AGE-ASSOCIATED ALTERATIONS IN THE IMMUNE SYSTEM OF NORMAL AND AUTOIMMUNE-SUSCEPTIBLE MICE

by

Aruna Seth

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APPROVED:

Prakash S. Nagarkatti, Chairman

Mitzi Nagarkatti

Ernest R. Stout

Gerhardt G. Schurig

Klaus D. Elgert

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(ABSTRACT)

In this study, the effect of aging on various cells of the immune system was investigated. The two experimental models used were normal young (1-2 months) and old (22-24 months) DBA/2 mice and autoimmune-susceptible young (1-2 months) and old (5-6 months) MRL-*lpr*/*lpr* (*lpr*) mice. Autoreactive T cell clones isolated from DBA/2 mice were used to study the age-induced differential responses of syngeneic T cells and B cells. These cell interactions were found to be greatly diminished in old DBA/2 mice, and this appeared to be due to an intrinsic defect in the cells from old mice. A decreased syngeneic mixed lymphocyte reaction (SMLR) was also found to be associated with these defects in T-T and T-B interactions. The decreased SMLR was due to a reduction in the production of interleukin-1 by macrophages from old mice. In the *lpr* mice, age-induced alterations in the cell surface characteristics of the abnormal T cells that accumulate in the lymph nodes were studied. The double-negative T cells from the lymph nodes of old lpr mice were found to express a cell surface marker, J11d, that is normally present only on immature T cells in the thymus. Futhermore, the number of double-negative J11d$^+$ T cells also increased in the thymus of old *lpr* mice. Autoreactive T cell clones isolated from DBA/2 and *lpr* mice exhibited the properties of both T_H1 and T_H2 subsets as the clones secreted IL-2, IL-4 and IFN-γ, and activated both B cells and macrophages. The current study indicates that with increasing age, the autoreactive T cell-induced immunoregulation is disturbed, which may account for reduced immune responsiveness to foreign antigens and increased susceptibility to autoimmune diseases.

Dedication

The author wishes to dedicate this work to the following people:

her grandmother, the late Mrs. Karam Devi ("Bhabiji")

her parents, Saroj and Nand Seth

her sister, Alka Seth

her uncle, Ved Prakash Chaudhry

and her friend, Hakim

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Table of Contents

List of Illustrations

List of Tables

Chapter 1: General Introduction and Specific Aims

The science of Immunology began with the ancient folk observation that once a person recovered from a disease, he/she does not normally get the disease again. Those who recovered from one disease thought that they would be protected from all other diseases. However, as time passed, people realized that this immunity was specific to the particular disease they had recovered from and would not protect them from other diseases. As early as the year 1700, the Chinese tried to protect people from contracting smallpox when they inoculated healthy people with material from a pustule of a person suffering from smallpox. However, this procedure was not widely used until the work of Edward Jenner in 1798, when he realized that milkmaids that had cowpox were spared from the disease smallpox. Jenner therefore deliberately introduced a mild form of cowpox in his patients so they would be protected from smallpox (Golub, 1987).

The early 1800s were important years for advances in the field of Microbiology. The works of Louis Pasteur and Robert Koch firmly established the germ theory of disease and during this time, the organisms responsible for causing many diseases were isolated. Pasteur and Koch also found that specific immunity could be developed in humans and animals against most of the disease-causing agents they had isolated. These disease-causing agents were termed **antigens**, and the term **antibody** was given to the substance that was responsible for

the specific immunity that appeared in the serum of the blood after a person had been inoculated with an antigen. Thus the first studies carried out in the field of Immunology were aimed at developing new immunization procedures and gradually, this science focused on the properties of antibodies and how antigens could induce their formation (Golub, 1987).

Immunity is resistance to infectious agents. Immunity can either be **innate** or **acquired**. Innate immunity implies a natural resistance to certain infectious agents and is usually non-specific, whereas acquired immunity is 'learned' upon exposure to a particular disease agent and is specific to that organism. Primitive organisms such as invertebrates possess innate immunity whereas vertebrates, in addition to innate immunity, are capable of developing specific immunity against a variety of disease agents.

The main criterion for the development of an immune response to a foreign agent is the ability of the host to distinguish **self** from **non-self**. The invertebrates' response to foreign agents is usually a cellular response, consisting primarily of engulfment (or **phagocytosis**) of the foreign agent. The immune system of vertebrates, on the other hand, is a complex network of interacting cells and soluble factors. Vertebrate responses to foreign antigens consist of two main types: **humoral** and **cellular** immunity. Humoral immunity involves the production of specific **immunoglobulins (antibodies)** by **B cells**, against the foreign agent. Cellular immunity is brought about by **T cells** which, by secreting a variety of soluble factors, can either directly destroy the foreign agent or activate other cells such as macrophages. T cells can also activate B cells to produce antibody molecules. Thus, the humoral and cellular components of the immune system interact closely with each other. Vertebrates, like the invertebrates, also possess the ability to phagocytose foreign particles, a function carried out by the macrophages and other cells of the mononuclear-phagocyte system (Roitt *et al*, 1989).

When the body is exposed to a foreign agent, the first event to occur is uptake of the antigen by specialized cells called **antigen-presenting cells (APC)**. Examples of APC include the B cells, dendritic cells and mononuclear phagocytes or macrophages. The antigen then

undergoes **processing** in which it is acted upon by various enzymes and broken down into small fragments. The majority of these fragments are destroyed but some of them are expressed on the surface of the APC in association with molecules of the **major histocompatibility complex** (**MHC**) (Unanue, 1984). The MHC is a set of genes located on chromosome 17 in the mouse and chromosome 6 in humans. Some of these genes encode cell surface antigens called class I and class II molecules. The MHC controls cell functions and interactions of the immune system (Dorf, 1981; Kimball, 1986).

The complex of antigen-MHC expressed on the APC surface can then be recognized by the T cells which are subsequently triggered to proliferate and secrete various growth factors known as **lymphokines**. Macrophages also secrete lymphokines such as Interleukin-1 that are considered to be essential for T cell activation.

B cells begin development in the fetal liver and complete this process in the bone marrow in mammals. These cells express immunoglobulin, specifically IgM, on their surface, which serves as the receptor for antigen. Upon contact with a foreign antigen and the lymphokines secreted by T cells, B cells are triggered to differentiate into terminal plasma cells that produce specific antibodies against the antigen. The resulting specific antibodies can activate a group of serum proteins known as the **complement system**, which leads to lysis of the antigen (Paul, 1984). B cells also have on their surface class II MHC antigens (the **Ia molecule**), which in association with the processed foreign antigen serves as a stimulus for T cells to proliferate. Some B cells may serve as **memory cells**. These are B cells that have been exposed to the antigen, have reverted back to their original small size and have the ability to 'remember seeing' the antigen when exposed to it a second time. B cell-mediated (or **humoral**) immunity is important in several bacterial and viral infections (Kimball, 1986).

T cells undergo maturation in the thymus. T cells recognize antigen in association with appropriate MHC molecules expressed on the surface of macrophages or B cells. This recognition **activates** the T cells to proliferate and secrete lymphokines which can provide help

to B cells to secrete specific antibody or other T cells and macrophages to provide cell-mediated immunity. T cell-mediated (or **cellular**) immunity is important in protection against intracellular pathogens, especially viral infections (Kimball, 1986).

Recognition of the antigen-MHC complex is carried out by a molecule on the surface of the T cell, the **T cell receptor** (**TcR**). The TcR is acquired by T cells during the process of differentiation and maturation in the thymus. The TcR is composed of two disulfide-linked polypeptide chains which may be of two different types -- the $\alpha\beta$ type or the $\gamma\delta$ type. The majority of the T cells found in the lymphoid organs express the $\alpha\beta$ TcR and are involved in antigen recognition. The function of the $\gamma\delta$ TcR^+ T cells is still being elucidated. Unlike the macrophage, both T cells and B cells proliferate only in response to a specific antigen.

On the basis of their function, T cells may be divided into **helper T cells** (T_H), which express the CD4 molecule on their surface, and the **suppressor/cytotoxic T cells** (T_S/T_C), which express the CD8 molecule. The T_H cells may further be divided on the basis of their lymphokine secretion pattern into **T_H1** and **T_H2** type of cells (Mosmann and Coffman, 1987). T_H1 cells produce IL-2 and IFN-γ but not IL-4, whereas T_H2 cells produce IL-4 and IL-5 but not IL-2 (Mosmann, 1988; Mosmann *et al*, 1986; Cherwinski *et al*, 1987). T_H1 type of T cells are thought to mediate delayed-type hypersensitivity (Vadas *et al*, 1976; Cher and Mosmann, 1987), whereas T_H2 type of T cells provide help to B cells in the production of specific antibodies (Kim *et al*, 1985; Boom *et al*, 1988; Coffman *et al*, 1988).

T cells found in the peripheral lymphoid organs, such as the spleen and lymph nodes, originate in the thymus. These thymocytes express a number of characteristic cell surface antigens, including (a) the TcR; (b) growth factor receptors; (c) the CD4 and CD8 antigens, which are thought to interact with monomorphic regions of the class II and class I MHC molecules respectively (Dialynas *et al*, 1983; Swain, 1983); (d) homing receptors (Gallatin *et al*, 1983; Reichert *et al*, 1984); and (e) molecules such as Thy-1, J11d, Lyt-1, etc., that as yet have no known function. Depending on their stage of development, thymocytes may express

different combinations of these markers (Scollay and Shortman,1983; Mathieson and Fowlkes,1984; Scollay *et al*, 1984; Ceredig and MacDonald,1985; Rothenberg and Lugo, 1985).

The majority of cells within the thymus are destined to die and are never exported to the peripheral lymphoid organs (Scollay and Shortman, 1984). Most of the cells in the cortical areas of the thymus are small and express Thy-1, Ly-1, and both CD4 and CD8 antigens (Scollay and Shortman, 1983; Mathieson and Fowlkes, 1984; Scollay *et al*, 1984; Ceredig and MacDonald, 1985; Rothenberg and Lugo, 1985; Shortman and Scollay, 1984). The majority of these cells die within the thymus. The cortical cells probably give rise to the mature single-positive cells, i.e. T cells expressing either CD4 or CD8.

T cells learn to distinguish between self and non-self in the thymus. The maturation of T cells occurs in stages. Initially, the T cells do not possess any of the markers found on mature T cells, such as the CD4 and CD8 molecules. This stage is called the **double-negative** (DN) stage. The cells then acquire both the CD4 and CD8 molecules on their surface (the **double-positive** stage). At this point, a selection process called **negative selection** occurs. In this process, the T cells that have a high affinity for self-antigens expressed on thymic stromal cells are deleted, whereas those that have a low affinity for self-antigens are spared and instead, are selected to proliferate (**positive selection**). Almost 99% of the T cells in the thymus are deleted and only about 1% are allowed to undergo maturation and are exported to the periphery. The next stage in development is the loss of either the CD4 or the CD8 molecule leading to mature thymocytes that express CD4 or CD8, never both.

As described in the last few pages, immune responses to foreign antigens are a result of the interactions of numerous cells and soluble factors of the immune system. As senescence has been shown to greatly affect immune responses, the current study was undertaken to investigate and compare the effects of aging on cell interactions and soluble factors in the immune system of normal and autoimmune-susceptible mice.

Specific Aims

With increase in age, immune responses to foreign antigens decrease substantially, whereas responses to self-antigens leading to autoimmune reactions increase. The reason for these immunological alterations seen in senescent animals is not well understood. The current studies were therefore initiated to investigate the mechanisms underlying the immunological defects seen with age in normal DBA/2 and autoimmune-susceptible MRL-lpr/lpr strains of mice. The specific aims can be broadly divided into:

Part I

1. Study of the T-T and T-B interactions in young and aged DBA/2 mice using autoreactive T cells isolated from normal DBA/2 mice.

2. Investigation and comparison of stimulatory capacity of B cells and macrophages from young and old DBA/2 mice for autoreactive T cells.

Part II

1. Phenotypic characterization of the abnormal double-negative T cells found in the lymph nodes of the MRL-lpr/lpr mice.

2. Investigation of the lymphokine secretion patterns of autoreactive T cell clones from normal DBA/2 and autoimmune MRL-lpr/lpr mice.

Chapter 2: Review of Literature

I. Aging and Autoreactivity

As an individual ages, the immune system undergoes major changes that lead to decreased immune responsiveness to foreign antigens and increased responses to self antigens. These age-related changes in the immune system play a major role in the overall biologic processes of aging (Walford, 1974). The maximum life span of individuals seems to be greatly affected by the MHC (Smith and Walford, 1977) as, in addition to controlling immune cell functions and interactions, the MHC gene family also regulates a variety of physiological processes which are altered with age (Harman, 1981). The major cause of the immune dysfunctions in aging individuals is a change in cell populations of the immune system (Makinodan and Adler, 1975). This change may involve cell proliferation and differentiation leading to decrease in cell number and function in one or more cell populations (Makinodan *et al*, 1976). Decreased immune responsiveness may also be due to a decrease with age in the number of precursors responding to antigenic stimulation, but the progeny of each cell precursor can proliferate normally and be fully immunocompetent (Miller, 1984).

Stem cells and other rapidly dividing cells are not much affected by the aging process (Callard, 1981; Kay and Makinodan, 1982). However, Harrison (1983) has shown that even though there is no intrinsic defect in the stem cells, there may be some defect in the genetic regulation of stem cell proliferation and differentiation.

As discussed earlier, macrophages process and present antigen on their surface in association with class II MHC molecules, and they also secrete IL-1. These two signals activate T cells to proliferate and secrete IL-2 (Unanue, 1984). When the functions of macrophages were investigated in relation to aging, no changes in phagocytosis or antigen presentation were seen in aged animals (Perkins, 1971; Heidrick and Makinodan, 1973; Callard, 1978). However, the activity of the lysosomal enzymes in the macrophages increases gradually with age (Heidrick, 1972) and this increased enzymatic activity enhances the rate at which antigen is removed from the system (Garvey *et al*, 1980; Nakano and Cinader, 1980).

Doria *et al* (1986) found that at all concentrations of antigen used to pulse macrophages, the macrophages from old mice were more efficient than those from young mice in presenting antigen to T cells. They did not find any change in IL-1 production by LPS-stimulated and -unstimulated macrophages from either young (2-3 months of age) or old (20-24 months of age) mice. Macrophages from old mice were found to express higher levels of Ia molecules than macrophages from younger mice, thus accounting for the higher efficiency of antigen presentation by macrophages from old mice.

Age-related changes in the functioning of B cells have also been observed when B cell responses to T-dependent antigens (Kishimoto *et al*, 1976; Callard and Basten, 1978) or T-independent antigens (Mason Smith, 1976; Callard *et al*, 1977) were studied. These defects were considered to be intrinsic as the total number of B cells and the number of B cells specific to a particular antigen remained unaffected as age progressed. Studies by Zharhary and Klinman (1983) have shown that although splenic B cells specific to DNP from old mice are capable of proliferating and producing normal amounts of anti-DNP antibody, the

proportion of B cells that respond to DNP in the spleen of old mice is greatly reduced compared to the spleens of young mice. However, aging does not seem to affect the proportion of B cells that respond to viral antigens (Zharhary and Klinman, 1984). Doria *et al* (1978) found that antibody responses to TNP and the affinity of these antibodies increase and decrease with age.

In the mouse, antigen-specific $CD4^+$ T cells are required for the production of antibodies by B cells to T-dependent antigens. This cooperation between T and B cells may be a **cognate** interaction which requires physical contact between the cells or a **non-cognate** interaction which does not require physical contact. Both these processes can occur simultaneously (Doria, 1982) and require lymphokines. Lymphokines are important soluble factors that act on B cells by initiating and enhancing their proliferation and differentiation (Farrar *et al*, 1982). B cells are first activated by antigen and then by the soluble factors from antigen-activated T_H cells. Their proliferation is enhanced by IL-1, IL-2 and IL-4 (Boom *et al*, 1988; Hofman *et al*, 1988) and their differentiation into antibody-secreting plasma cells is maintained by IL-5 and IL-2 (Boom et al, 1988).

Various defects have been found in the T_H cells of old mice (Callard and Basten, 1978; Krogsrud and Perkins, 1977). A decrease in the T-T interaction with age has also been noted and is thought to be due in part to a decrease in T_H cell activity (Doria *et al*, 1980; Doria *et al*, 1983). The decreased T_H activity has, in turn, been correlated with the involution of the thymus (Hirokawa and Makinodan, 1975) and decreased thymic factors required for T cell maturation (Goldstein *et al*, 1981). Miller (1984) did find that the T_H cell precursor frequency was decreased in aged mice but the T cells arising from these precursors were fully functional as mature T cells. Another factor responsible for the decreased T_H cell activity in aged mice is the decreased production of IL-2 by these cells (Thoman and Weigle, 1981), which has also been noted in aged rats (Gilman *et al*, 1982) and in humans (Gillis *et al*, 1981).

The activity of T_H cells and B cells is regulated by a subset of T cells called **suppressor cells** (Gershon and Kondo, 1970) and these cells are in turn regulated by **contrasuppressor T cells** (Gershon et al, 1981). Autoantibodies (Goidl *et al*, 1980; Meredith and Walford, 1979) and autoreactive T cells increase significantly with aging. This increase has been explained by a decrease in suppressor T cell (T_S) activity in aging NZB mice (Barthold *et al*, 1974) in contrast to the increased immunosuppression observed in other strains of mice (Goidl *et al*, 1976; Gerbase-De Lima *et al*, 1975; Segre and Segre, 1976). Doria *et al* (1982) found that antigen-specific Ts cells can be induced in old mice more easily than in young mice but lymphocytes from old mice lose their ability to respond to such immunosuppressive cells, which may account for the increased autoreactivity with age.

T cells in a normal individual respond only to foreign antigen in association with self Ia molecules. Autoreactive T cells are cells that respond to syngeneic Ia-bearing stimulator cells but not allogeneic stimulators (Lattime *et al*, 1980; Pasternak *et al*, 1980). The present view on autoreactive T cells is that they do exist in small numbers in an individual but are normally kept in check by suppressor cells. When this regulation becomes defective, it leads to activation and proliferation of the autoreactive T cells thus leading to a state of autoimmunity. Evidence for the existence of autoreactive T cells is provided by autologous mixed lymphocyte reactions (AMLRs) and syngeneic mixed lymphocyte reactions (SMLRs). A more direct evidence is the ability to isolate and characterize cloned autoreactive T cell lines that have been perpetuated for a long time *in vitro* (Zauderer *et al*). Autoreactive T cells have been found to produce the same soluble factors or lymphokines as other $CD4^+$ T cells (Clayberger *et al*, 1984; Shiohara *et al*, 1987a).

Nagarkatti *et al* (1985) isolated autoreactive T cell clones from normal unimmunized DBA/2 mice and used these to study the proliferation of normal splenic T cells. They showed that splenic $CD4^+$ T cells from normal DBA/2 mice responded directly to a syngeneic irradiated autoreactive T cell clone. As this proliferation did not require the presence of APC and Ia antigens, they hypothesized that the $CD4^+$ T cells proliferated in response to the autoreactive

T cell receptor. Using the autoreactive T cell clones from DBA/2 mice, they also studied the T-B cell interactions and found that the autoreactive T cells were able to induce proliferation and differentiation of antigen-specific B cells in the absence of antigen.

Why are autoreactive T cells not activated constantly *in vivo*? Nagarkatti *et al* (1988) studied the role of Ts cells in the regulation of autoreactive T cells in DBA/2 mice. They found that autoreactivity in a SMLR was greatly increased by removal of $CD8^+$ T cells either from responders or stimulators in the culture. Irradiated $CD8^+$ T cells were capable of inhibiting the proliferation of $CD4^+$ T cells to self-Ia antigens. This inhibitory effect was not observed with irradiated $CD4^+$ T cells. The *in vivo* depletion of $CD8^+$ T cells also led to enhanced *in vitro* autoreactive responses of splenic and lymph node T cells. Thus, they suggested that naturally occurring $CD8^+$ T_S cells may be responsible for the maintenance of self-tolerance *in vivo*.

II. Autoimmunity

Normally, the body does not mount an immune response against itself. Sometimes, however, the body can react strongly against its own components, leading to the diseased state known as **autoimmunity**. Autoimmune diseases may involve only particular organs or the whole body. Examples of human autoimmune diseases include rheumatoid arthritis, myasthenia gravis, multiple sclerosis and systemic lupus erythematosus (SLE). These diseases are characterized by the production of autoantibodies or autoreactive T cells that can cause pathological changes either throughout the body or in a specific organ. In SLE, autoantibodies are produced against self-antigens such as DNA, RNA, etc. leading to the formation of Ag-Ab complexes. Such complexes, which are normally cleared from the body by the mononuclear-phagocyte system, get deposited in various parts of the body, especially the kidneys, due to a greater difference in blood pressure. The deposition of Ag-Ab complexes

results in glomerulo-nephritic lesions which ultimately lead to death of the individual. The reasons for the body 'turning against itself' are still not clear. Tolerance to self-antigens was at first thought to be brought about by destruction of self-reactive lymphocyte clones during cell development (Burnet, 1959). Blackman, Kappler and Marrack (1990) have recently confirmed the clonal deletion hypothesis. However, some self-reactive clones may escape clonal deletion (Clagett and Weigle, 1973; Cohen and Wekerle, 1973; Bankhurst *et al*, 1973; Bankhurst and Williams, 1975; Sawada *et al*, 1977; Wigzell, 1977) and these clones may later lead to autoimmunity. It has also been observed that Class II MHC molecules are abnormally expressed on tissues in a number of target organs in organ-specific autoimmune diseases (Botazzo *et al*, 1983; Botazzo *et al*, 1985; Todd et al, 1984). These Ia-expressing cells can stimulate T cells to proliferate resulting in the inflammatory reactions typically observed in autoimmune diseases.

