC, a strong increase in the number of PFC was observed, not only at the single

dosage of thymocytes shown here (Figure 8.4B) but also at other doses of thymocytes (ranging from 0.5 to 2 x 10^5; data not shown). Thus, these results indicate that TES enhances the helper T cell function of thymocytes and has no effect on B cell function in the absence of thymocytes.

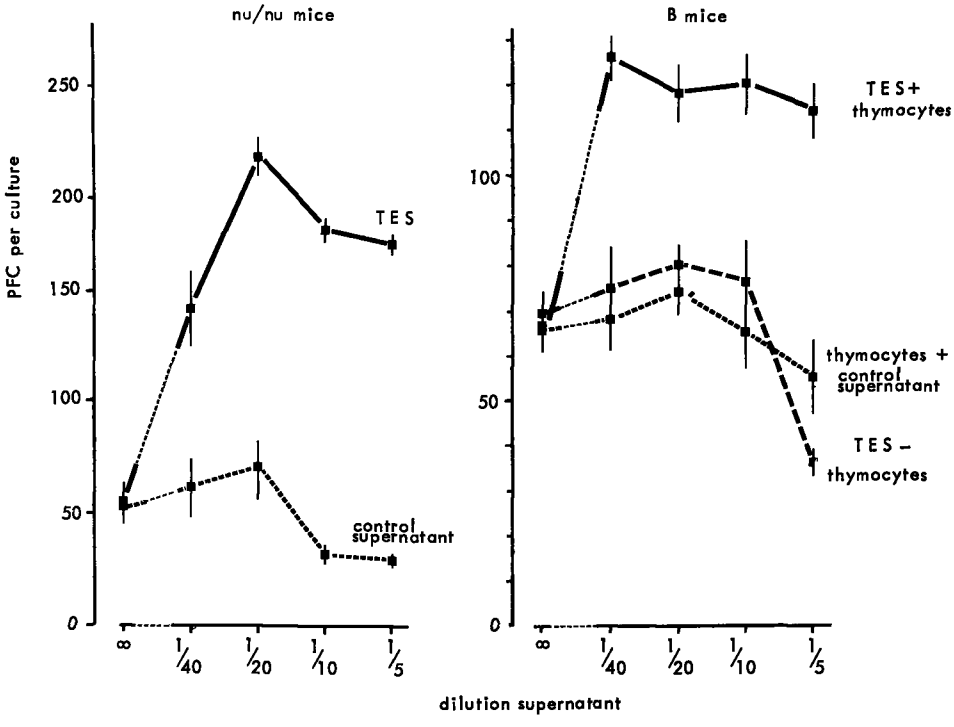

Figure 8.4

Effect of TES and kidney epithelial control supernatant on *in vitro* production of antibody forming cells to SRBC by spleen cells from athymic nu/nu or "B mice". Figures represent the mean ± standard deviation of four replicate cultures containing 1 x 10^6 spleen cells and 1 x 10^6 SRBC and are representative for five separate experiments with 5 different TES batches. Nude spleen cells were tested with TES and control supernatant. Spleen cells from "B mice" were cultured with TES either alone or in the presence of 1 x 10^5 thymocytes. The effect of control supernatant is shown only for spleen cells from "B mice" cultured in the presence of 1 x 10^5 thymocytes, similar values (data not shown) were obtained with spleen cells from "B mice" cultured alone. The number of PFC in control cultures (cultured without SRBC) was 16 ± 2 (nude spleen cells), 23 ± 2 (spleen cells from "B mice") and 30 ± 2 ("B mice" spleen cells plus 1 x 10^5 thymocytes) and was not affected by the addition of TES.

8.3.3 Effect of TES and several other substances on intracellular cyclic AMP levels

Table 8.2 shows the enhancing effect of TES on cyclic AMP levels in mouse thymocytes. No increase in cyclic AMP was induced by control preparations consisting of kidney epithelial culture supernatant (Table 8.2) or thymocyte supernatant (data not shown). Similar findings were observed in rat thymocytes (data not shown), but further experiments were always performed with mouse thymocytes. TES itself contains no detectable amounts of cAMP. Figure 8.5 shows that TES increased cyclic AMP in a dose-dependent manner after 5 min of incubation at 37°C (data obtained with a certain batch of TES with high activity).

We next investigated whether TES increased cyclic AMP levels by interaction with the β-adrenergic receptor present on thymocytes (22). For this purpose, it was investigated whether the increase induced by TES in cyclic AMP could be inhibited by propranolol, an antagonist of β-adrenergic activators. Table 8.2 shows that, as expected, the β-adrenergic activator isoproterenol strongly stimulated cellular cyclic AMP and that such stimulation could be completely prevented by the antagonist propranolol. In contrast, propranolol did not inhibit the effect of TES to any extent, indicating that TES did not act via the β-adrenergic receptor.

TABLE 8.2

EFFECT OF TES, ISOPROTERENOL AND PROPRANOLOL ON CYCLIC AMP LEVELS IN MOUSE THYMOCYTES

substances added	
no TES added (medium alone)	1.3 ± 0.9*
kidney supernatant (dilution 1:5)	1.1 ± 0.2
TES (dilution 1:5)	10.5 ± 0.3**
isoproterenol (10^{-5} M)	123.1 ± 5.0
propranolol (10^{-5} M)	1.3 ± 0.4
TES (1:5) + propranolol (10^{-5} M)	10.9 ± 0.5
isoproterenol (10^{-5} M) + propranolol (10^{-5} M)	1.2 ± 0.6

* Results expressed in pmol per 10^7 thymocytes (mean ± S.E. of four or more separate experiments). A particular batch of TES was used in these experiments (different from the batch used for the experiments shown in Fig. 8.5).

**The mean level of cAMP in thymocytes incubated with TES at a dilution of 1:5 was 14.3 ± 1.9 pmol/10^7 thymocytes (mean ± S.E. of 10 experiments, each performed with a different batch of TES).

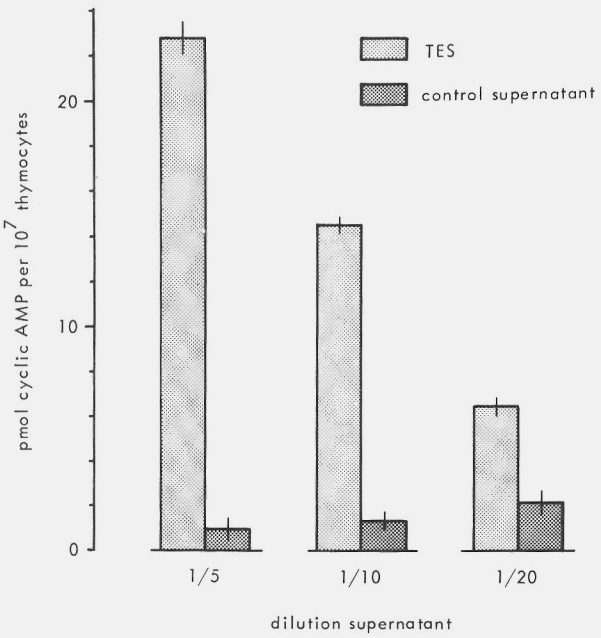

Figure 8.5

Effect of TES and kidney epithelial control supernatant on cellular cyclic AMP levels in mouse thymocytes. Figures represent the mean ± standard error of three separate experiments with one particular batch of TES; each experiment was performed in duplicate. The mean level of cAMP in thymocytes incubated with medium alone was 1.3 ± 0.9 (mean ± standard error of 10 separate experiments) i.e., not significantly different from the values obtained with control supernatant.

Finally, the effect of TES was tested on lymphoid cells from several organs and compared to PGE_1, an agent known to nonspecifically elevate cyclic AMP levels in lymphoid cells (116, 320). It appeared (Table 8.3) that TES exerted relatively greatest activity on cyclic AMP levels in thymocytes (i.e., increase by a factor of 15). Although the effects of TES on other lymphoid cells were relatively low (1.5 to 1.8 x increased), they were in absolute pmol sometimes (in lymph node cells) as high as those observed in thymocytes, but not as high as with PGE_1 (except for lymph node cells).

TABLE 8.3

EFFECT OF TES AND PGE_1 ON CYCLIC AMP LEVELS IN DIFFERENT TARGET CELLS

substances added	thymocytes	spleen cells	lymph node cells	PBL**
no TES added (medium alone)	0.9 ± 0.4*	7.7 ± 2.5	22.0 ± 5.0	9.4 ± 0.8
TES (dilution 1:5)	13.7 ± 3.1	12.9 ± 3.4	38.6 ± 2.0	14.0 ± 0.7
PGE1 (10^{-6} M)	97.4 ± 17.8	28.2 ± 3.8	35.2 ± 1.1	67.2 ± 12.2

* Results expressed in pmol per 10^7 cells (mean ± S.E. of three or more separate experiments).
**Rat peripheral blood lymphocytes; other target cells from mouse lymphoid organs.