As mentioned above, autoimmune diseases may result from the small number of self-reactive cells that escape clonal deletion. It is currently thought that autoreactive cells are normally prevented from proliferating by a variety of mechanisms and any change in these regulatory processes leads to the proliferation of autoreactive cells, leading to either temporary or permanent damage to the individual.

Various experimental models have been developed to address the problem of autoimmunity. One such system is the MRL- *lpr/lpr* strain of mice, which has been developed by the introduction of a spontaneous autosomal recessive mutation, *lpr* (lymphoproliferation) gene into the normal congeneic strain of mice, the MRL- +/+ strain (Murphy and Roths, 1978). The MRL-*lpr/lpr* strain serves as an excellent model for the study of autoimmune diseases as these mice spontaneously develop a lupus-like disease characterized by the production of autoantibodies, hypergammaglobulinemia and massive lymph node enlargement (lymphadenopathy) (Murphy and Roths, 1978; Rosenberg *et al*, 1984). In contrast, the MRL-+/+ mice, which lack the *lpr* gene, manifest a mild form of the disease and at a later age than do MRL-*lpr* mice (Murphy and Roths, 1978). Enlargement of lymph nodes (LN) in MRL-*lpr*

mice begins around 8 weeks of age and progressively increases until, by 16 weeks of age, the lymph nodes can be about 100 times the size of a normal lymph node (Murphy and Roths, 1978). These mice usually die of nephritis by about 6 months of age.

The BXSB (Murphy and Roths, 1979), NZB, NZW and NZBxW (Howie and Helyer, 1968; Bielschowsky and Goodall, 1970) mice are some other strains that spontaneously develop a disease similar to human SLE. All these autoimmune mice develop glomerulonephritis due to the deposition of antigen-antibody complexes in the glomerular basement membranes and this is the main cause of death (Andrews *et al*, 1978; Accinni and Dixon, 1978). The thymuses of these mice atrophy as they grow older. However, the extent of lymph node enlargement varies -- in MRL-*lpr/lpr* mice the LN's may enlarge up to 100 times whereas in NZBxW females, it may only be 2-3 times and in BXSB males, about 10-20 times the normal LN size. All such autoimmune mice have increased serum Ig levels, which most often include monoclonal γ-globulins, anti-DNA and anti-nuclear antibodies (Andrews *et al*,1978; Izui *et al*,1978; Izui and Eisenberg,1980; Eisenberg *et al*, 1978, 1979a,b). Some of the old MRL-*lpr* mice also develop rheumatoid factors of both IgM and IgG types (Andrews *et al*,1978; Izui and Eisenberg,1980; Eisenberg *et al*, 1979b) and thus these mice are also useful as a model for studying rheumatoid arthritis.

The lymphadenopathy in the MRL-*lpr* mice is the result of the accumulation of large numbers of abnormal lymphocytes (Lewis et al, 1981). These cells express certain B-cell surface antigens (Budd *et al*, 1985; Davidson *et al*, 1984) but are thought to belong to the T cell lineage as: (a) they rearrange T cell receptor genes while the Ig genes are still in the germline configuration (Nemazee *et al*, 1985; Davidson *et al*, 1986; Miescher *et al*, 1987); (b) they express Thy-1 and Lyt-1 surface antigens (Lewis et al, 1981; Cohen *et al*, 1986; Davignon *et al*, 1985; Ishida *et al*, 1987); (c) neonatal thymectomy prevents the appearance of these cells (Smith *et al*, 1983; Smathers *et al*, 1984); and (d) treatment of adult MRL-*lpr* mice with monoclonal antibodies to T cells reduces T cell proliferation and retards the autoimmune disease (Wofsy and Seaman, 1987). Phenotypic analyses of *lpr* DN cells have shown that the CD4 and CD8

surface markers, which characterize normal mature T cells, are absent in the accumulating lymph node cells of MRL-*lpr* mice (Davignon *et al*, 1985; Ishida *et al*, 1987; Wofsy *et al*, 1984).

LN cells of *lpr* mice can non-specifically help Ig production (Theofilopoulos *et al*, 1980) and can also help B cells to produce anti-Sm autoantibody (Cohen and Eisenberg, 1982). The exact mechanisms of B cell hyperactivation and enhanced autoantibody production in these mice are not clear. The causes may include a genetic defect in the B cells, a lack of regulation by suppressor T cells, or B cell help provided by the DN cells that infiltrate the LNs or by T cells with normal phenotype and characteristics. Theofilopoulos and Dixon (1981) and others (Theofilopoulos *et al*, 1980b; Primi *et al*, 1979) have shown that it is possible to induce Ts cells in autoimmune mice by antigen-specific and mitogenic activation and that these suppressor cells consequently suppress Ig production by the B cells. Theofilopoulos *et al* (1980b) also found that in MRL-lpr mice, the help provided by T cells from old animals to B cells from young syngeneic mice was 2-3 times more than the help provided by an equal number of T cells from young syngeneic mice or T cells from young and old normal mice having the same H-2 haplotype. The enhanced B cell activity in the *lpr* mice may therefore be due to an increased T cell activity (Theofilopoulos *et al*, 1979a; Sawada and Talal, 1979).

Fishbach (1984) and Rosenberg *et al* (1984) have shown that the DN T cells from lpr mice do not proliferate normally in response to antigen. Therefore, the lymphadenopathy in the MRL-*lpr* mice may be due to a massive accumulation of these T cells in the LNs rather than an *in situ* proliferation (Rosenberg *et al*, 1984). However, longterm T cell lines can be established from unstimulated 2-3 month old MRL-*lpr* spleens and lymph nodes. These lines are $CD4^+$ T cells which respond to stimulation with autologous Ia antigens (Cohen *et al*, 1986). The relationship between the autoreactive T cells and the DN T cells that accumulate in the LNs of *lpr* mice is still not clear.

The syngeneic mixed lymphocyte reaction (SMLR) represents the proliferative response of T cells to *in vitro* stimulation with syngeneic Ia-antigen bearing non-T cells (Battisto and Ponzio,

1981; Weksler *et al*, 1981). The SMLR has been investigated in the MRL-*lpr* mouse (Hom and Talal, 1982; Glimcher *et al*, 1980). In comparison with the normal proliferative response of congeneic MRL- +/+ T cells to syngeneic spleen cells, a decreased response was observed with T cells from MRL-*lpr* mice (Hom and Talal, 1987). However, long-term culture of LN cells from young *lpr* mice results in the isolation of autoreactive T cells, similar to the cell lines previously obtained from normal DBA/2 mice (Nagarkatti *et al*, 1985a, 1985b).

Wofsy *et al* (1981) suggested that the decreased autoreactivity in *lpr* mice may be due to the absence of IL-2, which is always produced during a normal SMLR. IL-2 is a lymphokine that is essential for the development and maturation of T cells and this lymphokine is not produced in detectable quantities by *lpr* cells (Davidson et al, 1984; Davignon *et al*, 1985; Katagiri *et al*, 1987; Altman et al, 1981; Naides, 1986). The *lpr* cells also do not respond to exogenous addition of IL-2 (Fischbach, 1984; Scott *et al*, 1984), indicating that these cells probably lack the receptor for IL-2 (Davignon *et al*, 1985; Scott *et al*, 1984). A pronounced decrease in response to T cell mitogens such as Con A and PHA has also been observed (Smathers *et al*, 1984; Santoro et al,1983; Yamashita, 1986). However, Rosenberg *et al* (1984) have shown that the IL-2 defect and Con A unresponsiveness is abolished after *in vitro* culture of these *lpr* lymph node cells.

A decrease in autoreactivity may also be due to a decrease in the expression of Ia antigens by APC. However, expression of Ia antigens in cells from *lpr* mice increases with age (Lu *et al*, 1982; Kofler et al, 1984; Dauphinee and Talal, 1984; Kelly and Roths, 1982). Since Cohen *et al* (1986) found that T cell lines respond similarly to Ia-bearing cells from *lpr* mice of different ages, they concluded that the increase in Ia does not lead to an increase in proliferation of autoreactive cells obtained *in vitro*.

As discussed above, aging affects immune responses in normal as well as autoimmune-susceptible mice. The present study specifically addresses the alterations with

age in immune cell networks and phenotype and functions of T cells in normal and autoimmune-susceptible mice.

Chapter 3: Impaired Autoreactive T cell-Induced T cell-T cell Interaction in Aged Mice

Introduction

T cells responding to self-Ia antigens have been designated autoreactive T cells. Such cells have been isolated and cloned from immunized (Finnegan *et al*, 1984; Clayberger *et al*, 1984; Quintans *et al*, 1986) or from normal unimmunized mice (Nagarkatti *et al*, 1985a) and have been shown to perform a variety of immunoregulatory functions (Finnegan *et al*, 1984; Clayberger *et al*, 1984; Quintans *et al*, 1986; Nagarkatti *et al*, 1985a, 1985b; Suzuki *et al*, 1986; Kennedy *et al*, 1986; Sano *et al*, 1987; Kotani *et al*, 1986). Recently it was demonstrated that the autoreactive T cells were uniquely able to induce the proliferation of naive syngeneic $CD4^+$ T cells (Nagarkatti *et al*, 1985b). This T cell-T cell (T-T) interaction was independent of antigen-presenting cells and was inhibited by anti-CD4 but not by anti-Ia antibodies (Nagarkatti *et al*, 1985b). Based on these findings it was suggested that the responding $CD4^+$ T cells were directly recognizing and interacting with the idiotypic determinants on the autoreactive T cells. Recently, several investigations have confirmed the existence of the autoreactive T

cell-induced T-T network *in vitro* (Suzuki *et al*, 1986; Kennedy *et al*, 1986) and extended these studies *in vivo* to demonstrate that, the autoreactive T cells induce an immune circuit recruiting T suppressor (Ts) cells which, in turn, suppress T helper (Th) cells with the same MHC-restriction specificity. All these studies suggest that the autoreactive T cell-induced T-T interaction may represent an important feature of the T cell idiotypic network postulated to exist in the normal immune system.

Aging in higher animals has been shown to lead to several dysfunctions associated with the T and B lymphocytes and an increased incidence of autoimmune disorders (reviewed by Makinodan and Kay, 1980; Walford *et al*, 1981; Meredith and Walford, 1979). Since T cell networks are believed to play an important role in maintaining normal immune system homeostasis and since any perturbation in these could lead to disturbed immunoregulation and development of autoimmune or lymphoproliferative diseases and neoplasia (Smith and Talal, 1982), in the current study, the effects of aging on autoreactive T cell induced T-T network was investigated. It was observed that the T-T interaction was severely depressed in aged mice when compared to young mice and furthermore, this defect was associated with the decreased autoreactive T cell response in aged mice.

Materials and Methods

Mice

Adult female DBA/2 mice, 8-12 weeks of age (young) were obtained from the National Cancer Institute, Bethesda Md. and 22-24 months old DBA/2 mice (aged) were obtained from the National Institute on Aging, Bethesda, Md.

Antisera and reagents

The following hybridoma cell lines were used in the present study: MKD6 (anti-I-A^d), 14-4-4 (anti-I-E^d), GK 1.5 (anti-CD4) and 3.155 (anti-CD8). All hybridoma cell lines were grown *in vitro* and the monoclonal antibodies were used as ammonium sulfate-precipitated fractions of the culture supernatants (Nagarkatti *et al*, 1985a, 1985b). Recombinant mouse interleukin 2 (rIL-2) and recombinant mouse interleukin 4 (rIL-4) were procured from Genzyme, Boston, MA. One unit of rIL-2 was defined as that amount of IL-2 which caused a half maximal incorporation of &sup.3H-thymidine, in $4x10^3$ CTLL cells in culture. The activity of IL-4 was expressed in proliferation units based on a co-proliferation assay using purified murine splenic B cells and immunoglobulin (Grabstein *et al*, 1986). The IL-2 and IL-4 were diluted in complete medium and were added to the T-T interaction assay.

Medium

The medium used consisted of RPMI 1640 (GIBCO Laboratories, Grand Island, NY) containing 2mM L-glutamine, $5x10^{-5}$ M 2-mercaptoethanol, 40 μg/ml gentamicin sulfate, 10mM HEPES, and 10% fetal bovine serum (GIBCO Laboratories, Grand Island, NY) henceforth referred to as complete medium.

Separation of $CD4^+$ T cells

The $CD4^+$ T cells were purified as described in detail elsewhere (Nagarkatti *et al*, 1988). Briefly, a single cell suspension was prepared from the spleen in complete medium. The erythrocytes were lysed using 0.83% ammonium chloride. The cells were washed 3 times and

passed over nylon wool columns to isolate enriched T cells. These cells were further depleted of contaminating B and accessory cells by treatment with 1:10 dilution of anti-I-A^d and anti-I-E^d antibodies plus complement (C). The $CD8^+$ T cells were next depleted by treatment with anti-CD8 antibodies plus C. The purity of the $CD4^+$ T cells isolated was usually >98% as determined by FACS analysis.

Syngeneic mixed lymphocyte reaction (SMLR)

To study the proliferation of autoreactive T cells in the SMLR, $4x10^5$ $CD4^+$ T cells were mixed with $8x10^5$ irradiated (2000 rad) syngeneic splenic adherent cells (SAC), in 96 microwell plates, in 0.2 ml of medium. The SAC were prepared by incubating spleen cells on 100 mm plastic petri-dishes (Corning Glassworks, Corning, NY) at 37°C for 2 hrs (Nagarkatti *et al*, 1985a). The non-adherent cells were discarded and the adherent cells were recovered by forceful pipetting. The assay was carried out in triplicate and after incubation at 37°C in a 5% CO_2/95% humidified air atmosphere for various periods of time, 2μci of ^{3}[H] thymidine was added to each well during the last 18hr of culture. The cells were then collected onto glass-fiber filters with a semiautomatic cell harvester (Skatron, Sterling, VA), and the radioactivity incorporated was determined in a liquid scintillation counter (Betatrac 6895, TM Analytic, Inc., Elk Grove Village, IL).

Autoreactive T cell lines

Cloned autoreactive T cell lines were established from normal DBA/2 mice as described at length elsewhere (Nagarkatti *et al*, 1985a). Briefly, T cells from DBA/2 mice were repeatedly stimulated with syngeneic SAC and 10% delectinated human T cell growth factor (TCGF) procured from Cellular Products, Inc. Buffalo, NY. The cell lines were cloned and 4 such

clones designated Auto D1.1, Auto D1.2, Auto D1.3 and Auto D1.4 were further characterized. All these clones responded to I-E^d determinants as analyzed by monoclonal antibody blocking studies and failed to respond to alloantigens (data not shown). Also, all the clones were $Thy1^+$, $CD4^+$, $CD8^-$ and Ia^-. The autoreactive T cell clones were maintained *in vitro* by culturing $5x10^5$ T cells with $3x10^6$ syngeneic spleen cells in 2ml complete medium containing 10% TCGF, in 24 well Costar plates. The autoreactive T cells were harvested 3-4 days after culture, and viable cells were purified on Ficoll-Hypaque gradient.

T-T cell interaction

The T-T interaction was performed as described elsewhere (Nagarkatti *et al*, 1985b). Briefly $CD4^+$ T cells isolated from young or aged DBA/2 mice were mixed with varying numbers of irradiated (2000 rad) autoreactive T cell clones. The proliferative response of the $CD4^+$ T cells was measured by ^{3}(H) thymidine incorporation assay as described earlier.

Statistical analysis

All assays were performed in triplicate and the mean ± standard error (S.E.) of different groups were compared by Student's t-test. P values less than 0.05 were considered to be statistically significant. In most experiments the data was depicted as Δcpm ± S.E. which represented the proliferative responses of $CD4^+$ T cells to the autoreactive T cells (in T-T interaction) or to syngeneic SAC (in SMLR), minus the sum of proliferative responses of the responder $CD4^+$ T cells alone and of the irradiated stimulator cells alone.

Results

Kinetics and dose response studies on the T-T interaction in young and aged mice

$CD4^+$ T cells from young and aged DBA/2 mice were stimulated with a syngeneic irradiated autoreactive T cell clone (auto D1.4) and the kinetics of the T-T interaction was studied. As seen from Table 3.1, the T-T interaction peaked on day 2 both in young and aged mice and it was observed that this response was deficient in old mice on all the days tested when compared to the response of young mice. In all subsequent experiments, therefore, the T-T interaction was studied on day 2. We next studied the T-T interaction using different concentrations of the responders and stimulator cells, and observed that the response of $CD4^+$ T cells in aged mice was consistently lower than that of $CD4^+$ T cells from young mice (Table 3.2). Considering the response of young $CD4^+$ T cells as 100%, the responses of $CD4^+$ T cells from aged mice in the T-T interaction in these experiments, ranged from 0 to 11% of the young $CD4^+$ T cell responses.

Effect of anti-CD4 and anti-Ia antibodies on T-T cell interaction

To further substantiate that the T-T interaction studied was elicited by $CD4^+$ T cells and that it was independent of Ia^+ accessory cells, blocking studies with monoclonal anti-CD4, anti-I-A^d and anti-I-E^d were carried out. It was observed that a 1:10 final dilution of anti-CD4 antibody supernatant blocked the T-T interaction in young and aged mice while a 1:50 dilution of a combination of anti-I-A^d and anti-I-E^d antibodies failed to inhibit the T-T interaction (Table 3.3).

It should be noted that these concentrations of anti-Ia^d antibodies inhibited the SMLR in a separate experiment (data not shown).

Table 3.1. Kinetics of T-T cell interaction in young and aged mice[a]

Responder cells ($4x10^5$)	Stimulator cells ($1x10^5$)	Cell proliferation (cpm ± S.E.) Days		
		1	2	3
CD4 (Young)	-	3,421 ± 375	4,351 ± 511	4,115 ± 213
CD4 (Young)	AutoD1.4	12,136 ± 1321	45,462 ± 5671	30,121 ± 4112
CD4 (Aged)	-	2,112 ± 311	3,225 ± 426	4,715 ± 912
CD4 (Aged)	AutoD1.4	6,211 ± 317	18,312 ± 2114	9,449 ± 728
-	AutoD1.4	2,113 ± 172	1,982 ± 175	1,843 ± 214

[a]T-T interaction was studied using $4x10^5$ responder $CD4^+$ T cells from young or aged mice and $1x10^5$ irradiated AutoD1.4 as stimulator cells. The cell proliferation was measured by adding 2μ Ci of ^{3}H-thymidine to each well during the last 18 hr of culture.

Table 3.2. T-T interaction in young and aged mice using varying concentrations of responder and stimulator cells.

Responder cells[a]	Stimulator cells	Cell proliferation[b] (Δ cpm ± S.E.)		Percent of young response[c]
		Young	Aged	
$CD4^+$ ($4x10^5$)	AutoD1.4 ($2x10^5$)	33,226 ± 403	3806 ± 364	11
$CD4^+$ ($4x10^5$)	AutoD1.4 ($1x10^5$)	22,785 ± 281	1753 ± 130	8
$CD4^+$ ($2x10^5$)	AutoD1.4 ($2x10^5$)	18,181 ± 326	-1282 ± 312	<0
$CD4^+$ ($2x10^5$)	AutoD1.4 ($1x10^5$)	11,401 ± 90	-1222 ± 76	<0

[a]$CD4^+$ T cells from young or old DBA/2 mice were cultured at $4x10^5$ or $2x10^5$ cells per well with $2x10^5$ or $1x10^5$ irradiated autoreactive T cell clone (Auto D1.4). The cultures were incubated at 37°C for 2 days and 2μCi of ^{3}H-thymidine was added to each well during the last 18 hr of culture.

[b]Δ cpm represents the proliferation response of $CD4^+$ T cells to the autoreactive T cells minus the sum of the proliferative responses of the responder $CD4^+$ T cells incubated alone and of the irradiated autoreactive T cell clone alone. The background cpms of $CD4^+$ T cells incubated alone were 3,126 ± 315 and 2,777 ± 251 for $4x10^5$ and $2x10^5$ young cells respectively and 2,691 ± 342 and 2,542 ± 192 for similar numbers of aged cells. Irradiated autoreactive T cells alone gave a mean cpm of 4,215 ± 926 with $2x10^5$ cells and 3,001 ± 215 with $1x10^5$ cells.

[c]The proliferative response of the $CD4^+$ T cells from aged mice was calculated by considering the response of young $CD4^+$ T cells as 100%

Table 3.3. Effect of anti-CD4 and anti-Ia^d antibodies on the T-T interaction in young and aged mice[a]

Responder cells	Stimulator cells	Antibodies added[b]	Cell proliferation[c] (Δcpm ± S.E.)	
			Young	Aged
CD4$^+$ (4x10^5)	AutoD1.4 (2x10^5)	-	45,715 ± 700	14,091 ± 260
CD4$^+$ (4x10^5)	AutoD1.4 (2x10^5)	Anti-CD4	4,302 ± 108	-597 ± 132
CD4$^+$ (4x10^5)	AutoD1.4 (2x10^5)	Anti-Ia^d	44,714 ± 259	17,255 ± 365

[a]The T-T interaction was studied as described in Table 3.2.

[b]Anti-CD4 (GK1.5) antibodies were added at a final dilution of 1:10. The anti-Ia^d antibodies consisted of 1:50 dilution of a combination of anti-I-A^d and anti-I-E^d antibodies.

[c]CD4$^+$ T cells alone from young mice gave a background cpm of 4,316 ± 247 and similar cells from old mice demonstrated a cpm of 3,924 ± 431. Irradiated autoreactive T cells alone incorporated 2,104 ± 312 cpm.