8.4 DISCUSSION

The data presented here indicate that TES is capable of enhancing two T cell properties in thymocytes: responder capacity in one-way MLR and helper cell reactivity in the *in vitro* immune response to SRBC, in addition to the previously reported effects on T cell mitogen responsiveness (Chapter VII, ref. 210). Furthermore, it is shown that TES increases cAMP levels relatively most in thymocytes, but also in other lymphoid cells.

Concerning activity of thymus-dependent factors in MLR, only enhancing effects with thymic extracts have been reported (6, 81, 358). The MLR of a brain-associated theta antigen positive subpopulation of nude mouse spleen cells (300) and of mouse thymocytes (257) could be enhanced by coculturing on monolayers of thymic epithelial cells; however, no evidence for effects of soluble factors from such thymic epithelial cultures was presented. In the present study, presence of TES in MLC with thymocytes as responder cells (Figures 8.1, 2 and 3) as well as preincubation of thymocytes with TES for 24 h (Table 8.1) was shown to lead to an increase in ^{14}C-TdR incorporation. The results obtained with preincubated thymocytes also indicate that the activity of TES in MLR is due to its effect on responder T cells. It remains to be investigated whether the enhancing effects of TES are due to an increase in the number of responsive cells (as suggested by the data shown in Figure 8.3) or mainly to selective survival or multiplication of preexistent responsive cells (see also Discussion of the previous and following Chapters).

A comparison between the effects of TES in MLR and similar studies performed with thymic extracts shows the following: continuous presence of thymic extracts in culture has been shown to both enhance (6, 81, with thymosin) and to inhibit (358, with THF) the response of mouse thymocytes to allogeneic stimulation. With TES, no such inhibitory effects have been observed. THF was shown to exhibit its enhancing activity only when the responder cells were preincubated for 1 h with the extract (358), but, with TES, 1 h preincubation of thymocytes had no effect (Table 8.1).

In contrast to THF (358), TES failed to increase the responder activity of spleen cells in MLR (Figure 8.3, Table 8.1); it also did not influence mitogen responsiveness in spleen cells. It should be noted that under certain conditions (Table 8.1) thymocytes and spleen cells exhibit similar TdR incorporation values when stimulated in MLC. Despite that, TES did enhance MLR of thymocytes but not in spleen cells, suggesting that target cells for the action of TES on MLR are mainly present in the thymus, as was reported for thymosin (6, 81).

Summarizing, the effects of TES in MLR are largely comparable to those reported for thymosin (6, 81), except for its lack of mitogenic activity; the conditions under which TES exerts its effect show no resemblance to THF (358), nor is it comparable to thymopoietin which was reported to enhance Con A responsiveness of spleen cells only (31).

An enhancing effect of thymosin-like factors on in vitro production of antibody forming cells to SRBC by nude mouse spleen cells has been demonstrated (6, 302). TES also enhanced the primary in vitro antibody response of nude spleen cells (Figure 8.4A). In addition, we provide evidence for the possible target cell for the action of TES in the in vitro SRBC response. Enhancing effects of any substance in this assay could, of course, reflect an influence on the function of either T cells, B cells or both. However, the fact that the addition of TES to spleen cells from "B mice" had no effect, while addition of TES together with thymocytes led to an increase in the response (Figure 8.4B), indicates the preferential activity of TES on precursor T cells. Since thymocytes, when added alone to cultures of spleen cells from "B" mice, did not lead to an increase in the number of PFC produced, these findings also suggest that TES acts on intrathymic precursors of helper T cells. Others (257) obtained increased helper cell function of thymocytes by culturing them for 48 h on thymic epithelial monolayers, but no evidence for release of (a) soluble factor(s) by these epithelial cells could be found. TES fails to increase levels of background anti-SRBC PFC in nude spleen cell cultures containing no antigen as was reported for a thymosin-like factor (6).

Differentiation of thymus-dependent lymphocytes is thought to be associated with intracellular cAMP production (25, 30, 201, 301, 302). Accordingly, the

effect of TES on cAMP levels in thymocytes was investigated. In the present studies, TES was shown to enhance cAMP levels in thymic lymphocytes (Tables 8.2 and 8.3), whereas it has relatively small effects on lymphocytes from other lymphoid organs (Table 8.3). However, the absolute increase in pmol induced by TES was comparable for all lymphoid organs, and in the case of lymph node cells similar to the effects induced by PGE_1. Thus, with regard to the target cel for the effect of TES on cAMP, no conclusion can be reached.

The effect of TES could not be inhibited by propranolol (Table 8.2), a substance known to inhibit the β-adrenergic activator isoproterenol; thus, it seems that TES (like SF, 7) acts on a membrane site distinct from the β-adrenergic receptor.

A direct effect of thymic factors on intracellular levels of cAMP in thymocytes *in vitro* has only been demonstrated with THF (201) and SF (7), whereas thymosin (8, 258) exhibited no such effect. However, when thymosin was injected into patients lacking SF activity, it induced the appearance of a serum factor which increased intracellular cAMP levels in thymocytes (8). The relationship (if any) between thymic factors in serum and extracts and in supernatants remains unknown: in the MLR and *in vitro* antibody production, the effects of TES and thymosin are comparable, but only TES exhibits an enhancing effect in the cAMP assay. In contrast, the effects of TES in MLR show no resemblance to those reported for THF (358), whereas both THF (201), SF (7) and TES exhibit cAMP-increasing activity. It seems possible that TES combines several activities.

It remains to be investigated whether the effects which TES apparently exerts on functional parameters of precursors of mature T cells such as thymocytes (i.e, increase in responsiveness to T cell mitogens and alloantigens and increased T helper cell activity) is related to the increased cAMP levels in these cells. Increased cAMP levels and functional T cell properties might also be unrelated events.

With regard to the target cells for TES, the following should be mentioned: thymocytes have been shown to differentiate *in vivo* into mature T cells under the influence of humoral thymic function (provided by thymus transplants in diffusion chambers (334, 335). Thus, our data obtained with TES and thymocytes would tentatively suggest that TES provides an *in vitro* analogue of humoral thymus function. However, the nude mouse is totally refractory to humoral thymus function *in vivo* (see Chapter 1.3.1). Therefore, the present and other (6, 43, 301, 302) findings on thymic factor induced acquisition of T cell markers and functions in nude spleen cells *in vitro* should not be overemphasized. It remains to be investigated whether the *in vitro* induction process represents a T cell differentiation phenomenon limited to thymic factor preparations.

CHAPTER IX

EFFECT OF THYMIC EPITHELIAL CULTURE SUPERNATANT ON KILLER T CELL FUNCTION, THY 1 DENSITY AND RESISTANCE TO CORTISONE OF THYMOCYTES

9.1 INTRODUCTION

The results presented in the previous Chapters suggest that, apart from enhancing the proliferative capacity of T cells, TES also induced helper T cell function in thymocytes. Data presented here are concerned with the question whether TES also affects another T cell function in thymocytes, i.e., killer T cell function towards allogeneic target cells. In addition, the effect of TES on two characteristics of thymocytes, Thy 1 antigen expression and resistance to cortisone, was studied. With regard to the different parameters which were investigated, the following should be mentioned:

1) Killer T cell function. As mentioned in the Introduction (section 1.3.5b), only a few cytotoxic effector (CTL) cells can be derived from thymocytes stimulated in mixed lymphocyte cultures (70, 365) and the few precursor killer cells are found only in the cortisone-resistant population (70, 365). It was investigated whether incubation of thymocytes with TES would render these cells capable of generating a higher CTL-response subsequent to stimulation in MLC. For this purpose a mouse CML-system was chosen because of the availability of the appropriate tumour cell lines to be used as target cells.

2) Resistance to corticosteroids. If TES enhances the number of CTL, a population which is *in vivo* found only among the hydrocortisone (HC) resistant cells, one could expect that TES also enhances the number of HC resistant cells. The HC resistant pool of mouse thymocytes seems to be derived from at least some of the HC sensitive intrathymic precursor T cells (106, 372, 374; see also Chapter 1.1.1b), suggesting a precursor-product relationship between these two subpopulations. This concept has been questioned by others (95, 312) who provided evidence for two independent thymocyte subclasses (i.e., the high Thy 1, HC sensitive and low Thy 1, HC resistant); however, there seems to be sufficient evidence to accept that more than one differentiation pathway exists, among which is the HC sensitive → HC resistant cells. Therefore, the effect of TES on the *in vitro* resistance of thymocytes to HC was tested.

3) Thy 1 density on thymocytes. The density of Thy 1 antigen on HC-resistant mouse thymocytes is lower than that on HC sensitive cells (199, 284, 325). Thus, a differentiation of HC sensitive into HC resistant cells would be accompanied by a decrease in the Thy 1-density on these cells (106). It is known (312) that, at relatively low antiserum concentrations, only the high Thy 1-density population of thymocytes is killed; this offers the possibility of selectively killing these cells. Therefore, the effect of TES on the sensitivity of thymocytes to anti-Thy 1 antiserum and complement was investigated.

8.2 MATERIALS AND METHODS

Animals and thymic epithelial cultures. Thymocytes from 6 to 10-week-old female C57BL/Rij mice (H-2b) were used in all experiments, except in the anti-Thy 1 antiserum cytotoxicity tests which were performed with AKR, C3H and C57BL/Rij mice. Spleen cells from 10-12-week-old female DBA/2 (H-2d) mice were used as allogeneic stimulator cells for mixed lymphocyte cultures. Cells from the spleens of C57BL/ Rij mice served as syngeneic controls. In some experiments, thymocytes from mice treated 3 days previously with 1.6 mg dexamethason (Merck and Co., Inc., Rahway, N.J.) or spleen cells were used as responder cells in MLC. Supernatants of thymic epithelial cultures (TES) were prepared from thymuses of 4-week-old WAG/Rij rats or C57BL/Rij mice as described earlier in detail (Chapter VII). Only TES from cultures which were shown by electron microscopy to consist mainly of epithelial cells was used. Control supernatants (referred to as CS) consisted of supernatants from rat kidney or pancreas epithelial cultures (Chapter VII, 210), mouse or rat thymic lymphocyte cultures (210) and mouse fibroblast cultures; none of these CS have ever exhibited effects in the assays described here or previously. Therefore, only data obtained with one particular CS will be shown in the figures and tables.

Determination of Thy 1-density and resistance to hydrocortisone. For determination of Thy 1-density, thymocytes from 3 mice were washed once in HEPES-buffered RPMI 1640 supplemented with 5% heat-inactivated foetal calf serum (FCS). One ml FCS was then layered under the cell suspension (362) and the cells were centrifuged at 400 g for 7 min and washed once more. Thymocytes were suspended at a concentration of 10×10^6 cells/ml in HEPES-buffered RPMI 1640 containing 5% FCS and TES or CS diluted 1:10 and incubated at $37^{\circ}C$ for 2 h in Falcon tubes (2058, Falcon Plastics, Oxnard, Cal.). At the end of the incubation period, the cells were centrifuged and the pellet was labelled with

100 µCi ^{51}Cr (Sodium Chromate, specific activity 100-350 mCi/mg; Radiochemical Centre, Amersham, England) at 37°C for 30 min. The labelled pellet was washed three times and resuspended at a concentration of 4 x 10^6 cells/ml (based on the initial number of cells). Twenty-five µl of cell suspension were transferred to U-shaped wells of microtitre plates (M 220-24 ART, Greiner, Würtingen, Germany) and a direct cytotoxicity test using C3H anti-AKR or AKR-anti-C3H antisera (Searle Diagnostic, High Wycombe, England) and guinea pig complement absorbed on agarose was performed. The specificity of the antisera was established by complete absorption of the killing activity with thymocytes of mice carrying the appropriate allele, by the failure to absorb the killing activity with thymocytes of mice carrying the other allele and by the failure to kill thymocytes of mice carrying the other allele. In the latter case, killing was always lower than 5%. ^{51}Cr release into the supernatant was measured and cytotoxicity was calculated as follows:

$$\% \text{ specific } {}^{51}Cr \text{ release} = 100 \times \frac{{}^{51}Cr \text{ release in presence of antiserum and complement} - {}^{51}Cr \text{ release with complement alone}}{\text{maximal } {}^{51}Cr \text{ release} - {}^{51}Cr \text{ release with medium}}$$

All determinations were performed in triplicate and standard deviations from the mean varied between 1 and 8%. In some experiments (see Results), the number of surviving cells (after addition of 0,2% trypan blue) was counted in a haemocytometer. Determination of resistance to hydrocortisone (HC) exposure in vitro was performed as described previously (10).

Mixed lymphocyte cultures (MLC) and generation of cytotoxic T lymphocytes (CTL). Mixtures of responding thymocytes and splenic stimulating cells, which had been irradiated with 1000 rad, were cultured in 16 mm diameter wells of tissue culture plates (Costar; Cambridge, Mass.). Each culture contained 5 x 10^6 responding and 5 x 10^6 stimulator cells in a final volume of 2 ml HEPES-buffered RPMI supplemented with 10% FCS, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, 5 x 10^{-5} M mercaptoethanol, 2 mM glutamine and extra glucose up to a final concentration of 4.5 mg/ml. For determination of labelled thymidine incorporation, triplicate 0.2 ml volumes of cell suspensions obtained from MLC at day 4, were transferred to U-shaped wells of microtitre plates (M 220-24 ART; Greiner), labelled with 0.15 µCi ^{14}C-TdR (by adding 50 µl of the labelling solution described in Chapter II) for 24 h and processed as described (Chapter III). Results are expressed as the mean cpm of triplicate cultures; standard deviations within triplicate cultures were between 4 and 15%.

Cell mediated cytotoxicity was assessed as follows: effector cells obtained from MLC on day 5 were added at various concentrations in a volume of 0.1 ml to 1 x 10^4 ^{51}Cr-labelled target cells in a volume of 0.1 ml in wells of U-shaped microtitre plates. The medium employed was HEPES-buffered RPMI 1640 supplemented with 5% FCS. Target cells were either P-815-X2 mastocytoma cells (syngeneic to DBA/2 and maintained *in vitro*) or ConA-stimulated C57BL/Rij lymph node cells (which served as control target cells) and were labelled with ^{51}Cr as described by Brunner et al. (53). After a 3.5 h incubation of effector-target cell mixtures, 0.1 ml of the supernatant was removed and the radioactivity released was counted in a gamma counter. All determinations were performed in triplicate and standard deviations of the mean varied between 0.05 and 5%. Data are expressed either as percentage specific ^{51}Cr release as a function of the effector: target cell ratio or as lytic units. Specific ^{51}Cr release is calculated as follows:

$$\% \text{ specific } {}^{51}\text{Cr release} = 100 \times \frac{{}^{51}\text{Cr release in presence of effector cells from allogeneic MLC} - {}^{51}\text{Cr release in presence of effector cells from syngenic control cultures}}{\text{maximal } {}^{51}\text{Cr release} - {}^{51}\text{Cr release in presence of medium}}$$

Lytic units (LU) are determined from dose response curves as described by Cerottini et al. (76). One LU is arbitrarily defined as the number of effector cells required to achieve 50% lysis of 1 x 10^4 ^{51}Cr-labelled target cells. Both the number of LU per 10^6 cells and per culture are given; the latter value was calculated on the basis of the number of surviving cells in a 5-day MLC.

9.3 RESULTS

9.3.1 Effect of TES on the generation of cytotoxic T lymphocytes (CTL)

Thymocytes from C57BL mice were cultured with DBA splenic stimulating cells in MLC with TES or CS. Figure 9.1 illustrates that, when TES was present during the MLC period, a strong increase in the CTL response was observed. Approximately 10-fold fewer effector cells from MLC with TES were required for lysis equivalent to that obtained with effector cells from MLC with CS.

The capacity of TES to elevate the CTL response of thymocytes is further illustrated when the results are expressed as the number of LU/10^6 cells or LU/culture, as shown in Table 9.1. A 7- to 30-fold increase in the number of LU/culture was obtained.