Correlation of decreased T-T interaction with deficient SMLR responses in aged mice

Studies were conducted to address whether the decreased T-T interaction observed in aged mice was related to any alterations in the functions of autoreactive T cells. For this purpose, T-T interaction and SMLR were studied simultaneously using the same population of $CD4^+$ T cells from young or aged mice. It was observed that mice which were deficient in T-T interaction, also demonstrated an impaired SMLR response (Table 3.4). We also used other autoreactive T cell clones (auto D1.1, auto D1.2 and auto D1.3 in these experiments) to investigate whether the defect in aged mice was generalized and not restricted to a single autoreactive T cell clone. It was observed that $CD4^+$ T cells from aged mice responded poorly to all the autoreactive T cell clones tested and these responses were 0-39% of the young $CD4^+$ T cell responses.

Effect of exogenous rIL-2 and rIL-4 on the T-T interaction in young and aged mice

Studies were conducted to determine the effect of addition of exogenous T cell growth factors such as IL-2 and IL-4 on the T-T interaction. Using two concentrations of rIL-2 (1000 or 500 u/ml), rIL-4 (500 or 200 u/ml) or a combination of these two factors, it was observed that rIL-2 alone failed to cause a significant increase ($p > 0.05$) in the T-T interaction involving the young responder cells (Table 3.5). IL-4, however, enhanced the T-T interaction significantly ($p < 0.025$) in the young mice. The combination of IL-2 and IL-4 failed to cause a synergistic effect, thereby suggesting further that only IL-4 was able to enhance the T-T interaction in young mice. In contrast, IL-2 and IL-4 failed to reconstitute the impaired T-T interaction in aged mice (Table 3.5).

Table 3.4. Correlation of SMLR and T-T interaction in young and aged mice.

Expt. No.	Stimulator cells	Responder cells[a]	Cell proliferation (Δ cpm ± s.e.)[d]			
			T-T interaction[b]	% of young response[e]	SMLR[c]	% of young response[e]
1	Auto D1.1	CD4 (young)	23,632 ± 591		37,947 ± 577	
		CD4 (aged)	-5991 ± 516	<0	18,218 ± 634	48
2	Auto D1.2	CD4 (young)	56,875 ± 1307		28,544 ± 2169	
		CD4 (aged)	22,122 ± 634	39	6,321 ± 2218	22
3	Auto D1.3	CD4 (young)	34,435 ± 674			
		CD4 (old)	7,616 ± 591	22		

[a]$CD4^+$ T cells from young or aged DBA/2 mice were used to study T-T interaction or SMLR at a concentration of $4x10^5$ cells/well.

[b]The T-T interaction was studied using irradiated autoreactive T cell clones, as stimulator cells, at a concentration of $1x10^5$ cell/wells. The irradiated autoreactive T cell clones alone demonstrated a mean cpm of 5,687 ± 1,500.

[c]SMLR was studied using $8x10^5$ irradiated SAC as stimulator cells which when cultured alone, demonstrated background counts of <3,000.

[d]Cell proliferation and Δcpm were studied as described in Table 3.2.

[e]The responses of cells from aged mice was calculated by comparing the responses obtained with young cells and considering the latter as 100%.

Table 3.5. Effect of rIL2 and rIL4 on the T-T interaction in young and aged mice.

Responder cells[a]	Stimulator cells[b]	Interleukin[c] and concentration		Cell proliferation[d] Δcpm ± s.e.	
				Young	Aged
$CD4^+$	AutoD1.4	—	—	33,100 ± 335	6,281 ± 139
$CD4^+$	AutoD1.4	rIL2	1000 U/ml	43,035 ± 188	6,868 ± 157
$CD4^+$	AutoD1.4	rIL2	500 U/ml	36,220 ± 165	7,136 ± 140
$CD4^+$	AutoD1.4	rIL4	400 U/ml	50,150 ± 198	8,607 ± 177
$CD4^+$	AutoD1.4	rIL4	200 U/ml	50,615 ± 266	8,559 ± 154
$CD4^+$	AutoD1.4	rIL-2 rIL-4	1000 U/ml 400 U/ml	52,413 ± 187	8,320 ± 194

[a]$CD4^+$ T cells from young or aged mice were used at a concentration of $3x10^5$ cells/well.

[b]Irradiated AutoD1.4 cells were used as stimulator cells at a concentration of $5x10^4$ cells/well.

[c]$CD4^+$ T cells incubated with rIL2 or rIL4 alone gave a mean cpm of $<3,000$.

[d]Cell proliferation and Δcpm were measured as described in Table 3.2.

Discussion

Earlier studies have demonstrated that autoreactive T cell clones induce the proliferation of syngeneic naive $CD4^+$ T cells (Nagarkatti *et al*, 1985b). In this report, similar proliferative responses by $CD4^+$ T cells from young mice to four new autoreactive T cell clones were demonstrated. This T-T interaction was inhibited by anti-CD4 antibodies but not by anti-Ia antibodies thereby suggesting that the T-T interaction involved the CD4 antigen but was independent of class II MHC antigens, similar to the earlier study (Nagarkatti *et al*, 1985b). Although it has been speculated that CD4 may interact with class II MHC antigens (Greenstein *et al*, 1984; Gay *et al*, 1987), several recent studies have suggested that anti-CD4 antibodies can inhibit responses of T cells activated in the absence of class II MHC-antigens (Bekoff *et al*, 1985; Wassmer *et al*, 1985), similar to the observations made in the present study, thereby suggesting that CD4 may be closely associated with the T cell receptor and may be involved in transmitting a signal which would regulate the T cell activation process. Based on these observations, it was speculated that the $CD4^+$ T cells were responding directly to the idiotypic determinants on the autoreactive T cells. Similar conclusions have been drawn by several recent studies (Suzuki *et al*, 1986; Kennedy *et al*, 1986; Sano *et al*, 1987). Although the functional roles of the $CD4^+$ T cells responding to the autoreactive T cells was not clear, recent studies have suggested that the T-T interaction can initiate a chain of regulatory interactions leading to either the stimulation or inhibition of an immune response (Suzuki *et al*, 1986; Kennedy *et al*, 1986; Sano *et al*, 1987; Kotani et al, 1986). Since the autoreactive T cell-induced T-T interaction appears to represent an important component of the T cell network, in the present study, the effect of aging on the T-T interaction was investigated. It was observed that $CD4^+$ T-cells from aged mice responded poorly to autoreactive T cell clones isolated from young mice. Furthermore, this defect appeared to be related to impaired SMLR in aged mice.

Several explanations can be considered to account for the age-associated decline in the T-T interaction. Firstly, this decline may be due to general defectiveness associated with aging such as defective lymphokine production (Thoman and Weigle, 1981), inability to respond to lymphokines (Thoman and Weigle, 1981; Gillis *et al*, 1981; Canonica *et al*, 1985) or a defective signal transduction mechanism. Secondly, the specificities of the $CD4^+$ T cell receptors recognizing the idiotypic determinants on autoreactive T cells from young mice might have been lost or altered due to aging. To address this question, autoreactive T cell clones from aged mice will have to be isolated and tested with young and aged $CD4^+$ T cells for T-T interaction. Isolation of autoreactive T cell clones from aged mice may, however, pose a problem since SMLR is deficient in these mice. Goidl *et al* (1980) have described similar age related changes in the B cell repertoire at the idiotypic level. Furthermore, different subsets of T suppressor cells from aged mice, constituting the T suppressor circuit, have been shown to be unable to interact with their counterparts in young mice or vice versa (Doria *et al*, 1987). Since Sano *et al* (1987) have demonstrated that autoreactive T cells induce a suppressor circuit *in vivo*, a deficient autoreactive T cell response and T-T interaction in aged mice may lead to impaired suppressor T cell functions as observed by several investigators (Amagai *et al*, 1982; Barthold *et al*, 1974; Krakauer *et al*, 1976; Gottesman *et al*, 1984).

A third possibility emerges if we consider the following. Recently, Sim and Augustin (1983) proposed that the internal images of MHC antigens on T cell receptors might play a role in the generation of the T helper cell repertoire. Based on this model, Nagarkatti *et al* (1985b) proposed that the autoreactive T cell may represent the first set of T cells (T_1) selected to proliferate by the thymic Ia molecules. These T_1 cells in turn, induce cells such as the responding $CD4^+$ T cells (T_2), having receptors that are internal images of self-Ia. This T-T interaction would result in the expansion of both the T cell types and could lead to somatic diversification, the end product of which would consist of autoreactive T cells and the T cells responding to them, having been modified from the original receptor repertoire. If this hypothesis is true, then, these subsets of T cells must be precisely regulated to maintain the

homeostasis of the immune system. However, in aged mice the level of autoreactive T cells (T_1) seems to have been altered as evident from impaired SMLR. This shift at the T_1 level in aged mice could have imbalanced the T-T interaction necessary for the maintenance of T_2. As a result, the repertoire of T_2 might have been changed or its generation itself may be down regulated in the aged mice. Further studies are necessary to distinguish these possibilities.

Recently both IL-2 and IL-4 have been shown to act as potent T cell growth factors (Grabstein *et al*, 1986; Fernandez-Botran *et al*, 1986; Mosmann *et al*, 1986; Mosmann *et al*, 1987). Since the factors involved in the T-T interaction have not been investigated previously, in the present study, attempts were made to determine the effect of addition of exogenous IL-2 and IL-4 on the T-T interaction and to study whether addition of these factors could reconstitute the response of aged $CD4^+$ T cells to the autoreactive T cells. Interestingly, it was observed that addition of IL-4 but not IL-2 could augment the T-T interaction in young mice. In contrast, similar concentrations of IL-4 failed to correct the impaired T-T interaction in aged mice. These findings suggested that IL-4 may represent an important growth factor involved in the autoreactive T cell-induced T-T interaction. It has been shown recently in our laboratory that the autoreactive T cell clones used in the present study produce IL-4, since their culture supernatants enhance the expression of Ia antigens on B cells (Kakkanaiah *et al*, 1990). The present study suggested that the decreased T-T interaction in aged mice may partly be due to impaired responsiveness of the $CD4^+$ T cells from aged mice to the IL-4 produced by the autoreactive T cells.

In the present study, it was also observed that SMLR was decreased in aged mice. Previous studies have shown that the normal $CD8^+$ T cell population has some endogenous suppressor cells which down-regulate autoreactive T cells (Nagarkatti *et al*, 1988). Thus, depletion of $CD8^+$ T cells *in vitro* or *in vivo* enhanced the autoreactive T cell responses (Nagarkatti *et al*, 1988). In earlier studies, defective SMLR in aged mice has been demonstrated using unseparated nylon wool non-adherent T cells as responders (Gutowski and Weksler, 1982). From these studies, therefore, it was not clear whether the impaired SMLR in aged mice was

due to a defect in the responder cells or due to enhanced suppressor activity by $CD8^+$ T cells. Our studies using $CD4^+$ T cells as responders and SAC as stimulators, clearly suggest a defect in the responder autoreactive T cells. Some of the possible explanations for the decreased SMLR in aged mice are, a low precursor frequency of SMLR-responsive T cells as reported in aged NZB mice (Bocchieri and Smith, 1982), an intrinsic defect associated with the recognition of self-Ia molecules or impaired response to growth factors (Thoman and Weigle, 1981). It has been shown that exogenous addition of interleukin-2 fails to correct the defective AMLR in aged humans (Canonica *et al*, 1985). Since autoreactive T cells are important in the generation and regulation of antigen-specific immune responses (Finnegan *et al*, 1984; Clayberger *et al*, 1984; Quintans *et al*, 1986; Nagarkatti *et al*, 1985a, 1985b; Suzuki *et al*, 1986; Kotani *et al*, 1986; DosReis and Shevach, 1981), and also evoke a T-T network (Nagarkatti *et al*, 1985a, 1985b; Suzuki *et al*, 1986; Kennedy *et al*, 1986; Sano *et al*, 1987), an impaired reactivity of the autoreactive T cells and their network may account for the age-related dysfunctions associated with the T and B cells and the increased incidence of autoimmune disorder in senescence (Makinodan and Kay, 1980; Walford *et al*, 1981; Meredith and Walford, 1979).

Summary

Self-Ia reactive (autoreactive) $CD4^+$ T cell clones have been shown earlier to stimulate the proliferation of syngeneic naive $CD4^+$ T cells and initiate a T-T interaction leading to the generation of immunoregulatory circuits. Since aging has been shown to be associated with a decline of the immune responsiveness, age-related alterations in the T-T interaction was investigated in the present study. Using several I-E^d-specific autoreactive T cell clones isolated from 2-3 month old (young) DBA/2 mice as stimulators, it was observed that $CD4^+$ T cells from 22-24 month old (aged) DBA/2 mice, failed to demonstrate a significant response to

the autoreactive T cells. In contrast, $CD4^+$ T cells from young mice responded strongly to the autoreactive T cell clones. The deficient T-T cell interaction in aged mice correlated with an impaired syngeneic mixed lymphocyte reaction (SMLR) in these mice, thereby suggesting that aging induces a defect both in the autoreactive T cells and in T cells which react with the autoreactive T cells. When exogenous recombinant interleukin 2 (rIL-2), recombinant interleukin 4 (rIL-4) or a combination of these was added to the interaction, it was observed that rIL-4 but not rIL-2 enhanced the T-T interaction in young mice. However, rIL-4 or a combination of rIL-2 and rIL-4 failed to correct the defective T-T interaction in aged mice. Since the T cell network is believed to play an important role in the maintenance of normal immune system homeostasis, the present study suggests that age-related alterations in T and B cell functions and increased susceptibility to autoimmune diseases with age, may result from a defect in the T cell network regulation.

Chapter 4: A Specific Defect in the Proliferative Capacity of B Cells from Old Mice Stimulated with Autoreactive T cells.

Introduction

The immune potential of an animal increases with age in ontogeny and reaches peak levels in adulthood. In senescent animals immune responses to external stimuli are decreased substantially whereas autoimmune reactions are increased (Makinodan and Kay, 1980; Segre and Smith, 1981; Meredith and Walford, 1979). In adult animals the thymus atrophies and consequently a variety of T lymphocyte-dependent responses such as graft rejection, lymphokine production, mitogen reactivity and suppressor function are altered in old age (Hirokawa and Makinodan, 1975; Menon *et al*, 1974; Bach, 1977; Hori *et al*, 1973; Chang *et al*, 1982; Thoman and Weigle, 1981; Dekruyff *et al*, 1980; Barthold *et al*, 1974; Kishimoto *et al*, 1979). Also, the autologous and syngeneic mixed lymphocyte reactions (SMLR) in humans and mice

respectively are found to decrease with age (Gutowski and Weksler, 1982; Bocchieri and Smith, 1982; Canonica *et al*, 1985; Smith and Talal, 1982). The defective SMLR was primarily due to a dimunition in the number of responding T cells, although alterations in the stimulating population were also found (Gutowski and Weksler, 1982; Bocchieri and Smith, 1982; Canonica *et al*, 1985).

Several investigators demonstrated that humoral responses of aged individuals were also diminished in quantity and were altered in quality (Makinodan and Peterson, 1962; Price and Makinodan, 1972; Goidl et al, 1976; Segre and Segre, 1976a; Segre and Segre, 1976b; Callard *et al*, 1977). Thus, idiotype constitution of the antibody response of the aged animals was different from that of the young animals (Goidl *et al*, 1980; Szewczuk and Campbell, 1980; Klinman, 1981; Goidl et al, 1983; Zharhary and Klinman, 1983, 1984, 1986). The alterations in the idiotype distribution were observed in the immune responses to several haptenic groups such as trinitrophenyl, phosphorylcholine and 5-nitro-3-iodo-1-phenylacetic acid coupled to T-independent (Ficoll) or T-dependent carriers (Goidl *et al*, 1980; Szewczuk and Campbell, 1980; Klinman, 1981; Zharhary and Klinman, 1983). In addition, the antibodies from old mice were of relative lower affinity than those from young animals (Goidl *et al*, 1976). However, both affinity maturation and the idiotype distribution were shown to be under the influence of T cells (Gershon and Paul, 1970). Therefore the defects in B cell responses of old mice may develop as a secondary consequence of the T cell deficiencies. In agreement with this, the total numbers of B cells were found to be nearly the same in young and old mice with only slight alterations in the density of the membrane immunoglobulins (Ig) (Kay *et al*, 1979; Woda and Feldman, 1979). Further, the mitogenic responses of B cells to stimulation with the B cell mitogen, lipopolysaccharide (LPS), were only marginally affected with age (Gerbase-Delima *et al*, 1974) although others found decreases in LPS responsive B cells with age (Anderson *et al*, 1977; Abraham *et al*, 1977). In addition, adoptive transfer studies by Kishimoto *et al* (Price and Makinodan, 1972; Kishimoto and Yamamura, 1971), analysis of clonal precursors for specific antigens (Klinman, 1981; Zharhary and Klinman, 1983; Zharhary and Klinman,

1986b ; Zharhary and Klinman, 1987) and studies of responses to thymic-independent antigens (Gerbase-Delima *et al*, 1974; Friedland and Globerson, 1978) suggested that, in aged individuals, B cells *per se* may be defective.

Recently it was observed that the relative ratio of cell surface IgM to IgD was altered idiosyncratically in individual old mice (Udhayakumar *et al*, 1986). The mitogenic response of these B cells to stimulation with monoclonal anti-Lyb-2 antibody, a B cell-specific mitogen, was also decreased, adding to the possibility that B cells from old mice might have intrinsic defects. For this study, B cell responses in aged mice were investigated using a unique model system. We took advantage of the demonstration that self-Ia reactive T cells stimulated polyclonal B cell proliferation and differentiation in young mice (Zauderer *et al*, 1984; Finnegan *et al*, 1984). Employing an autoreactive T cell clone (auto D1.4) isolated in our laboratory recently (Udhayakumar *et al*, 1988), it was observed that the B cells from young but not old DBA/2 mice proliferated normally to stimulation with the auto D1.4 cells. Also, polyclonal Ig secretion induced by the auto D1.4 cells was reduced in old B cells. Interestingly, after stimulation with the auto D1.4 cells, B cells from both old and young mice have increased their cell-surface Ia and size to the same extent, suggesting that B cells from old mice have a specific defect in their ability to progress into the S phase of the cell cycle.

Materials and Methods

Mice

Two-to four-month-old and 22-to 24-month-old DBA/2 mice were obtained from the National Institute of Aging, Bethesda, MD. C57BL/6 mice were obtained from the Jackson Laboratories (Bar Harbour, ME). Old mice were inspected for lymphoid abnormalities before use and were not included in the study if leukemia, lymphoma or any other tumor was suspected to be present.

Antibodies and lymphokines

Monoclonal anti-$I\alpha^d$ (MKD6), anti-IE^d (14.4.4), anti-Thy-1.2 (HO13-4-6), anti-CD8 (53-6.72) and anti-CD4 (GK1.5) antibodies were obtained from the American Type Culture Collection (Rockville, MD) (Ozato *et al*, 1980; Marshak-Rothsten *et al*, 1979; Ledbetter and Herzenberg, 1979; Dialynas *et al*, 1983). Affinity-purified goat anti-mouse κ was purified from goat anti-mouse IgG serum by methods described previously (Subbarao and Mosier, 1983). Alkaline phosphatase-conjugated goat anti-mouse IgM and IgG antibodies were obtained from Sigma Chemical Co. (St. Louis, MO). Purified interleukin-2 (IL-2) and recombinant interleukin-4 (IL-4) were obtained from Genzyme (Boston, MA).

Cell separation

Purified T cells were obtained by passing the spleen cells through a nylon wool column and collecting the nonadherent cells. Purified B cells were prepared by depleting the adherent cells by plastic adherence and killing T cells with a cocktail of antibodies (anti-Thy1.2, anti-CD4 and anti-CD8) plus rabbit complement. This treatment resulted in a preparation that consisted of >95% Ig^+ cells as judged by analysis on a cell sorter. In addition, the responses to the T cell mitogen concanavalin A were reduced by 90 to 95% in comparison to the responses of the untreated cells. The B cell purity was relatively the same in young and old mice derived cell populations which was probably due to the use of multiple anti-T cell antibodies for T cell depletion.

Cell cultures

Cloned D1.4 autoreactive T cells were obtained by methods described previously (Nagarkatti *et al*, 1985a). They were maintained by stimulation with syngeneic spleen cells (irradiated) weekly and supplemented with IL-2 every 3 days. Cell cultures were performed in RPMI 1640 medium supplemented with pyruvate, glutamine, 2-mercaptoethanol, and fetal calf serum. Cell proliferation was measured by tritiated thymidine incorporation. Cultures were pulsed with 1.0μCi of [^{3}H]thymidine (sp act 2Ci/mmol) for 4.0 hr and harvested on a Skatron cell harvester, and the filters were counted in a liquid scintillation spectrometer.

Immunofluorescence analysis

Cultured cells were washed with phosphate-buffered saline (PBS) containing 0.1% sodium azide and 1.0 x 10^6 cells were stained with monoclonal anti-Ia antibodies (1:10 dilution of a mixture of supernatants from the cell lines MKD6 and 14.4.4) and with $(Fab)_2$ fragments of fluoresceinated goat anti-IgG2 antibody (Cappel Labs, Inc., Cochranville, PA). After each incubation with antibodies for 45min at 4°C the cells were washed three times with PBS and were incubated for 10 min with propidium iodide just before analysis on the cell sorter. Fluorescence analysis was performed after gating out the dead cells on the basis of their red fluorescence. A Coulter EPICS 752 cytofluorograph was employed for fluorescence studies.