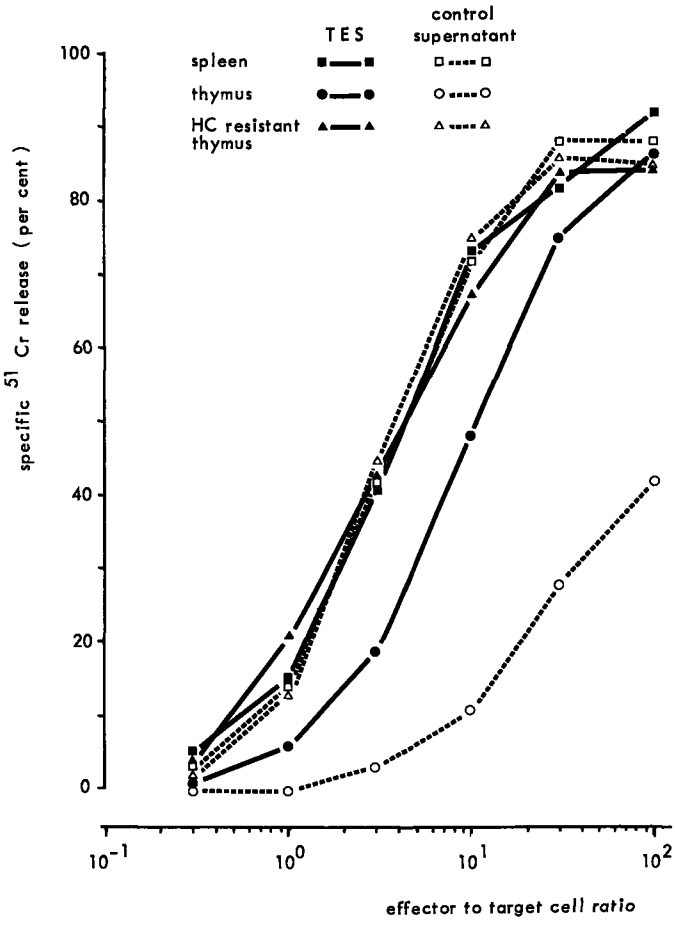

Figure 9.1

Effect of TES on the lysis of P815 (i.e., DBA) target cells by DBA stimulated thymocytes or spleen cells from C57BL mice.
Figures are representative for 5 separate experiments (for a summary, see Table 9.1). Five x 10^6 thymocytes or spleen cells from C57BL mice were cultured with 5 x 10^6 irradiated DBA spleen cells. On day 5, lymphocytes from the MLC were incubated with 1 x 10^4 ^{51}Cr-labelled P815 target cells for 3.5 h. Percent specific ^{51}Cr release is plotted as a function of the effector:target cell ratio. For control values (i.e., from syngeneic control cultures or with syngeneic target cells), see Table 9.1.

TABLE 9.1

EFFECT OF TES ON CTL-RESPONSE IN THYMOCYTES FROM C57BL MICE

experiment	stimulating spleen cells	target cells	LU/10^6 cells CS*	LU/10^6 cells TES*	LU/culture CS	LU/culture TES
1	DBA	DBA**	< 0.5	6	1	23
	DBA	C57BL***	< 0.2	< 0.1	< 0.4	< 0.4
	C57BL	DBA	< 0.1	< 0.4	< 0.1	< 0.4
2	DBA	DBA	1	13	2	58
	DBA	C57BL	< 0.1	< 0.1	< 0.2	< 0.5
	C57BL	DBA	< 0.4	< 0.3	< 0.5	< 0.2
3	DBA	DBA	3	15	6	42
	DBA	C57BL	< 0.3	< 0.1	< 0.6	< 0.3
	C57BL	DBA	< 0.2	< 0.1	< 0.1	< 0.1
4	DBA	DBA	3	14	5	39
	DBA	C57BL	< 0.1	< 0.2	< 0.2	< 0.6
	C57BL	DBA	< 0.2	< 0.1	< 0.3	< 0.1
5	DBA	DBA	6	21	12	84
	DBA	C57BL	< 0.2	< 0.2	< 0.4	< 0.8
	C57BL	DBA	< 0.2	< 0.3	< 0.1	< 0.1

* CS = control supernatant; TES = thymus epithelial culture supernatant
** As a source of allogeneic target cells, ^{51}Cr-labelled P815 X2 mastocytoma cells were employed.
***Control target cells (i.e., syngeneic to C57BL) were ^{51}Cr-labelled Con A-stimulated C57BL-lymph node cells.

The effect of TES on the CTL response was observed only after allogeneic stimulation. Lytic activity of lymphocytes from control cultures containing C57BL responder thymocytes and syngeneic (C57BL) stimulating cells remained unaffected (Table 9.1). In addition, TES only affected lysis of allogeneic target cells (Table 9.1). This indicates that the enhanced CTL response was specific for the sensitizing alloantigens.

The CTL response of thymocytes from *in vivo* HC treated mice was not further increased by the addition of TES (Figure 9.1), suggesting that TES affects the CTL response of HC sensitive thymocytes only. ^{14}C-TdR incorporation was also assessed in samples from the same cultures used for determination of CTL. TES clearly increased ^{14}C-TdR incorporation into MLC of thymocytes (Table 9.2), while the proliferative capacity of thymocytes in syngeneic control cultures remained unaffected.

TABLE 9.2

EFFECT OF TES ON ^{14}C-TdR INCORPORATION AND CTL-RESPONSE IN THYMOCYTES AND SPLEEN CELLS FROM C57BL MICE

C57BL responding cells	stimulating cells	^{14}C-TdR* incorporation CS	^{14}C-TdR* incorporation TES	target cells**	LU/culture CS	LU/culture TES
thymus	C57BL	604 ± 246	897 ± 141	DBA	< 0.2	< 0.4
	DBA	22924 ± 1833	62984 ± 5532	DBA	2	27
				C57BL	< 0.4	< 0.4
spleen	C57BL	6009 ± 573	7117 ± 573	DBA	< 0.1	< 0.3
	DBA	22903 ± 2768	24468 ± 1578	DBA	115	110
				C57BL	< 0.3	< 0.4

* ^{14}C-TdR incorporation was determined on day 4 in 0.2 ml samples taken from the same MLC cultures which on day 5 were used for determination of CTL responses.
**For explanation of target cells, see Table 9.1

Results obtained when spleen cells were used as responder cells are also shown in Figure 9.1 and Table 9.2. Spleen cells give rise to much stronger CTL responses than do thymocytes and these are not further intensified by the addition of TES. In addition, ^{14}C-TdR incorporation into spleen MLR is unaffected by TES (Table 9.2).

9.3.2 Effect of TES on Thy 1 density and resistance to *in vitro* HC exposure of thymocytes.

Thymocytes were incubated for 2 h at 37°C with TES or CS and then tested for sensitivity to lysis by anti-Thy 1 antisera and complement. The results of 4 experiments shown in Figure 9.2 indicate that both C3H and AKR thymocytes when incubated with TES show a decreased sensitivity to anti-Thy 1.2 and anti-Thy 1.1 antisera, respectively. Similar results (data not shown) were obtained with C57BL/Rij thymocytes and anti Thy 1.2 antiserum. The percentage of cells

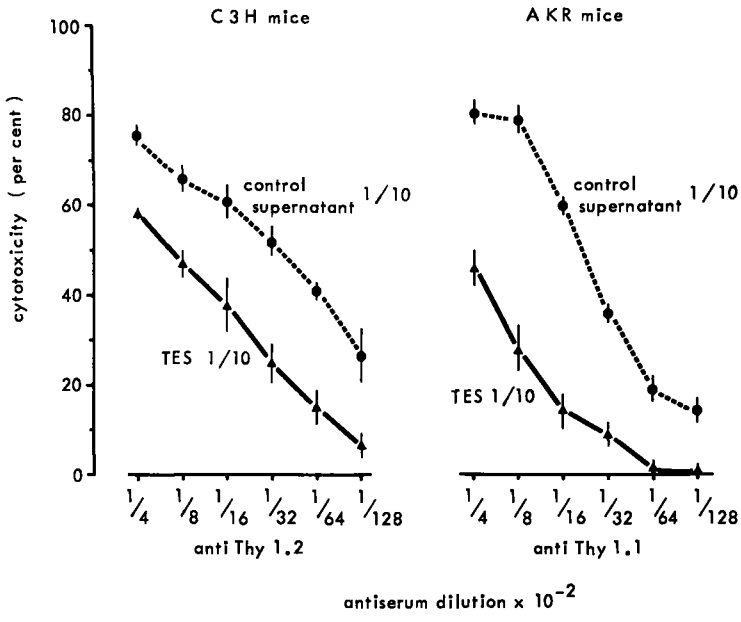

Figure 9.2

Effect of TES on the Thy 1 density of mouse thymocytes.
Figures represent the mean ± S.E. of 4 separate experiments. Thymocytes were incubated with TES or CS for 2 h at 37°C and then tested for sensitivity to lysis by the appropriate anti-Thy 1 antisera and complement in a ^{51}Cr-release assay. Background ^{51}Cr release values (i.e., from cells incubated with complement only) never exceeded 9%.

killed by these antisera in fresh thymocytes or thymocytes incubated with CS, as determined by counting the number of trypan blue stained cells, ranged from 75-85%. According to Shortman and Jackson (312), low-density Thy 1-positive cells represent ~ 16% of the total thymocyte population and can be killed only by high titre anti Thy 1 antisera. Thus, the above findings suggest that the antisera employed in the present study kill only the high Thy 1 cells and that this number is decreased after incubation with TES.