Enzyme-linked immunosorbent assay (ELISA)

IgM secreted in the cultures was measured by an ELISA assay. Briefly, affinity-purified goat anti-κ antibody (5.0 μg/ml) was coated onto the microwells of an ELISA plate and was blocked with phosphate-buffered saline plus 1% egg albumin plus 0.02% sodium azide. Wells were washed with PBS + 0.1% Tween 20 and were incubated with standard concentrations of purified IgM or varying dilutions of the test supernatants. The bound Ig was quantitated by the addition of 1:400 dilution of alkaline phosphatase-conjugated goat anti-mouse IgM and finally developed with *p*-nitrophenyl phosphate. The optical densities were measured on a Flow Multiskan MCC/340 reader and the concentrations of IgM were calculated by linear regression analysis using an IBM PC computer interfaced with the reader.

Statistics

Experimental determinations were performed in triplicates for cell cultures and in duplicates for ELISA measurements. Arithmetic mean ± standard error is reported.

Results

Proliferative response of B lymphocytes from young and old mice to stimulation with the autoreactive T cells

Self-Ia reactive T cells were demonstrated previously to induce polyclonal B cell activation (Zauderer *et al*, 1984; Finnegan *et al*, 1984; Nagarkatti *et al*, 1985a). Purified antigen-specific B cells were induced by the autoreactive T cells to proliferate and differentiate in the absence of added antigen (Nagarkatti *et al*, 1985a). In this study we employed the autoreactive T cell clone called auto D1.4, which was isolated in our laboratory recently (Udhayakumar *et al*, 1988). These auto D1.4 T cells were similar to our previously reported autoreactive T cell line PK4 in their ability to induce activation of B cells and proliferation of $CD4^+$ T cells (Udhayakumar *et al*, 1988; Nagarkatti *et al*, 1985a, 1985b). The characteristic responses of this auto D1.4 T cell clone are summarized in Table 4.1. The auto D1.4 T cells responded to stimulation with DBA/2 ($H\text{-}2^d$) stimulators but not with C57BL/6 ($H\text{-}2^b$) stimulators. Furthermore, the proliferative response of the auto D1.4 T cells was specifically inhibited by the monoclonal anti-IE^d but not anti-IA^d antibodies. It should be noted that these monoclonal antibodies, at the dilution used (1:100), significantly inhibited the syngeneic mixed lymphocyte

(anti-Ia^d) reaction but not the allogeneic (anti-Ia^b) responses of DBA/2 $CD4^+$ T cells (data not shown).

Stimulation of young mice derived B cells was achieved by culturing preactivated and irradiated auto D1.4 T cells with purified syngeneic B cells. Marked proliferation of B cells was observed after 2 days of stimulation with the autoreactive T cells (Table 4.2). Interestingly, purified B cells from old DBA/2 mice exhibited sharply reduced responses. The reduction in the B cell response was seen at all the different densities of B cells and T cells tested. As shown in Fig.4.1, the decreased growth of old mice derived B cells in response to stimulation with the autoreactive T cells was also not due to any alterations in the kinetics of the response.

Autoreactive T cell-induced differentiation of B Cells from old and young mice

Polyclonal activation of the B cells with the auto D1.4 T cells was measured by quantitating the amount of IgM secreted in an ELISA assay. As shown in Table 4.3, B cells from young mice responded to stimulation with the autoreactive T cells by secreting substantial amounts of IgM after 4 to 5 days of culture. Very little IgG_3 was seen in these cultures. Once again the B cells from the senescent animals secreted much less IgM than the young B cells after stimulation with the auto D1.4 T cells and this reduction in differentiation response was observed on both the fourth and the fifth days of the culture. The decrease in the IgM response was not due to increased class switch in the old mice derived B cells, since neither IgG_3 nor IgG_1 was detectable in these cultures.

It appears that this B cell defect is not unique to stimulation with a particular autoreactive T cell clone, since similar results were obtained if B cells were stimulated with uncloned

syngeneic T cells from young mice. In these studies B cell maturation was measured by the protein A plaque-forming cell assay which detects the total number of IgM-secreting cells. As shown in Table 4.4, old mice exhibited reduced PFC responses. However, the defect appeared to be specific to stimulation with syngeneic T cells, since their response to LPS was comparable to that of young mouse B cells in the same experiment.

Table 4.1. Characteristics of the AutoD1.4 T cell clone

Stimulator cells[a]	Anti-Ia antibody	Proliferation[b] (cpm x 10^3)
None	-	2,147 ± 326
DBA/2 spleen	-	35,715 ± 1,741
C57BL/6 spleen	-	2,889 ± 294
DBA/2 spleen	MKD6(IA^d)[c]	33,821 ± 2,111
DBA/2 spleen	14.4.4(IE^d)	5,422 ± 419

[a]D1.4 autoreactive T cells (2 x 10^5) were cultured with 5 x 10^5 irradiated spleen cells from DBA/2 or C57BL/6 mice in RPMI 1640 medium + 10% fetal calf serum.

[b]Triplicate cultures were pulsed on day 2 with tritiated thymidine and cultures were harvested 18hr later. Results are expressed as arithmetic mean ± standard error.

[c]Anti-Ia antibodies were added at a final concentration of 1:100 dilution of culture supernatant. Anti-IA^d antibody was shown to be functional by its ability to inhibit a syngeneic mixed lymphocyte reaction mediated by IA^d. The inhibition by 14.4.4 was specific since this antibody did not inhibit the IA^d-restricted SMLR or an allogenic mixed lymphocyte reaction to IA^b.

Table 4.2. Proliferation of B cells from young and old mice in response to stimulation with the Autoreactive T cell clone Auto D1.4

Source of B cells (no. of cells)[a]	Number of D1.4 cells	Proliferative response[b] (cpm)[c]	% of young response
Young(3×10^5)	1×10^5	30,945 ± 672	
Old(3×10^5)	1×10^5	7,469 ± 620	24
Young(3×10^5)	5×10^4	14,916 ± 356	
Old(3×10^5)	5×10^4	5,294 ± 526	35
Young(1.5×10^5)	1×10^5	25,641 ± 1,271	
Old(1.5×10^5)	1×10^5	4,360 ± 1,638	17
Young(1.5×10^5)	5×10^4	15,359 ± 615	
Old(1.5×10^5)	5×10^4	3,361 ± 1,134	22

[a]B cells from young or old DBA/2 mice were cultured with irradiated auto D1.4 cells at the indicated concentrations.

[b]Thymidine incorporation was measured on day 2. Values are arithmetic mean ± SD.

[c]cpm was obtained by subtracting the sum of responses obtained in cultures with B cells alone and irradiated D1.4 cells alone.

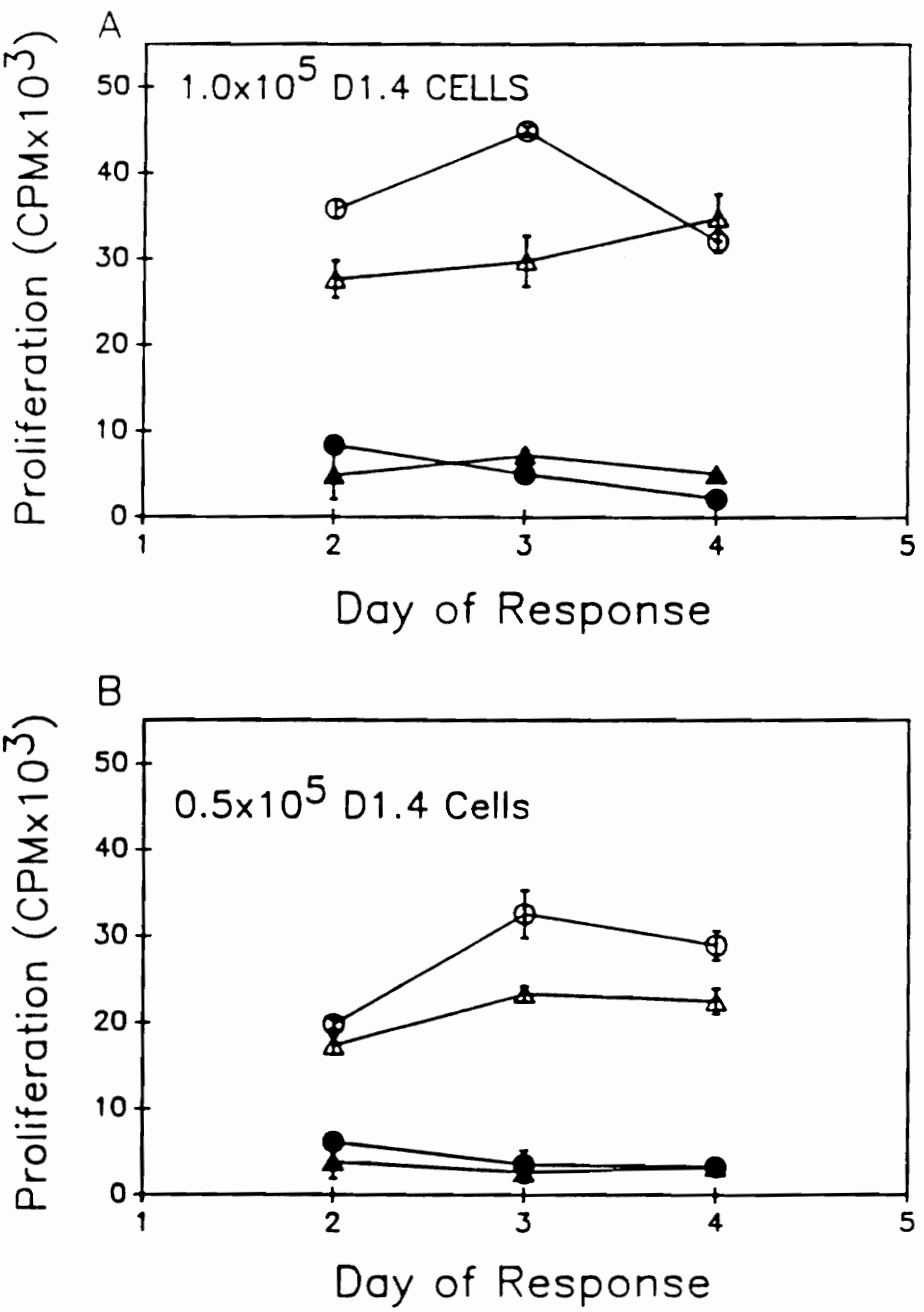

Figure 4.1. Kinetics of B cell proliferative response induced by the autoD1.4 T cells: Purified B cells from young or old mice were cultured with 1.0 x 10^5 (A) or 0.5 x 10^5 (B) irradiated T cells for 3, 4 and 5 days. Mean tritiated thymidine incorporation in triplicate cultures is plotted. Open and solid symbols, respectively, represent the responses of young and old mice. 3.0 x 10^5 (o) or 1.5 x 10^5 (Δ) B cells were employed in the cultures.

Table 4.3. Differentiation response of young and old B cells to stimulation with the T cell clone

Source of B cells	Addition of D1.4 cells	IgM secreted (μg/ml)[b]	
		Day 4	Day 5
Young	-	10.0 ± 1	44.6 ± 2
Young	+	131.0 ± 31	226.0 ± 89
Old	-	5.0 ± 1	16.0 ± 4
Old	+	49.6 ± 4	44.9 ± 3
% of young response[c]		37	16

[a]4.0 x 10^5 purified B cells from young or old DBA/2 mice were cultured with 4.0 x 10^5 irradiated auto D1.4 autoreactive T cells in 0.2ml of RPMI + 10% FCS.

[b]Amount of IgM secreted into the cultures was measured by an ELISA assay.

[c]Specific response of B cells from old mice is expressed as a fraction of the response of young B cells. Specific response induced by the T cells is obtained by subtracting the IgM secreted in the B cell cultures to which T cells were not added.

Table 4.4. Responses of young and old mouse B cells to stimulation with uncloned autoreactive T cells.

Source of B cells	Addition to culture	Protein A PFC/2 x 10^5 cells[a]	
		Expt 1	Expt 2
Young	-	10 (1.05)	29 (1.22)
Young	Syngeneic T cells (10^5)[b]	2406 (1.06)	741 (1.33)
Old	-	32 (1.18)	112 (1.08)
Old	Syngeneic T cells (10^5)	41 (1.27)	172 (1.15)
Young	LPS[c]	5674 (1.03)	21,011 (1.10)
Old	LPS	5349 (1.36)	20,358 (1.05)

[a]Total IgM-secreting cells were determined by using protein A-coupled sheep red blood cells and rabbit anti-μ antibodies. Results are geometric mean of triplicate cultures with SE in parentheses.

[b]T cells were purified from young C57BL/6 mice by passing spleen cells through a nylon wool column and treating the nonadherent cells with anti-Ia antibodies + complement. B cells were prepared as described under Materials and Methods. 2.0 x 10^5 B cells were cultured in IF12 + 5% fetal calf serum.

[c]LPS was employed at 50μg/ml.

Induction of B cell hyper Ia expression by stimulating with the Auto D1.4 T cells

Since B cells from old mice exhibited defects in both differentiation and proliferation, we wished to determine if these B cells received any stimulatory signals at all. One of the early measures of activation in B cells stimulated with anti-μ or mitogen was shown to be an increase in cell-surface Ia expression (Mond *et al*, 1981). Even though the induction of B cell proliferation and differentiation with the autoreactive T cells was previously reported, the effect of stimulation with self-Ia reactive T cells on B cell Ia expression was not investigated before. Here, we have demonstrated that the auto D1.4 T autoreactive T cells were able to induce hyper Ia expression in young mice derived B cells (Fig. 4.2A). B cells from old mice also responded by increasing their surface Ia after stimulation with the auto D1.4 cells (Fig. 4.2B) suggesting that the senescent B cells received at least some activation signals from the T cells. The extent of the response as measured by the increase in mean fluorescence intensity was about the same in young and old mice as shown in Table 4.5. Also, the kinetics of the induction of this hyper Ia expression were identical in young and old mice (data not shown). These experiments were repeated four times with consistent results.

Responsiveness of young and old B cells to stimulation with IL-4

The lymphokine interleukin-4 has been shown recently to be critically involved in T-dependent B cell activation (Paul and Ohara, 1987; Killar *et al*, 1987). Among the plethora of activities of IL-4 is its ability to enhance B cell Ia expression in resting B cells (Noelle *et al*, 1984). Therefore, we investigated if the defects in the old mice B cells were due to an alteration in their ability to respond to IL-4 that may be secreted by the auto D1.4 cells. B cells from young

and old mice were cultured with varying concentrations of recombinant IL-4 (rIL-4) and the increase in Ia expression was measured by flow cytometry. As shown in Table 4.5 and Figs. 4.3A and 4.3B, rIL-4 induced an increase in Ia expression in both young and old B cells compared to the B cells cultured without rIL-4. Once again, the magnitude of the response was identical in young and old mice (Table 4.5). Dose responses and the kinetics were nearly identical in young and old B cells ruling out differential sensitivity of senescent B cells to stimulation with IL-4. It should be noted that following stimulation with autoreactive T cells or IL-4, there was also an increase in the percentage of Ia^+ B cells which was similar in both young and old mice (Table 4.5).

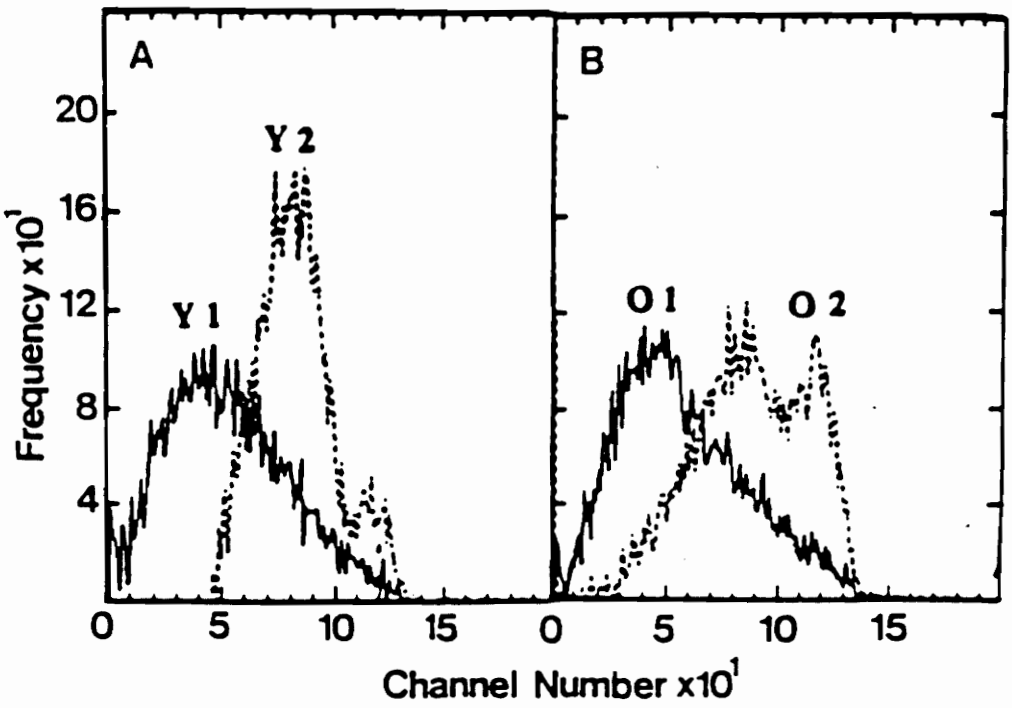

Figure 4.2. Induction of Ia expression on B cells by the autoreactive T cells: Cultures were set up as described in the legend to Fig.4.1. Ia expression was measured as detailed under Materials and Methods. The abscissa represents the green fluorescence on a linear scale and the ordinate represents the number of cells in a given fluorescence channel. Dotted lines (Y2, O2) represent Ia staining of B cells stimulated with T cells while the solid lines (Y1, O1) represent the staining of unstimulated B cells. (a) B cells from young mice; (b) old mice derived B cells.

Table 4.5. Percentage of Ia$^+$ cells, density of Ia expression, and size distribution of young and old B cells following stimulation with autoreactive T cells or IL-4[a]

Source of B cells	Stimulated with	Mean fluorescence intensity	Percentage of Ia$^+$ cells	Mean channel number (light scatter)[b]
Young	Medium	60.7 ± 24.2	49	14
Young	AutoD1.4	85.1 ± 17.2	64	21
Young	rIL-4	96.3 ± 19.5	82	20
Old	Medium	68.3 ± 25.7	50	13
Old	AutoD1.4	94.0 ± 21.5	64	21
Old	rIL-4	105.5 ± 18.0	81	22

[a]B cells from young or old mice were stimulated with auto D1.4 or rIL-4 and stained for Ia expression as described in the legends to Figs. 4.2 and 4.3. The stained cells were analyzed on a flow cytometer.

[b]Forward light scatter was taken as an approximate measure of the cell size.

Blastogenic response of B cells

Resting B cells are usually small in size and upon activation enlarge into blast cells before initiating DNA synthesis. The blastogenic response was measured by change in the forward angle light scatter of Ia^+ cells in our cultures using an EPICS flow cytometer. The results shown in Table 4.5 demonstrated that rIL-4 induced similar amounts of blastogenesis in B cells from young and old mice. Stimulation of B cells with the auto D1.4 T cells also yielded an identical blast transformation response.

Ability of B cells from old mice to stimulate the autoreactive T cells

Is the defective B cell response to the auto D1.4 T cell stimulation due to a defect in the stimulatory potential of the B cells from old mice? To address this question B cells from old and young mice were irradiated and were added to the cultures of the auto D1.4 cells and the proliferation of the auto D1.4 T cells was measured. The results summarized in Table 4.6 showed that B cells from old mice were as potent as those from young mice in inducing the proliferation of the self-Ia reactive T cells. In fact, the B cells from old animals induced better proliferative responses than the young mice derived B cells. This may be because the B cells from old mice express more cell surface Ia than their counterparts from young mice (our unpublished observations). These results suggested that the Ia antigens on old B cells appeared to be recognized by the autoreactive T cells in a normal fashion.

Table 4.6. Proliferative response of autoD1.4 T cell clone to stimulation with B cells from young and old mice

Auto D1.4 responder cells[a]	Source and no. of B cell stimulators	Proliferative response (cpm)[b]
+	-	18,134 ± 524
+	Young (7×10^5)	62,230 ± 1,237
+	Young (3.5×10^5)	45,495 ± 2,462
+	Old (7×10^5)	102,233 ± 1,811
+	Old (3.5×10^5)	83,345 ± 1,125

[a]Auto D1.4 responder cells were used at a concentration of 1×10^5 cells/well.

[b]The proliferative response was studied as described in Table 4.1.

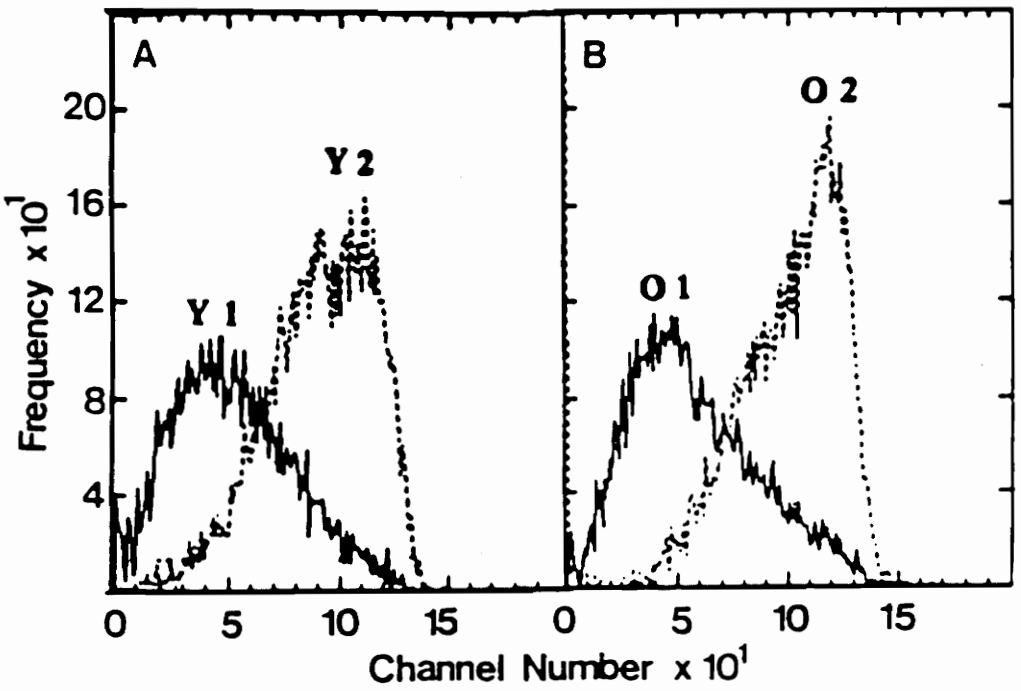

Figure 4.3. Responsiveness of B cells from young and old mice to stimulation with interleukin-4: Recombinant IL-4 was added at 100 units/ml to cultures of B cells from young and old mice. Staining with anti-Ia antibody was performed as described under Materials and Methods. Solid (Y1, O1) and dotted (Y2, O2) lines represent staining of B cells cultured without and with IL-4, respectively. (A) Young mice derived B cells; (B) old mice derived B cells.

Discussion

The origins of the hyporesponsive state of senescent individuals have been studied intensively for many years. Numbers of T cells decreased and helper T cell function declined with age leading to reductions in cell-mediated as well as humoral immunity (Makinodan and Kay, 1980; Segre and Smith, 1981). Also, it has been shown that the regulation might account for decreased humoral responses (Szewczuk and Campbell, 1980; Klinman, 1981; Goidl *et al*, 1983; Zharhary and Klinman, 1983, 1984, 1986). Although B cell numbers were not altered with age, immune responses to some thymic-independent antigens were reduced raising the possibility that B cells might exhibit intrinsic defects. To explain the decrease in antigen-specific B cell precursor frequency in the spleen but not in the bone marrow, Zharhary and Klinman also proposed that the majority of B cells in old mice may be intrinsically defective (Zharhary and Klinman, 1984, 1986, 1987). In this report, a system of T-dependent B cell activation has been established, and the ability of B cells from young and old mice to be activated by the same stimulus has been directly compared. An autoreactive T cell clone auto D1.4 was employed to induce polyclonal B cell proliferation and differentiation.

The results presented in this study demonstrated that B cells from young but not old mice proliferated well and differentiated into IgM-secreting cells. The possibility that the auto D1.4 T cells were receiving a stimulatory signal from young but not old mice derived B cells was ruled out. Thus, the B cells from senescent mice were as effective as the B cells from young mice in stimulating a proliferative response in the auto D1.4 T cells. Further, in all the B cell stimulation experiments, the autoreactive T cells were preactivated for 3 days with syngeneic

spleen cells from young mice and IL-2 before addition to cultures of purified B cells from young or old mice. Therefore, the failure of B cells from old mice to respond by proliferation appeared to be due to a defect intrinsic to B cells but not due to a failure of the T cells to provide the appropriate stimulus. The reduced differentiative response of old mouse B cells may be an additional deficiency or simply a secondary consequence of the B cell proliferative defect which limits clonal expansion, and therefore this aspect needs further investigation.

The current study does not directly address the possibility that two or more subsets of B cells are involved in the B cell response to stimulation with the autoreactive T cells. Then a deficient response by a major B cell subset and normal response by some B cells would also yield a result similar to that reported here. Although there is no direct evidence toward this idea, such a possibility can be directly tested by performing cell cycle analysis and determining the frequency of cells entering cell cycle. This view is consistent with an interpretation by Klinman and co-workers of their data from the clonal analysis of antigen-specific B cells in young and old mice (Zharhary and Klinman, 1987). Zharhary and Klinman found that in both young and old mice splenic B cells specific to various antigens had identical ability to produce a normal amount of antibody, multiplicity, and affinity although the frequencies of antigen-specific B cells in the spleen for TNP, NP and NNP but not for the hemagglutinin of the influenza virus or the hapten phosphorylcholine were decreased (Klinman, 1981; Zharhary and Klinman, 1984, 1986a, 1986b, 1987). Since the frequency of antigen-specific precursors in the Ig- cell compartment of bone marrow was unaltered or increased with age, it was suggested that a majority of splenic B cells in the old animals may be in an inactive state due to intrinsic defects or due to regulatory influences. However, other explanations such as decreased ability of the precursor B cells to emerge from the bone marrow into a mature B cell or increased turnover of aged animal's B cells are also consistent with these data (Zharhary and Klinman, 1987). If the antigen-specific B cells are heterogenous such that some respond near normally whereas a majority of the B cells are unresponsive, a significant decrease is expected in the polyclonal B cell proliferation and differentiation, which

is observed in our studies. The residual responses we observe may be due to the presence of a small number of normal B cells.

In young mice activation of resting B cells into the replication cycle is preceded by several biochemical and phenotypic changes which facilitate the movement of B cells from the G_0 into the G_1 and S states of the cell cycle (DeFranco *et al*, 1982; Cambier and Ransom, 1987; Bergstedt-Lindqvist *et al*, 1988). In the case ofantigen-specific cells, specific cell-cell interactions between T cells and B cells has been shown to be required for B cell activation and for the subsequent growth response to stimulation with lymphokines secreted by the activated T cells (Noelle *et al*, 1983). The cognate T-B contact results in induction of Ia expression in B cells and in the movement of resting G_0 B cells into the G_1 stage of the cell cycle by enlargement of the B cells (Bergstedt-Lindqvist *et al*, 1988; Swain and Dutton, 1987; Snow and Noelle, 1987; Noelle *et al*, 1983). These specific earlier changes in B cells have not been investigated previously for the autoreactive T cell-dependent B cell activation. The studies reported here demonstrated that similar changes in B cell surface Ia expression and in size were induced by the autoreactive T cells. Further, B cells from both young and old mice responded similarly in both Ia expression and blastogenic response. The B cells from old mice were deficient only in tritiated thymidine incorporation suggesting that they exhibited a specific defect in their ability to move from the G_1 state of the cell cycle into the S phase. We have also observed that B cells from young mice cultured with auto D1.4 T cells increase in number while B cells from old mice do not increase in number thereby supporting the concept that old B cells have a defect in the proliferative response (data not shown).

Although the direct effect of T-B interaction leads to similar early changes in the B cell phenotype, it is conceivable that the two types of B cells (from young and old mice) may differ in their ability to respond to the lymphokines that are secreted subsequently. Studies with young mice derived B cells showed that two lymphokines IL-4 and IL-5 secreted by the T_H2 type of T cells play a crucial role in the T-dependent induction of B cell proliferation and

differentiation (Paul and Ohara, 1987; Killar *et al*, 1987; Harriman and Strober, 1987; Swain, 1985; Harada *et al*, 1985). Of these two lymphokines, IL-4 acts on resting B cells by inducing an elevation in their surface Ia density as well as in their size. At a later stage in B cell activation, IL-4 augments IgG1 and IgE synthesis from LPS-stimulated B cells. The experiments on the direct effects of IL-4 on unstimulated B cells showed that B cells from both old and young mice responded equally well to IL-4 by increasing surface Ia expression and size. These results provide further evidence to the concept that B cells from old mice progress from the resting state into the G_1 state of the cell cycle but fail to undergo the transition into the S phase. The lymphokine IL-5 is thought to be important for induction of DNA synthesis and differentiation in B cells (Harriman and Strober, 1987; Swain, 1985; Harada *et al*, 1985). Therefore, it will be interesting to know if the B cells from old mice respond as well as their counterparts in young mice to stimulation with purified IL-5 and such studies are currently in progress.

In conclusion, the current study demonstrated that B cells from old mice have an intrinsic defect in their ability to proliferate when stimulated with an autoreactive T cell clone or autoreactive T cells.

Summary

B lymphocytes from aged mice were found to be defective in their ability to proliferate in response to stimulation with an autoreactive T cell clone D1.4. The differentiative response leading to antibody secretion was also impaired in the auto D1.4 T cell-stimulated B cells from old mice in comparison to similarly stimulated B cells from young mice. The B cells from old mice were competent in activating the autoreactive T cells such that the T cells were induced

to proliferate. The B cell defect appears to be restricted to a certain phase of B cell activation, since old mouse B cells responded to the auto D1.4 T cells by increasing cell surface Ia as well as size, but failed to incorporate tritiated thymidine. The responsiveness to interleukin-4 was found to be similar between B cells from young and old mice. It appeared that the B cells from old mice are specifically defective in progressing from the G_0 phase of the cell cycle into the S phase when stimulated with the auto D1.4 T cells.

Chapter 5: Macrophages but not B Cells from Aged Mice are Defective in Stimulating Autoreactive T cells In Vitro

Introduction

The syngeneic or autologous mixed-lymphocyte reaction (SMLR and AMLR)[3] has been defined as a T cell proliferative response to *in vitro* stimulation with syngeneic or autologous non-T cells bearing class II major histocompatibility complex (MHC) antigens (reviewed by Battisto and Ponzio, 1981; Weksler *et al*, 1981; Smith and Talal, 1982). The cells responding in the SMLR have been designated autoreactive T cells. Autoreactive T cell clones have been isolated earlier from normal, unimmunized mice and used to demonstrate that these cells perform several regulatory functions such as cytotoxic T lymphocyte differentiation, proliferation and differentiation of resting, unprimed B cells, participation in T - T cell interaction and enhancement of antigen-specific T cell proliferation (Nagarkatti *et al*, 1985a,

1985b; Udhayakumar *et al*, 1988; Nagarkatti *et al*, 1988; Nagarkatti et al, 1989). There is also ample evidence demonstrating that autoreactive T cells may perform other regulatory functions such as help (Chiorazzi *et al*, 1979; Damle *et al*, 1981), suppression (Clayberger *et al*, 1984) and cytotoxicity (Tomonari, 1980). These observations and the fact that SMLR is defective in patients with lymphoproliferative and autoimmune diseases (reviewed by Smith and Talal, 1982) suggest that autoreactive T cells may play an important role in maintaining normal immune system homeostasis.

Aging has been shown to be associated with several dysfunctions associated with the T and B lymphocytes and increased incidence of autoimmune disorders (Makinodan and Kay, 1980; Meredith and Walford, 1979; Chang *et al*, 1982; Dekruyff *et al*, 1980; Goidl *et al*, 1976; Klinman, 1981; Goidl *et al*, 1983). In an earlier study we observed that the autoreactive T cell induced T-T interaction was severely deficient in aged mice and this defect was associated with decreased autoreactive T cell response in aged mice (Udhayakumar *et al*, 1988). A decline in the SMLR with age has also been reported by others in humans and in normal or autoimmune-susceptible mice (Gutowski and Weksler, 1982; Canonica *et al*, 1985; Bocchieri and Smith, 1982; Smith and Pasternak, 1978; Moody *et al*, 1981; Fernandez and Michael MacSween, 1980; Gupta, 1984), although the defect in the individual cell type participating in the SMLR and the molecular basis of this defect is far from clear. Since it was observed earlier that the autoreactive T cells can respond to stimulation with both splenic B cells or splenic adherant cells (SAC) enriched for macrophages (Nagarkatti *et al*, 1985a, 1988, 1989), in the current study the SMLR response of purified $CD4^+$ T cells to stimulation with whole spleen cells, purified B cells and purified macrophages from aged mice was investigated to identify the defective cells and to further study the mechanism of the defect. The current study demonstrated that macrophages but not B cells from aged mice were defective in stimulating young autoreactive T cells. Furthermore, the defect in macrophages was not due to decreased Ia antigen expression or increased production of inhibitory factors such as prostaglandins but was probably due to decreased interleukin-1 production.

Materials and Methods

Mice

Adult female DBA/2 and CBA/ca mice, 8-12 weeks of age (young) and 22-24 months of age (aged or old) were obtained from the National Institute on Aging (Bethesda, MD).

Antisera and reagents

The following hybridoma cell lines were used in the present study : GK1.5 (anti-CD4), 3.155 (anti-CD8), MKD6 (anti-I-A^d), 14.4.4 (anti-I-E^d) , J11d (anti - B cell) and HO - 13.4.6 (anti-Thy 1.2.). All hybridoma cell lines were grown *in vitro* and the monoclonal antibodies were used as ammonium sulfate precipated fractions of the culture supernatants (Udhayakumar *et al*, 1988; Nagarkatti *et al*, 1988; Bocchieri and Smith, 1982). Fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG (Fab')2 fragment was purchased from Cappel Lab., Cooper Biomedical Inc., Melvern, PA. Recombinant interleukin 1 (r IL-1) was procured from Genzyme (Boston, MA). Five units/ml of rIL-1 was found to be the optimal dose of IL-1 that resulted in maximal proliferation in a thymocyte co-stimulation assay (Smith and Pasternak, 1978). This amount was therefore diluted in complete medium and added to the SMLR cultures. Indomethacin (SIGMA chemicals, St. Louis, MO) was dissolved in ethanol, diluted in complete medium and used at a concentration of 10^{-7} M.

Medium

The medium used consisted of RPMI 1640 (GIBCO Laboratories, Grand Island, NY) containing 2 mM L-glutamine, 50mM 2-mercaptoethanol, 40μg/ml gentamicin sulfate, 10 mM Hepes and 10% fetal bovine serum (GIBCO Laboratories), henceforth referred to as complete medium.

Purification of $CD4^+$ T cells

The $CD4^+$ T cells were purified as described in detail elsewhere (Udhayakumar *et al*, 1988; Nagarkatti *et al*, 1988). Briefly, a single-cell suspension was prepared from the spleen in complete medium. The erythrocytes were lysed with 0.83% ammonium chloride. The cells were then washed 3 times and passed over nylon wool columns to isolate enriched T cells. These cells were further depleted of contaminating B cells and accessory cells by treatment with a 1:10 dilution of a combination of J11d , anti-I-A^d and anti-IE^d antibodies plus complement (C). The $CD8^+$ T cells were next depleted by treatment with anti-CD8 antibodies plus C. The purity of the $CD4^+$ T cells isolated was usually greater than 95% as determined by flow cytometric analysis (Udhayakumar *et al*, 1988; Nagarkatti et al, 1988).

Preparation of splenic adherent cells (SAC)

SAC cells were isolated from the spleens as described elsewhere (Nagarkatti *et al*, 1985a). Briefly, the single-cell spleen suspensions were plated out on 100 x 20 mm tissue culture dishes (COSTAR, Cambridge, MA). After incubating the plates at 37° C for one-two hours in a 5% CO_2 incubator, the adherent cells were collected by forceful pipetting and used as

stimulators. Such SAC usually contained greater than 95% macrophages (Nagarkatti *et al*, 1985a).

Purification of B cells

Single-cell spleen suspensions were plated on 100 x 20 mm tissue culture dishes for one-two hours to remove macrophages. The non-adherent cells were collected and treated with anti-CD4, anti-CD8 and anti-Thy 1.2 antibodies for 30 minutes in cold, washed twice and incubated at 37° C with Complement (C) for 30-45 minutes. The resultant B cell suspension was washed 3 times and analysed by flow cytometry and was found to contain >95% surface Ig^+ cells (Nagarkatti *et al*, 1988; Nagarkatti *et al*, 1989).

Syngeneic mixed lymphocyte reaction (SMLR)

To study the proliferation of the $CD4^+$ T cells in the SMLR, varying numbers of $CD4^+$ T cells from young or aged DBA/2 mice were mixed with different concentrations of irradiated (2000 rad) syngeneic whole spleen cells, SAC or B cells in 96-microwell plates, in 0.2ml of medium (Nagarkatti *et al*, 1985; Udhayakumar et al, 1988; Nagarkatti *et al*, 1988). The assay was carried out in triplicate and after incubation at 37° C in a 5% CO_2/95% humidified air incubator for varying periods of time, 1μCi of ^{3}(H)thymidine was added to each well during the last 18 hr of culture. The cells were then collected onto glass-fiber filters with a semiautomatic cell harvester (Skatron, Sterling, VA), and the radioactivity incorporated was determined in a liquid scintillation counter (Betatrac 6895, TM Analytic, Inc., Elk Grove Village, IL).

Flow cytometric analysis

Single-color fluorescent staining for Ia antigen expression was performed as described elsewhere (Nagarkatti *et al*, 1989). Briefly, one million whole spleen cells, SAC or B cells were incubated with 100μl of 14.4.4 (anti-I-E^d) on ice for 30 minutes. The cells were washed twice and then incubated for a further 30 minutes with 1:10 diluted FITC-labeled anti-mouse IgG $(Fab')_2$ fragment. The cells were then washed 3 times and resuspended in 0.3 ml medium. During the entire staining procedure, the medium consisted of cold PBS containing 0.02% sodium azide. Controls for fluorescent staining consisted of cells which received normal mouse IgG in place of 14.4.4 (mouse IgG) and then incubated with FITC-conjugated anti-mouse IgG antibodies as described before. Fluorescence of 10,000 individual cells was measured using a flow cytometer (Epics V, model 752, Coulter Electronics, Hialeh, Fla.).

Statistical analysis

All assays were performed in triplicate and the means ± standard error (SE) of different groups were compared by the Student's *t* test. P values less than 0.05 were considered to be statistically significant. In most experiments the data were depicted as Δ c.p.m ± SE which represented the proliferative responses of $CD4^+$ T cells to syngeneic stimulators minus the sum of proliferative responses of the responder $CD4^+$ T cells alone and of irradiated stimulator cells alone (Nagarkatti *et al*, 1988; Nagarkatti *et al*, 1989).

Results

Aged $CD4^+$ T cells are defective in responding to stimulation with young or old splenic cells in the SMLR

Initial studies were carried out to investigate the effect of aging on $CD4^+$ T responder cells participating in the SMLR. To address this, young or old purified $CD4^+$ T cells from DBA/2 mice were cultured with whole spleen cells from young or old mice for 4 days. The data from several experiments have been summarized in Table 5.1. $CD4^+$ T cells from young mice demonstrated a good proliferative response when stimulated by either young or old spleen cells. In contrast, aged $CD4^+$ T cells showed a significantly decreased response to a similar stimulation. There was considerable variation in the defective responsiveness of aged $CD4^+$ T cells which ranged from 6-53% of the young $CD4^+$ T cell responses. In some of these experiments, addition of MK-D6 (anti-I-A^d) or 14.4.4 (anti-I-E^d) antibodies inhibited the $CD4^+$ T cell proliferation, thereby suggesting that the $CD4^+$ T cells were proliferating in response to stimulation with syngeneic Ia molecules (data not shown). To address whether the defect in the SMLR was restricted to only the DBA/2 strain or whether it was generalized, another strain, CBA/ca was also tested for its responsiveness in the SMLR. The data shown in Table 5.2 suggested that aged $CD4^+$ T cells from CBA/ca mice demonstrated decreased responsiveness when compared to the young $CD4^+$ T cells and furthermore, whole spleen cells, when used as stimulators did not demonstrate any defect but in fact stimulated significantly better than young whole spleen cells. Collectively, these data suggested that old $CD4^+$ T cells had an intrinsic defect in responding to syngeneic Ia molecules. In contrast, old spleen cells did not demonstrate any defect in their stimulatory activity and in some

experiments, they in fact, stimulated the autoreactive T cells better than the young spleen cells.

The defect in aged $CD4^+$ T cells is not due to a shift in the kinetics of the SMLR

Kinetics of the SMLR was studied to exclude the possibility that the defect observed with $CD4^+$ T cells from aged mice was not due to an early or delayed responsiveness by aged $CD4^+$ T cells in the SMLR. For this purpose, $CD4^+$ T cells from young or old mice (2×10^5) were cultured with irradiated young spleen cells (8×10^5) and the SMLR was studied on days 2,3,4 and 5 post-incubation. As shown in Fig.5.1, the young $CD4^+$ T cell response increased from 2-4 days, peaking on day 4 and declining by day 5. A similar kinetics of response was obtained using old $CD4^+$ T cells, although this response was markedly decreased on all days tested, when compared to the response of young $CD4^+$ T cells. Since the response for both young and old $CD4^+$ T cells peaked on day 4, in all subsequent experiments the SMLR was studied on this day.

Table 5.1. Age-induced defect in responder CD4$^+$ T cells but not in stimulator whole spleen cells in the SMLR of DBA/2 mice.

Expt	Source of whole spleen stimulator cells [b]	Cell Proliferation (Δ cpm ± S. E.)[a]	
		Young responders	Old responders
1	Young	44,914 ± 1,089	6,899 ± 427 (15)
	Old	46,812 ± 3,276	2,866 ± 162 (6)
2	Young	18,352 ± 1,364	9,884 ± 310 (53)
	Old	16,123 ± 417	5,436 ± 254 (33)
3	Young	24,187 ± 1,870	4,347 ± 937 (17)
	Old	37,335 ± 2,105	6,260 ± 513 (16)
4	Young	33,891 ± 1,514	12,638 ± 904 (37)
	Old	37,177 ± 1,248	12,463 ± 1,478 (33)
5	Young	14,462 ± 911	6,600 ± 417 (45)
	Old	17,360 ± 836	7,064 ± 243 (40)

[a]CD4$^+$ T cells from young or old mice were used as responder cells at 3-4x10^5/well. Cell proliferation was studied on day 4 by the uptake of ^{3}H-thymidine added during the last 18 hours of culture.