Furthermore, we investigated whether TES induced an increase in resistance to exposure to HC in vitro. Figure 9.3 shows that the presence of TES increases the number of HC-resistant cells in a fraction of the thymocyte population, an event which is significant from the 4th hour on.

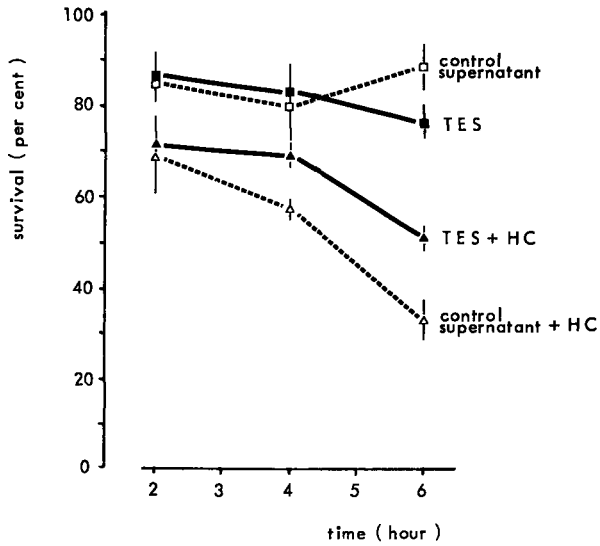

Figure 9.3

Effect of TES on the HC resistance of mouse thymocytes.
Figures represent the mean ± S.E. of 3 separate experiments. Five x 10^6/ml thymocytes were coincubated with TES or CS (diluted 1:10) and HC-succinate (20µg/ml) for a period of 6 h at 37°C and cell survival was determined at different times by means of Coulter counter analysis (ref.10)

9.4 DISCUSSION

The data presented here indicate that TES increases the CTL response of thymocytes and not that of spleen cells and HC resistant thymocytes. In addition, TES induces a decrease in Thy 1-density and HC sensitivity in a fraction of the thymocyte population. Inasmuch as the few immunocompetent T cells of the thymus reside within the HC resistant, low Thy 1-density subpopulation, the present findings might be interpreted as representing a T cell maturation process.

It is known that the generation of CTL is amplified by helper T cells (68, 70, 80, 363; see also Chapter 1.1.5a and b). Thus, the question arises whether the TES-induced enhancement of CTL responses of thymocytes is due to a direct induction of extra precursor CTL or to an induction of helper T cell function. On the basis of the following, it seems likely that the effect of TES on CTL responses is at least partially due to an effect on helper T cells:

1) It was previously demonstrated (212, Chapter VIII) that TES can induce thymocytes to help B cells in in vitro antibody production to SRBC. If the same type of helper T cell participates in both B and T cell responses, as seems probable on the basis of surface markers (both belong to the Lyt-1 positive cells)(54) , enhanced helper T function might also lead to an increased CTL response.

2) T cells which act as helper cells for the generation of CTL are probably responsible for at least part of the proliferative response measured in MLR (68, 70; see also Chapter 1.1.4b and 1.1.5a). Because TES increased ^{14}C-TdR incorporation into MLC's (212, Chapter VIII and this Chapter, Table 9.2), these findings also suggest an effect of TES on helper T cells.

In summary, no conclusive evidence can be given concerning the mechanism by which TES enhances the CTL response, but enhanced helper T cell function possibly contributes to the observed effects. The TES-induced decrease in the number of thymocytes exhibiting high Thy 1-density and high sensitivity to HC exposure might be explained as maturation of a fraction of the high Thy 1, HC sensitive thymocyte population on the basis of the following: Fathman et al. (106) and Weissman (372) demonstrated that labelling the thymus outer cortical DNA synthesizing cells by topical application of ^{3}H-TdR in situ initially labels only large, high Thy 1, HC sensitive thymocytes. Under conditions of ^{3}H-TdR "chase" in vivo, the label shifted proportionally and in parallel to the small, HC-sensitive, high Thy 1-population and to the medium-sized, low Thy 1 HC resistant cells. Therefore, though other subclass maturation pathways which involve nondividing cells in the cortex or dividing cells not in the cortex cannot be ruled out, these authors conclude that at least one of the differentiation lineages in the thymus involves the high Thy 1, HC sensitive → low Thy 1, HC resistant pathway. The present results suggest that this process can be induced in vitro by TES. Similarly, the human serum thymic factor SF (10) and the thymic extract THF (351) were shown to induce a reduction in the number of HC-sensitive cells in vitro.

An alternative explanation for the observed findings (i.e., multiplication of the low Thy 1 population, as suggested by others) (163) seems unlikely. In that case, the number of high Thy 1 thymocytes would have remained the same, so that no change in anti-Thy 1-induced cytotoxicity would have been observed.

Relative enrichment of low Thy 1-cells by differential death of the high Thy 1 cells (a population which is extremely labile in culture, see ref. 162, 312) could also explain the observed decrease in high Thy 1-positive cells. This, however, seems unlikely for the following reasons: the CTL response of

thymocytes resides in the low Thy 1, HC resistant population (70, 365). The TES induced increase in CTL response was not only observed when the results were expressed on a per cell basis (i.e., Figure 9.1) but also when the results were expressed on a total activity per culture basis (e.g., Table 9.1). Thus, the absolute number of killer T cells (presumably low Thy 1, HC-resistant cells) generated in the presence of TES is higher.

The only remaining interpretations of these findings seem to be either better survival of already responsive cells, due to the presence of TES, or true TES-induced maturation of previously unresponsive cells. In order to explain the Thy 1 data, this first interpretation would at the same time require a selective higher death percentage of high Thy 1 cells in the presence of TES, a rather unlikely phenomenon for which no evidence exists.

Nevertheless, conclusive evidence for true differentiation can be obtained only by using unresponsive cells. These might be obtained by separating thymocytes on density gradients (198, 199) or by selective removal of the population which is not agglutinated with peanut agglutinin (286), which seems to be equivalent to the low Thy 1, HC resistant population. This will be the subject of further studies.

It seems appropriate to finally refer to the studies of Shortman and coworkers (162, 163, 312), since their results suggest that the low Thy 1 and high Thy 1 subpopulations in the thymus represent largely independent lines of development rather than having a precursor-product relationship. Others (reviewed in 381) also favour this view. These findings appear to contradict those of Weissman and coworkers (106, 372, 374), suggesting a high Thy 1 → low Thy 1 maturation lineage, but, as mentioned above, their analysis was restricted to dividing cells in the thymic cortex. Our in vitro data accord with Weissman's in vivo findings, but it seems quite likely that, in addition to the high Thy 1 → low Thy 1 pathway, other pathways also will have to be considered.

CHAPTER X

GENERAL DISCUSSION AND CONCLUSIONS

10.1 AGEING AND CELLULAR IMMUNE COMPETENCE

Studies on the effects of ageing on the thymus-dependent immune system have been performed mainly with rodents. Numerous investigators reported on a decrease in mitogen- or alloantigen-induced T cell stimulation of peripheral lymphocytes, as determined by labelled thymidine incorporation. Our own studies indicated that this decline in thymidine incorporation reflected a decrease in the number of responsive cells (Chapter II), a finding which in the meantime has been confirmed by others (1, 167).

Results presented in Chapters IV and V indicate that a small increase in the number of macrophages in spleen cell suspensions (in those experiments derived from tumour-bearing rats) can cause a strong suppression of T cell proliferative responses. One might speculate that also in spleen cells from old rats such a change in the number of macrophages, rather than intrinsic defects in the T cells, contributes to the observed decrease of the response. However, differential counts of spleen cells from young and old (WAG x BN)F1 rats did not reveal any significant differences in the ratio of macrophages and lymphocytes (see Table 2.3). Also the composition of the spleen cell suspensions from rats from other strains was not different between old and young animals (unpublished observations). Thus, it seems that, unlike in tumour-bearing spleen, the depressed T cell proliferative response of spleen cells from old rats is not due to increased numbers of macrophages. The possibility that qualitative changes in the macrophage population from spleen cells from old rats occur (i.e., increased suppressor activity on a per cell basis or decreased helper function, see Chapter VI for further reading) was investigated by macrophage depletion and addition experiments (performed as described for young spleen cells in Chapter VI). Depletion of macrophages from spleen cells from old rats did not lead to enhanced responsiveness, whereas addition of macrophages from young rats to macrophage-depleted spleen cells from old rats did not lead to improvement either (M. van Hees and A.M. Kruisbeek, unpublished observations). Therefore, defective helper cell function or increased suppressor cell function of macrophages from old rats is not a likely explanation for the observed decrease in T cell proliferative capacity.