[b]Whole spleen cells from young or old mice were irradiated and used as stimulator cells at 10x10^5/well.

Table 5.2. Age-induced defect in responder CD4$^+$ T cells from CBA/ca mice

Source of whole spleen stimulator cells[b]	Cell Proliferation (Δ cpm ± S.E.)[a]		
	Young responders	Old responders[1]	Old responders[2]
Young	12,141 ± 1,168	3,124 ± 152 (25)[c]	3,014 ± 152 (24.8)
Old	23,338 ± 689	5,343 ± 350 (22.8)	2,801 ± 246 (12)

[a]CD4$^+$ T cells, isolated from a pool of 4 spleens from young CBA/ca mice, were used as responders at 6 x 10^5/well. Old CBA/ca mice were divided into 2 groups and in each group, spleen cells from 2 different mice were pooled and CD4$^+$ T cells were isolated.

[b]Whole spleen cells from young or old CBA/ca mice were irradiated and used as stimulator cells at 10 x 10^5/well.

[c]Numbers in parentheses represent percent of the control response obtained using young responders, considering the latter as 100%.

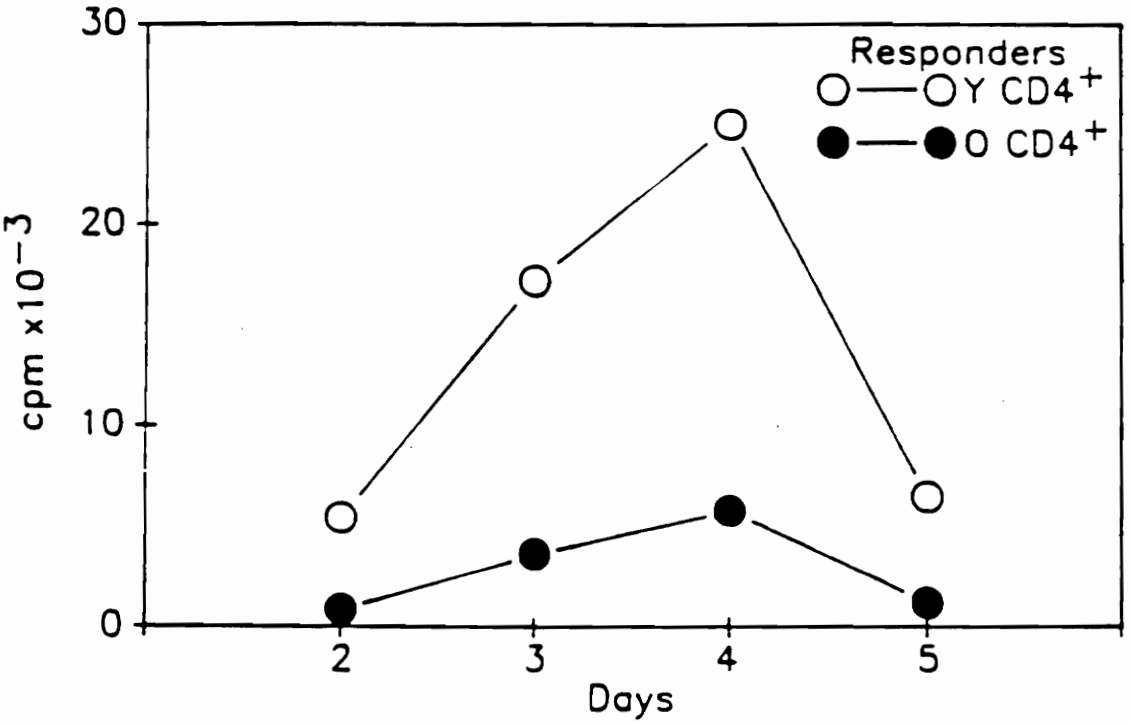

Figure 5.1. Kinetics of SMLR in young and aged mice: $CD4^+$ T cells from young (open circles) or old (closed circles) DBA/2 mice (2×10^5) were cultured with irradiated young spleen cells (8×10^5) and the SMLR was studied on different days post-incubation by pulsing the plates with 1 μCi of ^{3}H-thymidine during the last 18 hours of culture.

Purified SAC but not whole spleen cells from aged mice have defective stimulatory activity in the SMLR

Since Ia$^+$ macrophages constitute an important stimulatory cell in the SMLR (Nagarkatti *et al*, 1985a), and represent only a minor subpopulation in the splenic cells, further studies were conducted to investigate whether purified SAC from aged mice had normal stimulatory activity in the SMLR. In this experiment young CD4$^+$ T cells were used as responders and were stimulated with whole spleen cells or purified SAC from young or aged mice. The data depicted in Fig.5.2 showed that young and old spleen cells had similar stimulatory activity as seen before. Interestingly however, old SAC were markedly deficient in stimulating autoreactive T cells when compared to the young SAC. It should be noted that young SAC induced stronger SMLR than young whole spleen cells consistent with our earlier observation (Nagarkatti *et al*, 1985a, Udhaykumar *et al*, 1988). Taken together, these data suggested that using the same CD4$^+$ T cells as responders, a defect was demonstrable in old SAC but not when whole spleen cells were used as stimulator cells.

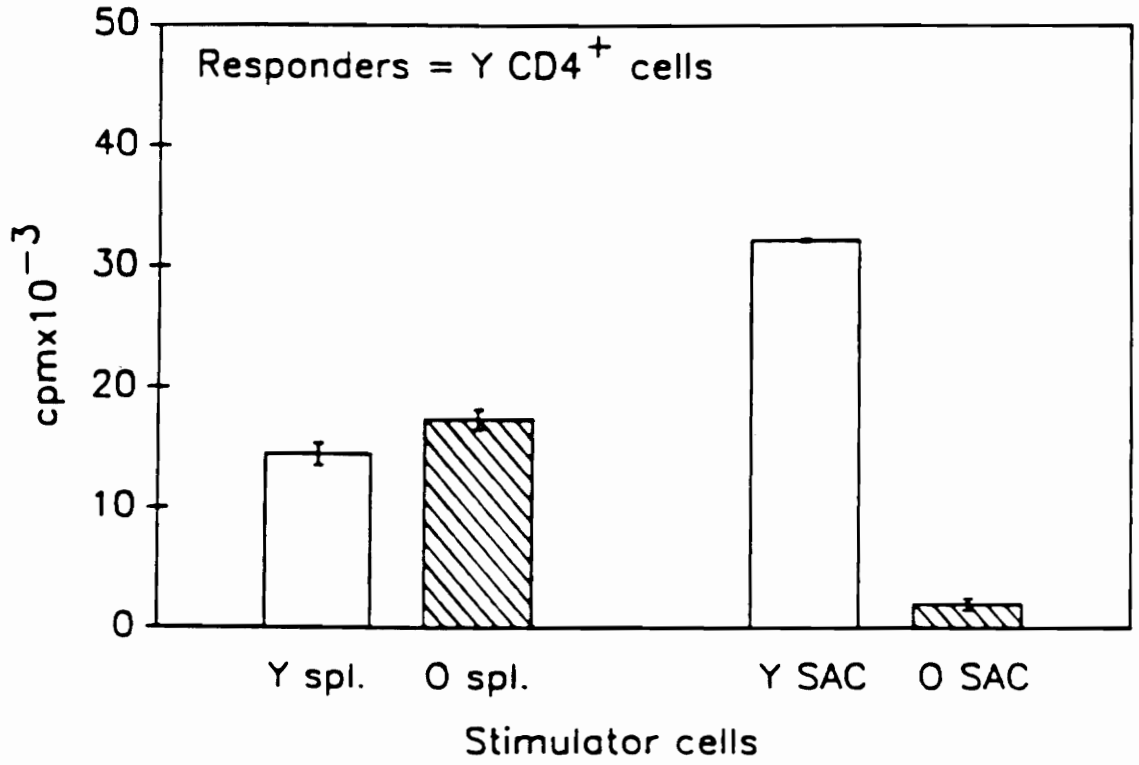

Figure 5.2. Purified SAC but not whole spleen cells from old DBA/2 mice have defective stimulatory activity in the SMLR: Young $CD4^+$ T cells (2×10^5) were used as responders and irradiated young (Y) or old (O) whole spleen cells or SAC (8×10^5) were used as stimulators, in the SMLR which was studied on day 4 as described before.

The defect in $CD4^+$ T cell responders and SAC stimulators from aged mice in the SMLR is consistently demonstrable at different responder : stimulator cell densities

To further confirm the defect in responder $CD4^+$ cells and stimulator SAC from aged mice, several experiments were carried out using different responder : stimulator cell ratios in the SMLR, of which three representative experiments are depicted in Table 5.3. In experiment 1, purified $CD4^+$ responder T cells (4 or 8 X 10^5) from young or old mice were mixed with 5 or 10 x 10^5 irradiated young stimulator spleen cells. The data demonstrated a consistent decrease in the proliferative response of aged $CD4^+$ T cells which was 12-17% of the control response of young $CD4^+$ responder T cells. In experiment 2, a constant number (4 x 10^5) of $CD4^+$ T cells from young or aged mice were mixed with two different doses (5 or 10 x 10^5) of young or old stimulator spleen cells. Once again, aged $CD4^+$ T cell responders demonstrated a consistent decrease in their proliferative response. In the same experiment, old spleen cells did not demonstrate any stimulatory defect. In experiment 3, a constant number (4 x 10^5) of responder $CD4^+$ T cells from young or old mice were mixed with 4 or 8 x 10^5 irradiated stimulator SAC from young or aged mice. The results showed that old $CD4^+$ T cells when compared to young $CD4^+$ T cells showed decreased reactivity to stimulation with 4 x 10^5 young SAC (53% of the control response) and to stimulation with 8 x 10^5 young SAC (48% of the control response). Old SAC were severely deficient in stimulating young and old $CD4^+$ T cells when used at 4 or 8 x 10^5/well and the SMLR generated was only 2-13% of the control response obtained using similar numbers of young SAC. In summary, these data suggested that at all responder : stimulator cell densities tested, old $CD4^+$ T cells demonstrated an intrinsic defect in responding to syngeneic Ia antigens and old SAC consistently failed to activate autoreactive T cells.

Table 5.3. Studies on stimulator cell defect in old DBA/2 mice using different responder : stimulator cell ratios in the SMLR[a]

Responders (cell numbers)		Stimulators (Source and cell numbers)		Cell proliferation of responder $CD4^+$ T cells[b] (Δ cpm ± S.E.)	
				Young	Old
Expt. 1	$4x10^5$	Young spleen	$5x10^5$	16,123 ± 966	2,764 ± 333 (17)[c]
	$8x10^5$	Young spleen	$5x10^5$	NT	6,239 ± 361
	$4x10^5$	Young spleen	$10x10^5$	44,914 ± 1,089	6,899 ± 427 (15)[c]
	$8x10^5$	Young spleen	$10x10^5$	62,888 ± 696	7,301 ± 452 (12)[c]
Expt. 2	$4x10^5$	Young spleen	$5x10^5$	16,123 ± 966	2,764 ± 333 (17)[c]
	$4x10^5$	Old spleen	$5x10^5$	30,947 ±916	2,919 ± 372 (9)[c]
	$4x10^5$	Young spleen	$10x10^5$	44,914 ± 1,089	6,899 ± 427 (15)[c]
	$4x10^5$	Old spleen	$10x10^5$	46,812 ± 3,276	2,866 ± 162 (6)[c]
Expt. 3	$4.x10^5$	Young SAC	$4x10^5$	24,377 ± 490	12,921 ± 924 (53)[c]
	$4x10^5$	Old SAC	$4x10^5$	461 ± 238 (2)[d]	1,715 ± 193 (13)[d]
	$4x10^5$	Young SAC	$8x10^5$	37,947 ± 4,098	18,218 ± 449 (48)[c]
	$4x10^5$	Old SAC	$8x10^5$	3,650 ± 297 (10)[d]	2,136 ± 202 (12)[d]

[a]Purified $CD4^+$ T cells ($4\text{-}8x10^5$) from young or old mice were stimulated with varying numbers of young spleen, young SAC or old SAC as described in Fig. 5.1.

[b]Cell-proliferation was studied on day 4 by the uptake of ^3H-thymidine added during the last 18 hours of culture.

[c]Numbers in parenthesis represent percent of the young response (control) considering the latter as 100%.

[d]Numbers in parenthesis represent percent of response obtained using young SAC as stimulator cells (control), considering the latter as 100%.

SAC but not B cells from old mice are defective in stimulating autoreactive T cells in the SMLR

Using an autoreactive T cell clone as the responder, we had earlier demonstrated that B cells from aged mice had no defect in stimulatory activity (Nagarkatti *et al*, 1989). In fact B cells from aged mice stimulated autoreactive T cells better than young B cells (Nagarkatti *et al*, 1989). To investigate whether this was demonstrable in primary SMLR cultures, comparative studies using SAC and B cells as stimulators were carried out. In this experiment, young $CD4^+$ T cells served as responders and SAC or B cells from young or old mice served as stimulator cells. The data depicted in Table 5.4 showed that as before, old SAC were less stimulatory and yielded only 62% of the control response. In contrast, old B cells were similar to young B cells in their stimulatory activity and induced 91% of the control response ($p > 0.05$).

Table 5.4. Splenic adherant cells but not purified B cells from old DBA/2 mice are defective in stimulating autoreactive T cells in the SMLR.

Responders (Young $CD4^+$ cells)	Stimulator cells (Source and Number)		Cell proliferation[a] (cpm ± S.E.)
$4x10^5$	—		9,796 ± 631
$4x10^5$	Young SAC	($8x10^5$)	111,568 ± 4,839
$4x10^5$	Old SAC	($8x10^5$)	69,679 ± 1,057 (62)[b]
$4x10^5$	Young B cells	($8x10^5$)	58,261 ± 3,176
$4x10^5$	Old B cells	($8x10^5$)	53,123 ± 2330 (91)[b]

[a]Cell proliferation was studied as described in Fig. 5.1 on day 4.

[b]Figures in parenthesis represent percent of the control response obtained using young stimulator cells considering the latter as 100%.

Defect in stimulatory activity of old SAC was not due to decreased Ia antigen expression

Since Ia antigens are the primary molecules involved in the stimulation of autoreactive T cells, we next investigated whether the decreased stimulatory activity of SAC was associated with decreased expression of Ia antigens by the SAC. To test this possibility, whole spleen cells, SAC and splenic nonadherant cells (as a source of B cells) were stained for the presence of Ia antigens using anti-I-E^d antibodies. The single-color fluorescence analysis depicted in figure 5.3 demonstrated that the percent Ia^+ cells in the whole spleen cell population in young mice (30%) was similar to that found in old mice (33%). Also, the intensity of Ia staining represented as mean channel number was slightly increased in old whole spleen cells when compared to the young whole spleen cells (mean fluorescence intensity was 73 for young spleen cells and 81 for old spleen cells). Similar results were obtained using nonadherant spleen cells as a source of B cells or using SAC. Together, these data suggested that the decreased stimulatory activity of aged SAC was neither due to a decrease in Ia^+ SAC nor due to any decreased density of Ia antigen expression.

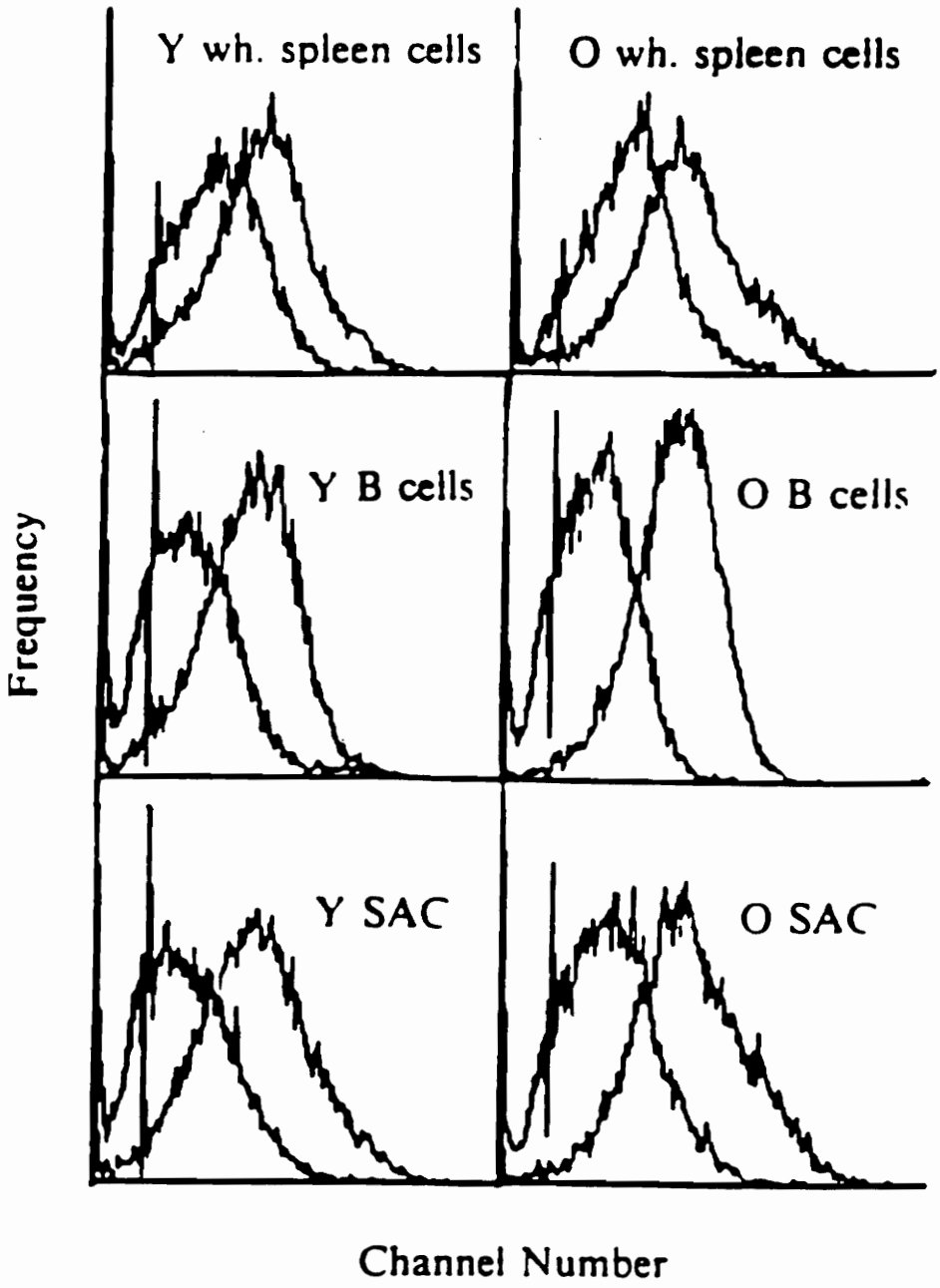

Figure 5.3. Analysis of Ia antigen expression by flow cytometry: Young (Y) or old (O) whole spleen cells, non-adherant spleen cells as a source of B cells or SAC from DBA/2 mice were stained with monoclonal anti-IE^d antibodies followed by FITC-conjugated anti-mouse IgG and the cells were analyzed by flow cytometry. The first profile, close to the Y axis, depicts the negative control in which cells were incubated with normal mouse IgG followed by FITC-conjugated anti-mouse IgG and the second profile depicts cells positively stained with anti-IE^d antibodies.

Demonstration of suppressor SAC in aged mice using cell mixing assay in vitro

Cell-mixing experiments were performed to investigate whether old SAC contained any suppressor macrophages which may actively inhibit the SMLR. $CD4^+$ T cells from young mice (2×10^5) were mixed with 4×10^5 SAC from young mice, in the absence or presence of 1, 2 or 4×10^5 irradiated SAC from young or old mice (referred to as regulatory cells). The data shown in figure 5.4 suggested that $CD4^+$ T cells stimulated with 4×10^5 young SAC gave a good proliferative response in the absence of any regulatory cells added (closed bars, 43,717 $\pm$ 2936). This response, following addition of 1, 2 or 4×10^5 irradiated young regulatory SAC, increased in a dose-dependent manner (open bars). In contrast, addition of similar numbers of old regulatory SAC caused a dose-dependent suppression of the SMLR, with maximum suppression demonstrable using 4×10^5 old SAC (hatched bars). These data clearly suggested that old SAC were not only deficient in stimulating autoreactive T cells but also could inhibit the SMLR induced by young SAC.