It seems that the decreased T cell proliferative capacity in old age is thymus-related, since a more pronounced defect was observed in animals suffering from more severe thymus atrophy (Chapter III). Subsequent investigations into the possible role of the thymus in this age-related decline in T-cell proliferative capacity were necessarily rather limited, since they were performed in a period during which a large number of aged animals was lost due to an outbreak of respiratory disease. Thus, only few animals were available for performing the studies described in this particular part of this thesis. Nonetheless, some conclusions emerge from these experiments.

Thymocyte responses of aged rats to mitogen- or alloantigen-induced stimulation exhibited a wide variation (Chapter III), ranging from depressed to enhanced responses as compared to young animals. This is in contrast to the changes observed in lymphocytes from peripheral lymphoid organs of aged rats, which all exhibit depressed responses (Chapters II and III). The experiments with thymocytes indicate that thymus involution follows different patterns in different animals of the same inbred strain, i.e., in some of the aged rats (those exhibiting depressed responses), a relative decrease in the number of responsive cells must have occurred, whereas in others (those showing enhanced responses), a relative increase in the number of responsive cells had occurred. Hence, these results emphasize that pools of thymocytes cannot be used in studies concerned with ageing, since totally opposite age-related changes may be observed.

Several studies had already demonstrated that with increasing age intrinsic changes in the thymus occur, which render it incapable of restoring thymus-dependent immune functions after transplantation into young thymectomized recipients (156-158). One possible reason for this defect might be a decline in intrathymic precursor T cells (Chapter III), which under the influence of thymic humoral factors must still differentiate into mature T cells. This would fit in with the finding that with increasing age a loss of thymocyte precursors in the bone marrow or a decrease in proliferative capacity of these cells occurs (356, 357). However, a decline in prethymic precursor T cells in the bone marrow cannot be the only explanation of age-related immune defects, since grafting old mice with young bone marrow was not found to restore their depressed immune functions (159, 246), whereas a combination of young thymus and young bone marrow does (159). In other words, future research should be directed to the question why the old thymus no longer provides a suitable environment for the influx of bone marrow derived precursor T cells. In addition, more basic research is needed to reveal whether there is a direct relation between diminished thymus function and a decrease in prethymic precursor

T cells in the bone marrow, as suggested by some authors (359, 386-388) but negated by others (281).

It has been suggested that reconsitution of depressed thymic factor production in old age (19, 161) by thymic factor treatment might restore T cell dysfunctions. It seems appropriate at this point to emphasize that thymic humoral factors should not be considered too much as a panacea for the reconstitution of T cell functions in old age. A prerequisite for a beneficial effect of these factors is that the target cells for their action still be present. For reasons outlined above, one could question whether this is indeed always the case. However, it should be realized that the exact role of thymic factors in the T cell differentiation process, especially with regard to their site of action, is still uncertain (see below). Thus, besides more research in the causes of age-related dysfunctioning of T cells, more basic research on T cell differentiation processes is required before manipulation of the defects can be envisaged. It is anticipated that elucidation of these processes may also reveal at which stages defects can occur and thus provide a better rationale for attempts at reconstitution.

10.2 T CELL FUNCTIONS IN TUMOUR-BEARING ANIMALS

The findings reported in Chapters IV and V indicate that macrophages are responsible for the diminished T cell proliferative response observed in tumour-bearing rats. While these studies were conducted similar suppressive effects of macrophages were described in many different tumour systems by a number of laboratories (110, 131, 185, 186, 189, 261, 276, 361). These systems included a variety of spontaneous neoplasms (i.e., the studies described in Chapters IV and V) as well as viral- and carcinogen-induced tumours of mice and rats. While the suppressive effects of macrophages were nonspecific in nature, there seems to be sufficient evidence that tumour-specific immune functions, such as the mixed lymphocyte tumour cell interaction or the generation of specific killer T cells, were also inhibited (131, 189, 261).

The question is whether the macrophages found in tumour-bearing animals represent a particular subclass of suppressor macrophages in a state of increased activation. In our experiments, the suppressed T cell proliferative response in tumour-bearers was correlated with an increased number of macrophages. In addition we found (Chapter V) that the suppression caused by macrophages in spleen or blood from tumour-bearing rats could be mimicked by appropriate numbers of peritoneal exudate macrophages from normal rats. Taken together these findings strongly suggest, but do not prove directly, that the depressed response in

tumour-bearers was due to a quantitative rather than a qualitative change in the spleen or blood macrophages from tumour-bearing animals. The only way to prove this hypothesis is to separate macrophages from spleen cells of normal and tumour-bearing animals and to test their respective suppressive effects on T cell proliferation on a per cell basis. Such experiments have been reported recently (99, 370) and demonstrated that normal and tumour-bearing splenic macrophages exhibited similar suppressive effects on a per cell basis, thus supporting the conclusions advanced above, which were based on indirect evidence.

One of the studies mentioned above (99) also provided evidence for a population of suppressor T cells, which can be detected only in macrophage-depleted tumour-bearing spleen, but remains undetected in untreated tumour-bearing spleen. In the latter situation, the strong suppressive effect of macrophages overruled suppressive effects of other cell types and macrophages may therefore be erroneously charged as being the sole suppressor cells. Also in other tumour models suppressor T cells have been shown to interfere with specific anti-tumour immune reactions (119, 190, 318, 343). Because the suppressor T cells in the spleen were found to be corticosteroid-sensitive in vivo (99), it has been postulated that they originated from the thymus cortex. Thus, one could further postulate that this explains the depletion of lymphocytes in the thymus cortex observed in tumour-bearing animals (Chapter V). However, it remains to be investigated whether such cortisone-sensitive suppressor T cells also operate in tumour systems other than the particular one mentioned above (99) in which a methylcholanthrene-induced fibrosarcoma was used.

In summary , it is apparent that tumours cause a general suppression of the immune system, which seems to be mediated by macrophages in many different tumour systems, and, more specifically, by increased numbers of macrophages in the tumour models we employed. As was outlined already in Chapter 1.2, no conclusive evidence concerning the mechanism by which macrophages exert their suppressive effects has been obtained so far. In addition, in certain tumour models suppressor T cells are operative which may exert their influence independently (119, 190, 318, 343) or interact with the suppressive effect of macrophages (99). If immunological therapy is to be employed for cancer patients, it is of critical importance to further evaluate the cellular nature and mechanisms of suppressor cells in tumour bearers. The recent findings of suppressive effects of macrophages (37, 63, 381) or T cells (381) in blood from cancer patients supports the notion that the above experimental systems provide suitable models for approaching this problem.

10.3 THYMIC FACTORS AND THEIR *IN VITRO* EFFECTS

Factor(s) present in thymic epithelial culture supernatants (TES) could be shown to enhance in thymocytes T cell proliferative capacity induced by mitogens and allogeneic cells (Chapters VII to IX). In addition, helper and killer T cell function (Chapters VIII and IX) were enhanced by TES. The observed effects on functional T cell parameters are accompanied by changes in certain characteristics (Chapter IX) which can be regarded as reflecting a T cell differentiation process (106, 372, 374). Thus, all of these findings are suggestive for a TES-induced maturation process of thymocytes. However, it is clear that conclusive evidence for maturation can only be obtained when nonresponsive target cells are used instead of a mixed population of responsive and nonresponsive cells. Technical advances which have recently become available will allow such experiments in the near future.

Thymocytes remain an important experimental tool for studying T cell maturation induced by thymic factors, since most of the cortical thymocytes represent nonfunctional T cells, albeit cells that have already undergone thymic influence, in that they have acquired the Thy 1-antigen and Lyt 123-antigens. As outlined in Chapter IX, two different concepts for intrathymic T cell differentiation have emerged: one, that of Shortman and co-workers, suggesting that the high Thy 1, HC-sensitive and low Thy 1, HC-resistant thymocytes represent independent lines of differentiation (95, 162, 163, 312) and the other, that of Weissman and co-workers, that the low Thy 1, HC-resistant cells are a differentiation product of the high Thy 1, HC-sensitive cells (106, 372, 374). This discrepancy might, for the moment, be best explained by assuming that there exists more than one single T cell differentiation pathway and that different experimental designs reveal different maturational sequences. TES was shown to decrease Thy 1 density and sensitivity to HC in part of the thymocytes.