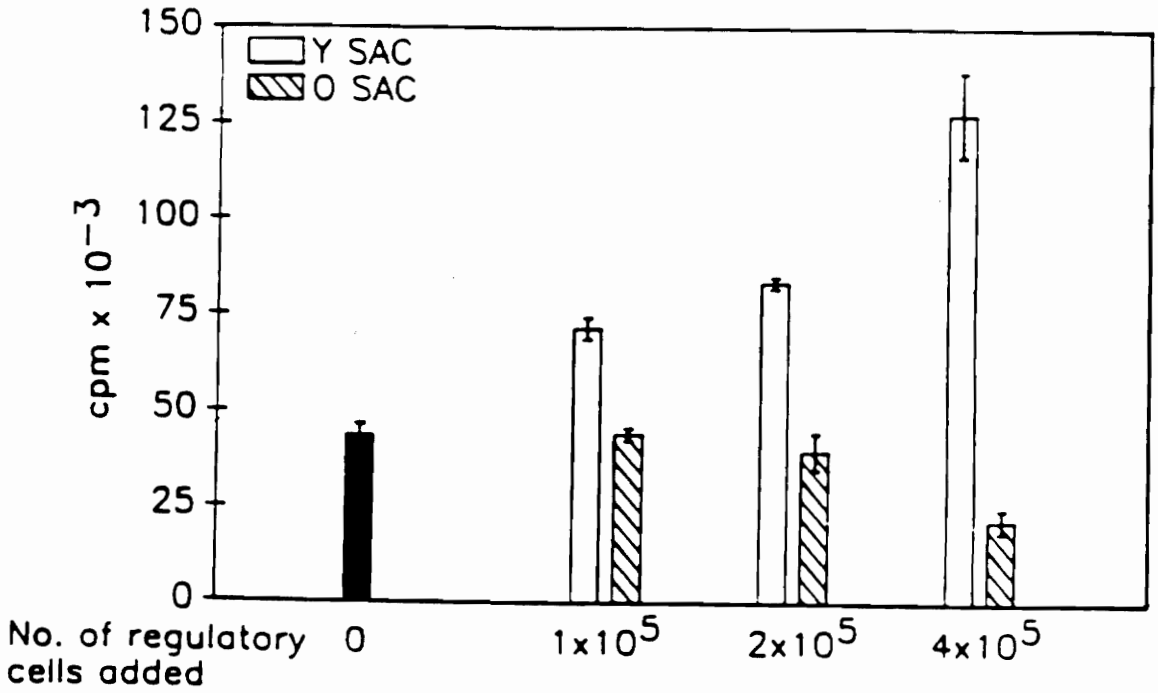

Figure 5.4. Demonstration of suppressor SAC in aged DBA/2 mice using cell-mixing experiments: $CD4^+$ T cells from young mice (2 x 10^5) were mixed with 4 x 10^5 SAC from young mice in the absence or presence of 1, 2 or 4 x 10^5 irradiated SAC from young (Y) or old (O) mice, referred to as regulatory cells. The SMLR was studied on day 4 as described in Fig.5.1.

Decreased stimulatory activity of old SAC is not due to increased prostaglandin production but due to decreased IL-1 synthesis

Several studies have demonstrated that macrophages mediate suppression by producing increased amounts of prostaglandins (Rocklin *et al*, 1983; Aune, 1987). It is also known that addition of indomethacin to the cultures which inhibits prostaglandin synthesis can prevent the inhibitory activities of macrophages and thereby increase the *in vitro* response (Inamizu *et al*, 1985). In the present study it was therefore investigated whether the inability of the macrophages from old mice to stimulate autoreactive T cells was due to suppressor macrophages producing prostaglandins. The data shown in Table 5.5 suggested that addition of indomethacin had no significant effect on the defective stimulatory activity of old SAC. In contrast, addition of exogenous IL-1 which is an important factor produced by the macrophages and involved in T cell interaction, could completely reconstitute the SMLR response stimulated by old SAC. In fact, addition of IL-1 increased the responses of both young and old SAC-mediated SMLR to a similar extent.

Table 5.5. Effect of indomethacin and IL-1 on the SMLR in DBA/2 mice

Responders (Young $CD4^+$)	Stimulators (4×10^5)	Indomethacin (10^{-7} M)	IL-1 (5 u/ml)	Cell proliferation[a] (Δ cpm $\pm$ S.E.)
+	Young SAC	-	-	34,820 ± 2,744
+	Old SAC	-	-	18,474 ± 2,461 (53)[b]
+	Young SAC	+	-	33,405 ± 2,260
+	Old SAC	+	-	17,552 ± 1,669 (52)
+	Young SAC	-	+	95,578 ± 354 (274)
+	Old SAC	-	+	90,291 ± 284 (259)

[a]Purified $CD4^+$ T cells from young mice ($4x10^5$) were stimulated with $4x10^5$ young or old SAC stimulators for 4 days in the SMLR as described in Fig. 5.1.

[b]Numbers in parentheses represent percent of the response obtained using young SAC considering the latter as 100%.

Correlation between inhibitory activity and decreased IL-1 production by old SAC in the SMLR

We next investigated whether the suppressor activity of aged SAC and decreased IL-1 production were related or independent events which contributed to decreased stimulatory activity of old SAC. The data has been summarized in Table 5.6. In Experiment 1, old SAC were found to be less stimulatory when compared to young SAC. Addition of IL-1 could completely reconstitute the defective stimulation by aged SAC, in agreement with earlier data presented in Table 5.5. Experiment 2 of Table 5.5 shows data from a cell-mixing experiment similar to that described in figure 5.4. In this experiment, 2×10^5 young $CD4^+$ T cell responders were stimulated with irradiated young SAC (4×10^5), in the presence of 4×10^5 irradiated young SAC or old SAC (referred to as regulatory stimulator cells). As before, addition of old SAC inhibited the SMLR response to 56% of the control response. Interestingly, addition of exogenous IL-1 abolished the inhibitory effect and the response increased to 99% of the control response. These data suggested that IL-1 could not only correct the defective stimulatory activity of old SAC but also that such SAC failed to exert suppressor activity in the presence of exogenous IL-1 in the SMLR.

Table 5.6. Addition of exogenous IL-1 abolishes the suppressor activity of the aged SAC in the SMLR of DBA/2 mice

Expt.	Young $CD4^+$ Responders (2×10^5)	Stimulators (4×10^5)	Regulatory Stimulators (4×10^5)	IL-1 (5 u/ml)	Cell proliferation[a] (Δ c.p.m.)
1	+	Young SAC	-	-	22,393 ± 1,439
	+	Old SAC	-	-	13,264 ± 1,382 (59)[b]
	+	Young SAC	-	+	35,365 ± 4,627 (157)
	+	Old SAC	-	+	32,585 ± 2,871 (145)
2	+	Young SAC	Young SAC	-	26,102 ± 259
	+	Young SAC	Old SAC	-	14,857 ± 113 (56)
	+	Young SAC	Old SAC	+	25,866 ± 264 (99)

[a]Cell proliferation was studied on day 4 as described in Fig. 5.1.

[b]Numbers in parentheses represent percent of the control young response considering the latter as 100%.

Discussion

Several studies have demonstrated that aging is associated with a variety of defects in T and B lymphocytes and in other cells such as macrophages and NK cells (Udhayakumar *et al*, 1988; Nagarkatti et al, 1989; Makinodan and Kay, 1980; Meredith and Walford, 1979; Chang et al, 1982; Dekruyff *et al*, 1980; Goidl *et al*, 1976; Klinman, 1981; Goidl et al, 1983; Doria *et al*, 1986). In the present study the effect of aging on the SMLR was investigated, in which $CD4^+$ autoreactive T cells respond to Ia^+ non-T cells resulting in the generation of important immuno-regulatory functions (Battisto and Ponzio, 1981; Weksler et al, 1981; Smith and Talal, 1982; Nagarkatti *et al*, 1985a, 1985b; Udhayakumar *et al*, 1988; Nagarkatti *et al*, 1988; Nagarkatti et al, 1989; Chiorazzi *et al*, 1979; Damle *et al*, 1981; Clayberger *et al*, 1984; Tomonari, 1980). It was observed that purified $CD4^+$ T cells from aged mice clearly demonstrated a defect in responding to syngeneic Ia antigens expressed on young spleen cells. During these studies, an interesting observation was made suggesting that whole spleen cells or B cells from aged mice when used as stimulator cells elicited a normal SMLR response by young $CD4^+$ T cell responders. However, SAC enriched for macrophages were deficient in their stimulatory activity in the SMLR. The defect in the SAC of aged mice was not due to decreased Ia expression nor was it due to increased prostaglandin production, but was found to be associated with a defective IL-1 mediated regulation by the aged SAC. Thus addition of exogenous IL-1 completely restored the defective stimulatory activity of aged SAC.

Gutowski and Weksler (1982) have earlier shown that the SMLR in Balb/c mice declines with age. In these studies whole T cells were used as responders and whole spleen cells as stimulators and the authors found a defect in both the responder and stimulator cells in 24 month old mice. The deficient SMLR was further characterized to be due to suppressor cell activity. It should be noted that our earlier studies have demonstrated that normal $CD8^+$ T cell population has endogenous suppressor cells which down-regulate autoreactive T cells

(Nagarkatti et al, 1988). Thus, depletion of $CD8^+$ T cells *in vitro* or *in vivo* enhanced the autoreactive T cell responses (Nagarkatti *et al*, 1988). Therefore, in earlier studies which used unseparated nylon wool non-adherant T cells as responders (Gutowski and Weksler, 1982), the defect in the SMLR may have been contributed by increased endogenous suppressor activity by the $CD8^+$ T cells present in the responder population. In the present study, since purified $CD4^+$ T cells were used as responders and since such cells from old mice were deficient in the SMLR response, irrespective of whether spleen cells, B cells or macrophages from young or old mice were used, the current study has unequivocally demonstrated for the first time that there is an intrinsic defect in the ability of responder autoreactive T cells from aged mice.

In the present study, it was observed that although SAC demonstrated decreased stimulatory activity, whole spleen cells when used as stimulators had normal activity. This may be because Ia^+ macrophages in the SAC constitute a small percentage of the whole spleen cell population and therefore a decrease in stimulatory activity in such cells may not be reflected in the whole spleen cell population. Alternatively, since B cells from aged mice were normal or in fact showed enhanced stimulatory activity to autoreactive T cells (Nagarkatti *et al*, 1989), the B cells may compensate for the deficiency of the aged macrophages. For example, it was demonstrated in this study that, addition of exogenous IL-1 could reconstitute the stimulatory activity of SAC. Thus, B cell-derived factors may help to reconstitute the defective macrophage-mediated stimulation when whole spleens were used. In this context it is interesting to note that Gupta (1984) recently reported that the AMLR induced by macrophages were defective in aging humans when compared to young individuals. In contrast, the AMLR induced by B cells was higher in aging subjects when compared to the young group. These data are consistent with the present study in the murine system.

In the present study, three major mechanisms to explain why old SAC were deficient in stimulating autoreactive T cells were investigated: a) old SAC expressed decreased number or density of Ia antigens b) the cells were producing prostaglandins and mediating active

suppression and c) SAC cells were deficient in IL-1 production. It was observed that whole spleen, B cells and macrophages were expressing normal or slightly increased levels of Ia antigen expression. Similar observations were made by others while investigating the resident peritoneal macrophage population using antibodies directed against Ia antigens plus complement that there was indeed higher expression of I-A molecules in macrophages of aged mice when compared to the young (Doria *et al*, 1986). These authors however noted that the peritoneal macrophages from aged mice were more efficient in presenting a foreign antigen to the specific T cells when compared to the young mice. This difference was found not to be due to increased Ia expression (Doria *et al*, 1986). It should be noted however that in these studies, splenic macrophages were not compared. Also, stimulation of antigen-specific T cells by macrophages involves foreign antigen processing whereas stimulation of autoreactive T cells by macrophages does not involve any antigen-processing step (Finnegan *et al*, 1985).

Macrophages have been known to produce several non-specific suppressor factors including prostaglandins (Goodwin and Ceuppens, 1983;Aune, 1987). Earlier studies have demonstrated that aged peritoneal macrophages do not produce increased prostaglandins and therefore this may not account for decreased IL-1 synthesis (Aune, 1987). In the present study, it was noted that addition of indomethacin, a specific inhibitor of prostaglandin synthesis failed to correct the defective stimulatory activity of the macrophages, thereby suggesting that the macrophage defect was not associated with increased prostaglandin synthesis. Since macrophages are known to produce several different non-specific inhibitory molecules (Aune, 1987) and since cell-mixing experiments in the present study indicated the presence of suppressor macrophages, the possibility still remains that aging may induce suppressor macrophages producing inhibitory factors other than prostaglandins.

Interleukin -1 is an important T cell activating factor produced by the antigen-presenting cells (Mizel, 1982). Recent studies have demonstrated that IL-1 production by peritoneal

macrophages from old mice was reduced when compared to that produced by young mice (Inamizu *et al*, 1985). The fact that exogenous IL-1 can reconstitute the old macrophage stimulatory defect, support the above studies and further suggest that such IL-1 deficient macrophages are also demonstrable in the spleens of aged mice.

It was also interesting to observe in the present study that the suppressor effect of the aged macrophages was abolished following addition of exogenous IL-1. It can be speculated that in cell-mixing experiments, the defective old SAC were competing with the young SAC for binding to autoreactive T cell receptors and since they were deficient in IL-1 production, they caused an apparent suppression of the young SAC-induced SMLR. Thus, provision of excess of IL-1 exogenously allowed the activation of $CD4^+$ T cells that had bound the old SAC and therefore restored the SMLR responses to normal levels. An alternate possibility was that the aged macrophages were producing a suppressor factor inhibiting the IL-1 activity and therefore addition of excess of exogenous IL-1 could restore the SMLR. Suppressor factors released from certain human glioblastoma cell lines have been shown to inhibit T cell proliferation mediated by IL-1 (Schwyzer and Fontana, 1985). Inhibitors of IL-1 activity have also been detected in human urine (Liao *et al*, 1985). Thus growth-inhibitory factors directed against a specific growth factor exist (Aune, 1987). It is also possible that the exogenous IL-1 added in the cultures in the present study, actively inhibited the activity of the suppressor factor produced by aged macrophages. Evidence in support of this was recently shown by Aune (1985) who reported that growth factors such as IL-1 and IL-2 could prevent or reverse the inhibition in antibody secretion caused by soluble suppressor factors.

In contrast to the macrophages, purified B cells from old mice, in the present study were not defective in stimulating autoreactive T cells in primary SMLR. This was consistent with our earlier observation that aged B cells could stimulate an autoreactive T cell clone very well, in fact better than normal B cells (Nagarkatti *et al*, 1989). Although B cells from aged mice had normal stimulatory activity, they failed to proliferate and differentiate when stimulated by

autoreactive T cell clones (Nagarkatti *et al*, 1989). In these studies the aged B cells were normal in their initial stages of activation inasmuch as they demonstrated increased Ia expression as well as size comparable to young B cells. Also, the responsiveness to IL-4 was found to be similar in both young and old B cells. These data suggested that B cells from aged mice were specifically defective in progressing from G_0 phase of cell cycle into S phase, when stimulated with autoreactive T cells (Nagarkatti *et al*, 1989). These observations collectively suggested that B cells from aged mice can express normal levels of Ia antigens and may secrete optimum levels of growth factors necessary for T cell activation, although such cells may have an intrinsic defect in responding to the factors produced by the activated autoreactive T helper cells such as interleukin-5 and interleukin-6. Further studies are essential to delineate whether the B cells involved in the activation of autoreactive T cells and those that respond to such T cells, represent the same population of B cells or whether they belong to different subpopulations.

In the present study, an intrinsic defect in the responsiveness of autoreactive $CD4^+$ T cells from aged mice consistent with earlier observations (Udhayakumar *et al*, 1988) was also observed. This defect was demonstrable whether whole spleen cells, purified B cells or SAC were used. Earlier studies have shown that this T cell defect could be due to defective T cell responsiveness to IL-2 (Canonica *et al*, 1985). In the autoimmune susceptible NZB strain of mice, the decline in the SMLR has been shown to be due to decreased lymphokine production, excessive T cell activation and decreased precursor frequencies of autoreactive T cells (Bocchieri and Smith, 1982). Earlier studies have demonstrated that the defective T cell responsiveness from aged mice cannot be corrected by addition of exogenous IL-2, IL-4 or a combination of IL-2 and IL-4 (Udhayakumar *et al*, 1988). In summary, in the present study autoreactive T cells were used to demonstrate a unique defect in macrophages but not in B cells from aged mice. Whether a similar differential defect exists in the antigen-processing abilities of macrophages and B cells remains an interesting issue to be resolved.

Summary

In the present study the effect of aging on the capacity of Ia$^+$ cells to stimulate autoreactive T cells in the SMLR was investigated. Using young CD4$^+$ T cells as responders, it was observed that unseparated whole spleen cells from aged mice had normal stimulatory activity comparable to that of young spleen cells. Interestingly, however, when purified splenic adherent cells (SAC) enriched for macrophages or splenic B cells were used as stimulators, aged SAC but not aged B cells were found to be defective in stimulating autoreactive T cells. This defect in aged SAC was not due to decreased expression of Ia antigens since the percentage of Ia$^+$ SAC and density of Ia antigen expression was similar in both young and old mice. Also, the B cells from aged mice expressed normal levels of Ia antigens. Aged SAC, when mixed with young SAC could also actively suppress the normal SMLR. However, this suppression was not due to increased prostaglandin production but was found to be associated with interleukin-1 (IL-1) regulation, inasmuch as addition of exogenous IL-1 could completely reconstitute the defective stimulatory activity of aged SAC and also abolished the suppressor activity of the SAC. Aged mice also demonstrated an intrinsic defect in the CD4$^+$ T cells responding in the SMLR. Together, the current study on the SMLR demonstrates an age-related defect in responder autoreactive T cells and in stimulator splenic macrophages but not in the stimulatory activity of B cells.

Chapter 6: Expression of the J11d Marker on Peripheral T Lymphocytes of MRL-lpr/lpr Mice

Introduction

MRL-*lpr/lpr* (hereafter called *lpr*) mice spontaneously develop massive lymphadenopathy and an autoimmune lupus-like disease. The lymphadenopathy results from the expansion of a unique population of Thy-1$^+$ cells which are CD4$^-$ and CD8$^-$, referred to as double-negative cells, the exact nature of which is not clear (Wofsy *et al*, 1984). The antibody J11d has been shown to define a differentiation antigen found on cortical thymocytes but not on mature and functional peripheral CD4$^+$ or CD8$^+$ T cells (Bruce *et al*, 1981; Crispe and Bevan, 1987). Recent studies have demonstrated that the J11d marker was precisely excluded even from those subpopulations of thymocytes that functioned like peripheral T cells (Crispe and Bevan, 1987; Ceredig *et al*, 1987). Normal adult thymus has been shown to constitute about 3% double-negative T cells, while, in a 15-day old fetus , ~90% of the thymocytes are double-negative (Ceredig *et al*, 1983;Kisielow *et al*, 1984; Mathieson and Fowlkes, 1984). Furthermore, double-negative thymocytes contain J11d$^+$ and J11d$^-$ subpopulations (Crispe

and Bevan, 1987) of which the J11d$^+$ thymocytes expressed CD3$\gamma\delta$ type of TCR (Crispe *et al*, 1987); the J11d$^-$ thymocytes expressed the CD3$\alpha\beta$ TCR (Ceredig *et al*, 1987; Crispe *et al*, 1987).

Since the predominant cells in the peripheral lymph nodes of *lpr* mice are phenotypically double-negative, it was considered interesting to investigate whether these double-negative cells expressed the J11d marker. The current study demonstrates for the first time that a significant proportion of the peripheral T cells of lpr mice expressed J11d antigen, present normally on immature thymocytes, and absent on mature and functional thymocytes and peripheral T cells.

Materials and Methods

Mice

MRL-lpr/lpr (lpr) and MRL-+/+ (+/+) mice, originally procured from the Jackson Laboratory, Bar Harbor, Maine, were bred in our animal facilities in a sterile environment (Animal storage Isolators, Nuaire, Plymouth, MN) and only female mice were used in this study.

Reagents

The mAb GK 1.5 (rat IgG2b, anti-CD4[L3T4]), 3.155 (rat IgM, anti-CD8[Lyt 2]) and J11d (rat IgM), were grown *in vitro* and used as antibody concentrated from the culture supernatants (Nagarkatti *et al*, 1985a, 1985b). FITC-conjugated anti-rat IgG and FITC-conjugated anti-rat IgM

were procured from Cappel Laboratories, Cooper Biomedical Inc., Melvern, PA. Affinity purified anti-CD8(Lyt 2) (53-6.7, rat IgG), biotin-coupled anti-Thy 1.2 (30-H12), and PE-avidin (PE) conjugates were purchased from Becton Dickinson, Mountain View, CA. Highly purified rat Ig was obtained from Jackson Immuno Research Laboratories, West Grove, PA.

Purification of double-negative T cells

Single cell suspension of lymph nodes were prepared using a laboratory blender (Stomacher, Tekmar Company, Cincinnati, Ohio) in RPMI 1640 supplemented with 10% FCS (GIBCO Laboratories, Grand Island, NY), 10mM HEPES, 1mM glutamine and 40 μg/ml gentamicin sulfate. The red cells were lysed by treatment with 0.83% ammonium chloride and the cells were washed twice. The LN cells were suspended at approximately $2x10^7$/ml in anti-CD4 (GK 1.5) and anti-CD8 (3.155) antibody containing supernatants, diluted 1:10. The cells were incubated for 45 min. on ice, followed by centrifugation, washing and resuspension in 1:10 diluted rabbit complement (Low-Tox, Cederlane Lab., Hornby, Canada). The cells were next incubated at 37°C for 45 min and washed twice before use. The viable cells were counted by the trypan blue dye exclusion test.

Staining of cells

Two-color fluorescent staining for J11d and Thy1 or J11d and CD8 markers was performed as follows. One million cells were incubated with 1:10 diluted J11d on ice for 30 min. The cells were washed once followed by staining with 1:10 diluted FITC-labeled anti-rat IgM. After an additional 30 min incubation on ice, the cells were washed twice and were treated with biotin-coupled anti-Thy 1 or biotin-coupled anti-CD8 antibodies (1:25, final dilution). The cells were incubated on ice for 15 min followed by one washing and staining with phycoerythrin (1:5

final dilution). The cells were washed finally twice and resuspended in 0.5 ml medium. During the entire staining procedure, the medium consisted of cold PBS containing 0.02% sodium azide. For two-color staining with J11d and anti-CD4 antibodies, a similar protocol was adapted as above, except that after J11d treatment, the cells were incubated with phycoerythrin-conjugated anti-CD4 antibodies (1:20 final dilution).