The question remains whether maturation of thymocytes in *in vitro* studies with thymic factors represents an analogue of *in vivo* intrathymic or extrathymic differentiation. As was extensively discussed in Chapter 1.3.1, it seems that the bulk of peripheral T cells are derived from immature HC-sensitive thymocytes, which have migrated to the peripheral lymphoid organs where they undergo further maturation (335, 373). There is no detectable evidence for export of the intrathymic HC-resistant mature T cells to peripheral lymphoid organs (335, 373). Others (100, 101) showed that the PHA-responsive, HC-resistant thymocytes in a thymus graft are of native thymus origin more than a year after grafting, lending further support to the above observations (335, 373). Taken together, these findings suggest that the immunocompetent set of

intrathymic T cells represents a resident population which does not contribute to peripheral T lymphocyte function. The functional relevance of this population is still unknown. The immunocompetent T cells in peripheral lymphoid organs seem to be directly derived from the immature thymocytes. Thus, one could tentatively regard maturation of thymocytes in vitro induced by TES as representative for postthymic precursor T cell maturation in vivo. This view is supported by the observation of Stutman (335, 339) that HC-sensitive thymocytes, when injected into neonatally thymectomized mice, can develop into fully competent T cells in combination with thymic humoral function, provided by thymus transplants in a millipore chamber.

It seems of critical importance to investigate whether TES (or any other putative analogue of thymic humoral function) can also induce differentiation of peripheral postthymic precursor T cells, as found, for example, in the spleen. In unseparated spleen cells, enhancing effects of TES on T cell functions are probably masked by the high response of already mature T cells (see also discussions of Chapters VII and VIII). Preliminary experiments indicate that when this population of responsive cells is removed, TES enhances the MLR and CTL-response of mouse spleen cells. In these particular experiments, spleen cells were separated on discontinuous bovine serum albumin (BSA) gradients (198, 199); competent T cells were found mainly in the high buoyant density layers (29-35% BSA) and TES had no effect on the response of these cells. In contrast, TES did enhance the response of the cells with lower buoyant density found in the upper layers (23-26%). These findings are in line with the observations of Stutman (335) who showed that the postthymic precursor cells are found in the less dense bands of similar BSA gradients. Other characteristics of these cells must be investigated, however, before these findings can be regarded as evidence for a "maturation" process.

It is still uncertain whether prethymic precursor cells can be induced to differentiate into incompetent T cells by thymic factors. Results obtained from in vivo experiments argue against this, as was discussed when the concept of Stutman concerning T cell differentiation was introduced (Chapter 1.3.1). In vitro experiments from several laboratories suggest that some functional T cell properties can be induced in nude mouse spleen cells with thymic extracts (6, 43, 135, 247). TES enhances the in vitro anti-SRBC response of nude mouse spleen cells (Chapter VIII) but certainly not to the level observed in normal mouse spleen cells. Also Thy 1-antigen can be induced on nude mouse spleen cells with TES (209), but the relevance of this event is questionable (for arguments, see Chapter 1.3.1 and 1.3.8).

The idea that for prethymic precursor cells traffic through the intact thymus is a prerequisite for differentiation into fully competent T cells is

strongly supported by recent reports of Zinkernagel and co-workers (383-385). It has been known for some time that killer T cells, developed in mice infected with certain viruses, would kill only virus-infected target cells sharing H-2K or H-2D antigen with the infected host, but not cells infected with the same virus but sharing neither H-2K nor H-2D with the host (reviewed in ref. 94). This phenomenon of "H-2 restriction" has also been found by others in the T cell mediated lysis of hapten-modified target cells and of targets differing from the immunized donor by minor (non-H-2) transplantation antigens (reviewed in ref. 94, 384). Zinkernagel et al. (383-385) recently provided evidence that this H-2 restriction is acquired during differentiation of prethymic precursor T cells within the thymus. During this differentiation process the cells acquire the capacity to recognize those H-2 antigens which are expressed on the thymic epithelial cells, independent of the lymphocytes' own H-2 specificity and independent of antigenic stimulation. These findings indicate that specificity of certain cellular immune reactions is determined by the H-2 antigens of the thymic epithelial cells, and make it highly unlikely that thymic factors alone are sufficient for development of full T cell immunocompetence in prethymic precursor cells. Thymic factors might induce appearance of certain T cell surface antigens, alloreactivity and responsiveness to mitogens in prethymic precursors, but these events may not represent the full repertoire of T cell immunocompetence, inasmuch as they do not reflect the capacity of T cells to recognize H-2 self antigens. The latter capacity may be a crucial requirement for protection against viral infections. Thus, in order to assess T cell functions which are more relevant for the host's immune protection, it seems necessary to determine also antigen-specific responses for which H-2 restriction has been demonstrated (384).

The above experimental results and interpretations also clarify why for intrathymic and postthymic precursor T cells (i.e., cells which probably have already acquired H-2 recognition structures) thymic factors alone can induce further differentiation. However, also for these target cells it seems relevant to extend the test parameters with those involving H-2 restriction. The question remaining concerning the differentiation of prethymic precursor cells (i.e., requirement for thymic factors and/or thymus microenvironment) could possibly be solved by comparing the effects of cocultivation on syngeneic or allogeneic epithelial monolayers viz. effects of TES on antigen-specific, H-2 restricted T cell functions.

10.4 CONCLUDING REMARKS

Thymus-dependent immune functions in ageing and tumour-bearing animals were found to be depressed. The causes of this dysfunction, however, were completely different in the two models. In aged animals, a decline in the number of T cells capable of eliciting a response was found, whereas in tumour-bearing animals increased numbers of macrophages caused suppression of T cell proliferative responses. Thus, studies using different approaches aimed eventually at manipulation of these defects should be sought.

Both our data and those from the literature, reviewed in Chapter 1.1, seem to support the notion that in aged animals the thymus no longer provides the proper environment for T cell differentiation. Moreover, a defect in bone marrow derived prethymic precursor T cells has been found. Therefore, future research should be directed at unravelling the process(es) causing these changes in aged animals.

Increased numbers of macrophages in spleen and blood from tumour-bearing animals not only interfere *in vitro* with T cell functions, but also seem to compromise T cell functions *in vivo* (reviewed in Chapter 1.2). Hence, in attempts aimed at reversal of this immunosuppression, protocols that lead to *in vivo* macrophage depletion may merit consideration in the future. In addition, it is necessary to evaluate in such experiments not only nonspecific T cell functions, but also specific anti-tumour responses and tumour growth, in order to investigate whether tumour-induced immunosuppression influences tumour growth. The latter matter has not been dealt with so far in this type of study.

Possible application of thymic factors in restoration of thymus-dependent immune dysfunctions requires much more basic information concerning the site of action and nature of thymic factors before a rational approach can be started. Awaiting this information, it seems that *in vitro* studies with thymic factors will help to unravel the problems in the field of thymic factors and T cell differentiation.

SUMMARY

Studies described in this thesis are concerned with changes in cellular immunocompetence in ageing and tumour-bearing animals. The possible therapeutical application of thymic humoral factors in restoration of diminished T cell functions initiated the more fundamental studies on the humoral function of the thymus also described here.

In Chapter I, these three different subjects are introduced. First of all, techniques used to evaluate several aspects of T cell immunocompetence are reviewed. In addition, the effects of ageing on thymus-dependent immune parameters are described. The possible causes of diminished T cell functions in old age remain unclear. Both a decrease in the number of precursor T cells in the bone marrow and intrinsic defects in the control of T cell differentiation by the thymus might be responsible for the observed defects.

The phenomenon of diminished T cell functions in tumour bearing animals is discussed in the second part of Chapter I. Evidence from the literature is presented which suggests that this dysfunction, as observed with in vitro techniques, is probably due to suppressive effects of macrophages rather than to defects in the T cells. In addition, it appears that suppressive effects of macrophages in vitro are correlated with immunosuppression in vivo.

Finally, the last part of the introduction is devoted to the information available on the possible role of thymic factors in T cell differentiation. No conclusions can be reached as to the target cells for the action of thymic factors, although the postthymic precursor cells seem to be likely candidates. It is also clear that the relationship between the different factors is still unknown. Comparison of their biological effects reveals both similarities and differences and comparison of their chemical nature only yields support for the notion that totally different principles have been isolated so far.

Experiments designed to define more precisely the depressed T lymphocyte proliferative capacity in old age are described in Chapter II. The conclusion is reached that fewer T cells are capable of responding to T cell mitogens.

In Chapter III some investigations regarding the role of the thymus in age-related defects in T cell proliferative capacity are described. After it is shown that the diminished T cell responses of peripheral lymphocytes are related to the extent of thymus atrophy, data are presented which suggest that thymocytes from old rats are no longer sensitive to the action of a certain source of thymic factors, i.e., supernatants from thymic epithelial cultures.

The effects of tumour bearing on T cell proliferative capacity of spleen cells are presented in Chapter IV. A correlation was found between depressed responsiveness to T cell mitogens and an increased number of macrophages. The depressed responsiveness could be fully restored by depleting the spleen cells from macrophages. The data reported in Chapter V extend the previous findings to other tumour models and other lymphoid organs. In addition, the concept that enhanced numbers of macrophages were responsible for the observed suppression is supported by data showing that the restorative effect of macrophage depletion can be abolished by adding macrophages from normal individuals.

In Chapter VI, the effects of macrophage depletion and addition on T cell proliferative responses of spleen, thymus and blood lymphocytes from normal rats are reported. A model for the relationship between T lymphocyte responsiveness and macrophage to lymphocyte ratio in the culture is presented. This model explains why some authors report helper and others mainly inhibitory effects of macrophages on lymphocyte proliferation.

From Chapter VII on, the rest of this thesis is devoted to studies on the effects of thymic factors. As a source of thymic factors, supernatants from thymic epithelial cultures (TES) are employed. A detailed description of the preparation of TES is given in Chapter VII. In addition, it is shown that TES enhances ^{14}C-TdR incorporation into mitogen-stimulated thymocytes, at least partially by increasing the number of responsive cells.

In Chapter VIII it is shown that TES also enhances ^{14}C-TdR incorporation into mixed lymphocyte cultures of thymocytes. Furthermore, TES seems to induce in thymocytes T helper cell function for *in vitro* production of anti-SRBC antibodies by B cells, as well as an increase in intracellular cyclic AMP levels.

In Chapter IX, evidence is presented that TES also enhances a specific effector T cell function in thymocytes, i.e., T killer cell function. No conclusion can be reached as yet as to whether this enhancement is due to a direct effect of TES on precursor killer T cells or on helper T cells. In addition, it is shown that TES reduces the number of thymocytes with a high Thy 1 density and with high sensitivity to cortisone. Arguments are presented which suggest that these findings reflect a T cell differentiation process, but the possibility that other T cell differentiation pathways exist remains still open.

In Chapter X the results are discussed in relation to recent findings reported in the literature.

SAMENVATTING

Het in dit proefschrift beschreven onderzoek is gewijd aan de effecten van veroudering en tumoren op verschillende aspecten van de cellulaire immuniteit bij proefdieren. Met het oog op mogelijke toepassing van humorale thymus factoren, bij pogingen tot herstel van afgenomen T cel functies, zijn tevens meer fundamentele experimenten omtrent de humorale functie van de thymus uitgevoerd.

In Hoofdstuk I worden bovengenoemde drie onderwerpen geintroduceerd. In de eerste plaats wordt een overzicht gegeven van technieken die gebruikt kunnen worden om het T cel-afhankelijke immunologisch reactievermogen te bepalen. Daarnaast wordt het effect van veroudering op de hiertoe gebruikte parameters beschreven. Uit de literatuurgegevens kan geen eensluidende conclusie over de oorzaken van de leeftijdsgebonden afname in T cel functies getrokken worden. Zowel een afname in het aantal voorloper T cellen in het beenmerg alsmede intrinsieke defecten in de regulerende functie van de thymus op het T cel differentiatie proces lijken mede verantwoordelijk te zijn voor de waargenomen defecten.

Bij tumordragende dieren wordt een sterke daling in verschillende T cel functies waargenomen, zoals uiteengezet in het tweede deel van Hoofdstuk I. Deze vorm van immuundeficientie is, althans op grond van resultaten verkregen met in vitro technieken, waarschijnlijk te wijten aan suppressieve effecten van macrophagen en niet aan een afname in reactiviteit van de T cellen zelf. Er zijn aanwijzingen dat suppressieve effecten van macrophagen in vitro gecorreleerd zijn met immunosuppressie in vivo.

Het laatste deel van de introductie is gewijd aan de mogelijke rol van thymus factoren in het T cel differentiatie proces. Hoewel er geen eensluidende conclusie omtrent het celtype waarop thymus factoren hun invloed uitoefenen bereikt kan worden, lijken de z.g. "postthymic precursor cells" (geintroduceerd in Figuur 1.1) de meest waarschijnlijke kandidaten. De relatie tussen de op dit moment bekende thymus factoren is nog niet bekend. Er bestaan zowel overeenkomsten als verschillen tussen hun respectievelijke biologische effecten, terwijl uit vergelijking van de chemische eigenschappen blijkt dat tot dusverre totaal verschillende producten zijn geisoleerd.

Met het doel om de bij veroudering optredende afname in T cel proliferatie capaciteit beter te definieren werden de experimenten beschreven in Hoofdstuk II uitgevoerd. Uit de resultaten blijkt dat er een afname is in het aantal T cellen dat in staat is tot proliferatie te worden aangezet door middel van stimulatie met mitogenen.

In Hoofdstuk III worden enkele aspecten van de mogelijke rol van de thymus in de leeftijdsgebonden afname in T cel proliferatie capaciteit behandeld. De afname in T cel reactiviteit in milt en bloed lijkt gerelateerd te zijn aan de mate waarin de thymus geatrophieerd is, althans bij één bepaalde rattestam. Verder wordt aangetoond dat de T cel proliferatie capaciteit van thymocyten van oude dieren niet meer verhoogd kan worden met factoren uit supernatanten van gekweekt thymus epitheel. Bij jonge dieren kunnen zulke supernatanten meerdere T cel functies in thymocyten verhogen of induceren.

Bij tumor-dragende dieren wordt een sterke daling van de in vitro respons tegen T cel mitogenen van milt en bloed lymphocyten waargenomen (Hoofdstuk IV en V). Dit blijkt gepaard te gaan met een stijging in het relatieve aantal macrophagen. Door milt en bloed lymphocyten suspensies te behandelen met technieken die leiden tot macrophagen-depletie kan de verlaagde respons volledig hersteld worden. Dit suggereert dat de verlaagde respons het gevolg is van het toegenomen aantal macrophagen en niet van afwijkingen in de T cellen zelf. Het feit dat het suppressieve effect van toevoeging van macrophagen van normale dieren het effect van tumoren kan nabootsen (Hoofdstuk V) ondersteunt dit concept.

De experimenten die in Hoofdstuk VI behandeld worden hadden tot doel het effect van macrophagen-depletie en -toevoeging op de in vitro T cel proliferatieve respons van milt, thymus en bloed lymphocyten van normale dieren te onderzoeken, mede omdat over dit onderwerp in de literatuur nogal wat controverses bestaan. De resultaten hebben geleid tot de constructie van een model omtrent de relatie tussen de proliferatieve respons van T cellen en dé verhouding tussen macrophagen en lymphocyten in de kweek. Dit model verklaart waarom sommige onderzoekers vinden dat macrophagen een helper cel functie uitoefenen bij lymphocyten proliferatie en anderen voornamelijk suppressieve effecten van macrophagen waarnemen.

Vanaf Hoofdstuk VII is de rest van dit proefschrift gewijd aan in vitro experimenten met thymus factoren, en wel die welke aanwezig zijn in supernatanten van gekweekte thymus epitheel cellen (TES). De bereidingswijze van TES wordt behandeld in Hoofdstuk VII. TES blijkt in staat te zijn de ^{14}C-TdR incorporatie in thymocyten die met T cel mitogenen worden gestimuleerd sterk te verhogen.

In Hoofdstuk VIII wordt aangetoond dat ook de door allogene stimulatie geïnduceerde proliferatie van thymocyten door TES verhoogd kan worden. Tevens lijkt TES in een deel van de thymocyten T helper cel functie te induceren, alsmede een toename in het intracellulaire cyclisch AMP niveau. Ook een specifieke "effector" T cel functie, n.l. T "killer" cel functie, kan door TES in thymocyten sterk verhoogd worden (Hoofdstuk IX). Bovendien reduceert TES het aantal thymocyten met een hoge dichtheid van Thy 1 antigeen en een hoge gevoeligheid

voor corticosteroiden. Er zijn een aantal aanwijzingen die suggereren dat deze resultaten een T cel differentiatie proces vertegenwoordigen, maar het blijft mogelijk dat er nog alternatieven bestaan.

In Hoofdstuk X tenslotte worden de resultaten met elkaar in verband gebracht en tevens vergeleken met recente ontwikkelingen in de respectievelijke vakgebieden.

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THYMUS DEPENDENT IMMUNE COMPETENCE

EFFECTS OF AGEING

TUMOUR-BEARING AND THYMIC HUMORAL FUNCTION

ADA M. KRUISBEEK