Double staining for CD4 and CD8 markers was performed by incubating cells with anti-CD4 antibodies, followed by FITC-anti-rat IgG, biotin-coupled anti-CD8, and, finally, staining with PE-avidin. The other steps were similar to those described before. Because all antibodies used for double-staining were of rat IgG or rat IgM origin, controls for fluorescent staining were set in which cells received normal rat Ig (25 μg/ml) or medium (in some experiments) in the place of antibodies against different markers. The cells were next stained with FITC-conjugated anti-rat IgG or IgM, followed by staining with PE-Avidin. In some experiments, single-color staining was performed to detect the presence of CD4 and CD8 markers. The cells were similarly treated with anti-CD4 or anti-CD8 antibodies followed by staining with FITC-conjugated anti-rat IgG antibodies.

Flow cytometry

Fluorescence of individual cells was measured by flow cytometry. The analysis was performed on an Epics V, Model 752 (Coulter Electronics, Hialeah, Fla.), laser flow cytometer and cell sorter. The four parameters per cell that were studied were: forward angle light scatter, 90° light scatter, green fluorescence (GFL) and red fluorescence (RFL). Laser excitation was normally 300 mW at 488 nm using a 5-W Innova 90 Argon Laser (Coherent Inc., Palo Alto, Ca.). All data collection and analysis were done with the multiparameter data acquisition and display system and the Easy 88 microcomputer analysis system of Coulter Electronics. Forward angle light scatter was collected by linear integral; 90° light scatter, GFL

and RFL were collected by log integral. Histograms showing cell number per channel as a function of fluorescence were collected at a resolution of 256 channels and gated on forward angle/90° light scatter and GFL/RFL dual parameter histograms of 64 X 64 channels resolution, defining the cell population of interest. The total number of counts for each was 10,000 per sample.

Results

Simultaneous expression of J11d and Thy-1 on peripheral T cells of lpr mice

To investigate the expression of J11d antigen on peripheral T cells in lpr mice, lymph node cells from 3, 8 and 20 week-old mice were individually screened for the simultaneous expression of J11d and Thy 1 Ag. As a control, the LN cells of +/+ mice were similarly screened. In each experiment, negative controls for individual cell suspensions were included which consisted of cells incubated with the medium or with normal rat Ig instead of J11d and anti-Thy-1 antibodies and stained with FITC-conjugated anti-rat IgG and PE-avidin. Both of the controls showed similar results with >98% of the cells being negative for RFL and GFL. Thymocytes of +/+ mice were included in each experiment as a positive control for the simultaneous expression of J11d and Thy-1.

A representative experiment of the two color FACS analysis of different cell populations has been shown in Figs. 6.1 and 6.2 and the data of several similar experiments has been summarized in Table 6.1. The upper left quadrant of Figure 6.2 depicts cells with RFL (Thy-1$^+$), the upper right quadrant depicts RFL and GFL (Thy 1$^+$ J11d$^+$), the lower left quadrant depicts

cells negative for both RFL and GFL, and the lower right quadrant shows cells positive for GFL ($J11d^+$) only. Figure 6.1A demonstrates a representative negative control in which thymocytes were screened for background fluorescence by staining with FITC and PE conjugates only. The thymocytes did not demonstrate background fluorescence and 99% of the cells were negative for GFL and RFL. By using LN cells, the background fluorescence was slightly higher, although very negligible (0.04 to 2.82%). These values were subtracted from the experimental values to obtain the percentage of cells stained specifically for J11d and Thy 1. The thymocytes from +/+ mice contained ~87% $J11d^+$ Thy 1^+ cells (Table 6.1, Fig. 6.1B), whereas the +/+ LN cells had <3% of such cells (Table 6.1, Fig. 6.1C). These data were consistent with the previous studies that J11d is expressed by all immature cortical thymocytes but not by mature peripheral T cells (Bruce *et al*, 1981; Crispe and Bevan, 1987).

Similar studies in *lpr* mice led to interesting observations. The LN cells of 3-to 4-wk-old *lpr* mice had <4% $J11d^+$ Thy 1^+ cells (Fig. 6.2A, Table 6.1), similar to the LN cells of +/+ mice (Fig. 6.1C, Table 6.1). However, with increasing age and coinciding with the appearance of lymphadenopathy (8 to 10 wk), the *lpr* LN cells expressed ~34% $J11d^+$ Thy 1^+ cells (Table 6.1, Fig. 6.2B). Once the lymphadenopathy had set in, the percentage of cells expressing J11d and Thy 1 remained constant and did not increase further with age (Table 6.1, Fig. 6.2C). Eight- to 10-wk-old lpr mice had two subpopulations of $J11d^+$ cells, one expressing high density Thy-1 and the other expressing lower levels of Thy-1 (Fig. 6.2B). In contrast, 20- to 24-wk-old lpr mice demonstrated a single population (Fig. 6.2C). As the above studies did not exclude the possibility that J11d was also expressed on $CD4^+$ or $CD8^+$ peripheral T cells of *lpr* mice, further investigations were carried out by simultaneous staining of cells with the J11d and anti-CD4 or J11d and anti-CD8 antibodies. The data from three individual experiments have been summarized in Table 6.2 and a representative experiment is depicted in Fig.6.3. In all of these experiments, the negative controls demonstrated that >96% of the cells were negative for both GFL and RFL. A representative negative control, depicted in Fig.6.3A, demonstrated that 97% of the cells were negative for both GFL and RFL (see legend to Fig.6.3

for detailed analysis). In these experiments, a positive control was also included, consisting of thymocytes from MRL +/+ mice (FACS analysis not shown).

The LN of 20-wk-old *lpr* mice, when simultaneously stained for CD4 and CD8 markers, demonstrated ~11% $CD4^+CD8^-$ cells and ~3% $CD4^-CD8^+$ cells. The remaining 86% of the cells were DN (Table 6.2, Fig.6.3D). When the same cells were stained for J11d and CD8 markers, it was observed that ~0.5% of the cells were $J11d^+CD8^+$, whereas ~30% were $J11d^+CD8^-$ (Table 6.2, Fig.3B). Similar results were obtained when the cells were examined for the simultaneous expression of J11d and CD4 Ag. Only 0.7% of the cells were $J11d^+CD4^+$, whereas ~30% of the cells were $J11d^+CD4^-$ (Table 6.2, Fig.6.3C). Therefore, these studies clearly demonstrated that the J11d Ag was absent on $CD4^+$ or $CD8^+$ peripheral T cells and that it was expressed only by the abnormal DN T cell population.

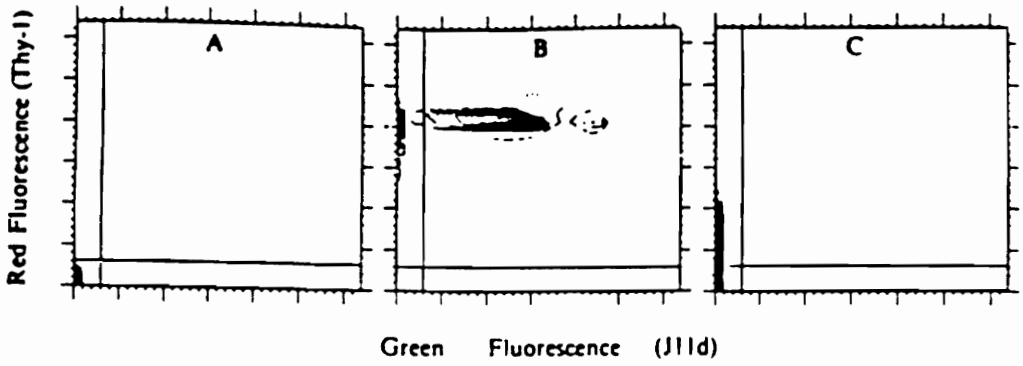

Figure 6.1. Two-color FACS analysis of J11d$^+$ Thy 1$^+$ cells in +/+ mice: Thymocytes (B) or lymph node cells (C) from +/+ mice were stained with J11d, FITC-labeled anti-rat IgM, biotin-coupled anti-Thy 1 and phycoerythrin as described in Materials and Methods. A represents the negative control in which thymocytes were stained as described above except that they did not receive J11d and anti-Thy 1 antibodies. The upper left quadrant depicts cells stained for Thy-1 alone, the upper right quadrant, Thy 1 and J11d, the lower left quadrant, cells negative for both Thy-1 and J11d and the lower right quadrant, cells stained for J11d alone. In A (negative control), 99% of the cells were negative for RFL and GFL. In B (thymocytes), 16% were Thy-1$^+$, 83% J11d$^+$ Thy-1$^+$, and 1% J11d$^+$. In C (LN cells) 76% were Thy-1$^+$, 2% were J11d$^+$ Thy-1$^+$, 20% were negative for both markers and 2% were positive for J11d alone.

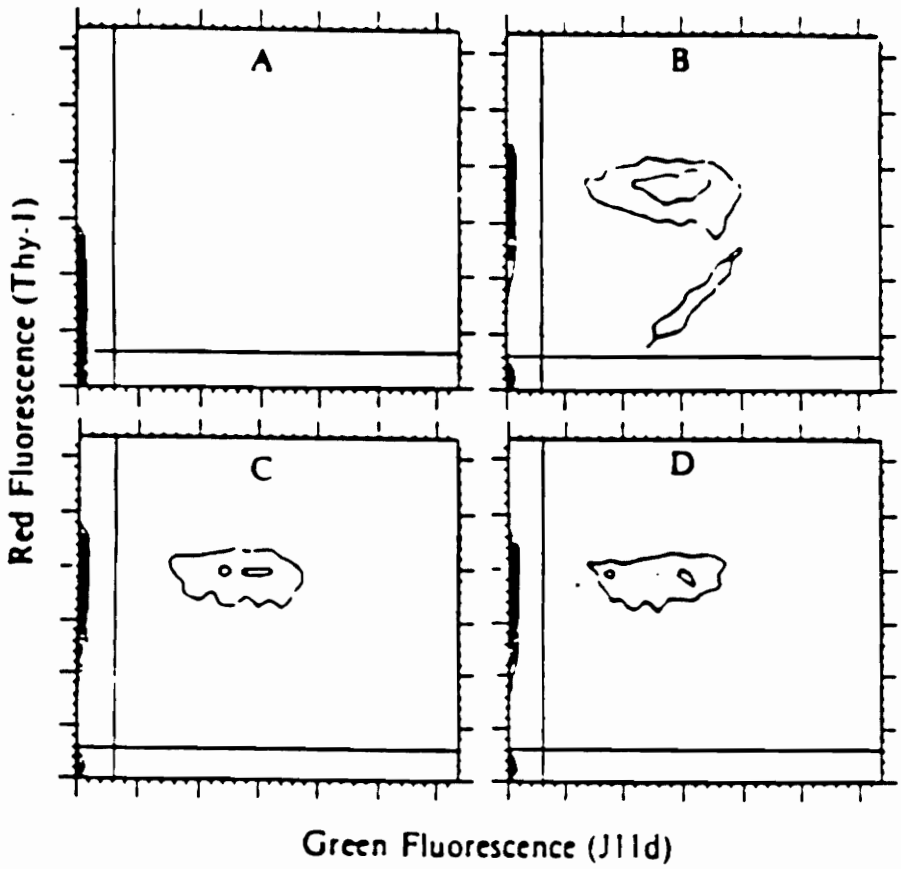

Figure 6.2. Two-color FACS analysis of J11d$^+$ Thy-1$^+$ cells in lpr mice: LN cells from 3- (A), 8- (B) and 20-wk old (C) lpr mice were screened as described in Figure 6.1. In D, LN cells from 20-wk-old lpr mice were further purified into double-negative cells and similarly screened. The LN cells from 3-wk-old lpr mice had 84% Thy 1$^+$ cells, 4% J11d$^+$ Thy 1$^+$ cells, 10% negative for both markers and 2% for J11d alone (A). The LN cells from 8-wk-old lpr mice contained 55% Thy 1$^+$ cells and 40% J11d$^-$ Thy 1$^+$ cells, 2% were negative for both markers and 3% for J11d alone (B). The 20-wk-old lpr mice had 70% Thy 1$^+$ cells, 27% J11d$^+$ Thy-1$^+$ cells, 1% negative for both markers and 2% positive for J11d alone (C). The double-negative cells of 20-wk-old lpr mice contained 69% Thy 1$^+$ cells, 27% J11d$^+$ Thy-1$^-$ cells, 1% negative for both markers and 3% positive for J11d alone.

Table 6.1. Expression of J11d and Thy 1 Ag on the LN cells of +/+ and lpr mice of different ages[a]

Strain of mice and organ		Age (wk)	No. of expts. J11d + Thy 1	% of Cells Expressing
+/+	Thymus	8	4	87 ± 4
+/+	LN	4-10	5	2.8 ± 1
lpr	LN	3-4	4	4.1 ± 1
lpr	LN	8-10	6	34 ± 5.7
lpr	LN	20-24	6	35 ± 4.3

[a]Two-color FACS analysis of J11d$^+$ Thy 1$^+$ cells was carried out as described in the legend to Figure 6.1. Values represent mean ± S.E. of 4-6 experiments carried out with individual mice.

J11d is expressed by CD4$^-$ CD8$^-$ cells from lpr mice

Whether the J11d antigen is expressed by the abnormal double-negative T cells of the *lpr* mice was next investigated. The double-negative T cells were purified by treatment of 20- to 24-wk-old *lpr* LN cells with anti-CD4 and anti-CD8 antibodies and complement. Such cells were >99% Thy 1$^+$ and CD4$^-$ CD8$^-$ (data not shown). When these double-negative T cells were screened, we observed that ~27% of these cells were J11d$^+$ Thy 1$^+$ (Fig. 6.2D), thereby suggesting that J11d was expressed by the double-negative T cells of older mice.

Increased number of double-negative J11d$^+$ Thy 1$^+$ cells in the thymus of old lpr mice

To determine whether the double-negative cells in old *lpr* mice migrated from the thymus, the number of such cells in the thymus was enumerated in *lpr* and +/+ mice using the double staining technique with anti-CD4 and anti-CD8 antibodies. Four-week-old *lpr* mice and +/+ control mice had ~4% double-negative Thy 1$^+$ cells in the thymus. In contrast, 20-wk-old *lpr* mice had ~31% double-negative Thy 1$^+$ cells, of which ~51% were J11d$^+$ (FACS analysis not shown). These data suggested that, with increasing age, the double-negative T cell population increases in the thymus and that the J11d$^+$ Thy1$^+$ double-negative peripheral cells in old *lpr* mice may originate from a similar population found in the thymus.

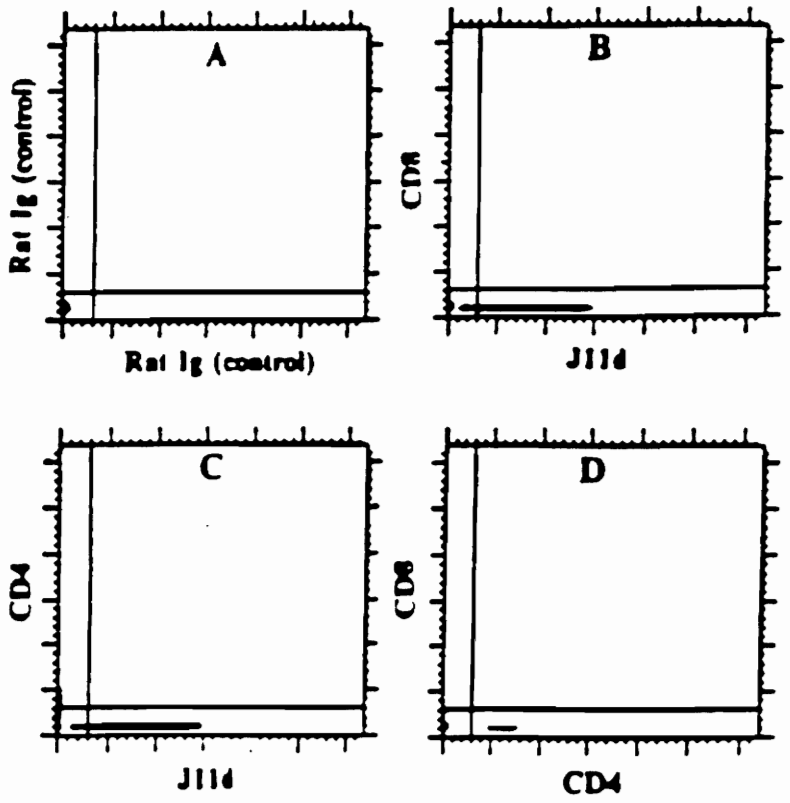

Figure 6.3. Two-color FACS analysis of J11d$^+$ CD8$^+$, J11d$^+$ CD4$^+$, or CD4$^+$ CD8$^+$ cells in the LN of 24-wk-old lpr mice: Cells were stained and screened as described in Figure 6.1. Negative controls consisted of cells incubated with normal rat Ig in the place of J11d, anti-CD4, or anti-CD8 antibodies. The cells were stained with FITC-anti-rat IgM (or IgG) and PE-avidin. A shows a representative negative control in which the cells were incubated first with normal rat Ig followed by FITC-anti-rat IgM and PE-avidin and demonstrated 97% of the cells to be negative for both GFL and RFL, 2% positive for GFL alone, 1% positive for both RFL and GFL, and 0% for RFL alone. When the LN cells were treated with J11d and anti-CD8 antibodies (B), 4% were found to be CD8$^+$ J11d$^-$, 0% CD8$^+$ J11d$^+$, 67% CD8$^-$ J11d$^-$ and 29% were CD8$^-$ J11d$^+$. Double-staining with J11d and anti-CD4 antibodies revealed (C) cells which were 9% CD4$^+$ J11d$^-$, 0% CD4$^+$ J11d$^+$, 62% CD4$^-$ J11d$^-$ and 29% CD4$^-$ J11d$^+$. When stained with anti-CD4 and anti-CD8 antibodies (D), 2% were found to be CD8$^+$ CD4$^-$, 0% CD8$^+$ CD4$^+$, 87% CD8$^-$ CD4$^-$, and 11% CD8$^-$ CD4$^+$.

Table 6.2. Subpopulations of LN stained simultaneously for J11d and CD8, or CD4 and CD8 markers[a]

	% of Cells Simultaneously Expressing Two Surface Markers			
	$CD4^+$	$CD4^-$	$CD8^+$	$CD8^-$
$J11d^+$	0.7 ± 1.2	30 ± 1.7	0.5 ± 1	30.7 ± 2.3
$J11d^-$	9 ± 2	60.3 ± 2.5	3.3 ± 1.1	65.5 ± 1
$CD4^+$			0.7 ± 0.6	11 ± 1
$CD4^-$			2.7 ± 1.5	86 ± 2.6

[a]LN of 20-wk-old *lpr* mice were analyzed for surface markers by using two-color immunofluorescent staining technique as described in Figure 6.3. The data represent the percentage mean ± SD of three individual experiments.

Discussion

In the present study it was demonstrated that a significant number of double-negative T cells accumulating in the peripheral LN of MRL-*lpr/lpr* mice express the J11d marker which has been characterized previously to be present only on immature thymocytes and absent on mature peripheral T cells and on thymocytes which function like peripheral T cells (Bruce *et al*, 1981; Crispe and Bevan, 1987). These studies support the hypothesis that the abnormal double-negative T cells in the *lpr* mouse may be originating from the double-negative subpopulation of thymocytes and migrating abnormally to the periphery prior to complete maturation (Budd *et al*, 1987).

Subpopulations within the thymocytes have been studied using three mAb, B2A2, M1/69 and J11d. These antibodies have been shown to share many common properties (reviewed by Crispe and Bevan, 1987). Recently, with the use of M1/69, which detects a heat-stable Ag (HSA), Davidson *et al* (1986) reported that a significant number of old *lpr* LN cells were heat-stable Ag^+. To our knowledge, the presence of B2A2 on the *lpr* double-negative LN cells has not been reported. It should be noted that although the three antibodies share many properties (Crispe and Bevan, 1987; Springer *et al*, 1978; Symington and Hakomori, 1984), there are several important differences in the expression of these markers. For example, based on their presence or absence, B2A2 and M1/69 subdivide $CD8^-CD4^+$ thymocytes, whereas J11d is absent on these cells. Also, based on the presence of J11d or B2A2, the $CD8^+CD4^-$ thymocytes can be divided into 2 groups, whereas M1/69 fails to divide this population (Crispe and Bevan, 1987). Furthermore, whereas the HSA recognized by M1/69 was proposed to be carbohydrate in nature (Springer *et al*, 1978), the J11d Ag was resistant to treatment intended to degrade carbohydrates (Symington and Hakomori, 1984). Thus, Crispe and Bevan (1987) suggested that the distinct reactivity patterns of B2A2, M1/69 and J11d might be due to the fact that these antibodies recognize different determinants on

carbohydrate side-chains of various glycosylated cell surface molecules. It will be interesting to investigate further the reactivity pattern of *lpr* double-negative T cells with the three antibodies.

Several recent studies have shown, first, that the double-negative cells in the *lpr* mice belong to the T cell lineage and not to the B cell lineage although these cells express several B cell surface characteristics. The abnormal cells rearrange and transcribe the T cell receptor genes while their Ig genes are in the germline configuration (Davidson *et al*, 1986; Nemazee *et al*, 1985; Mountz *et al*, 1986; Morse *et al*, 1982). Second, the disease of *lpr* mice is thymic dependent, inasmuch as neonatal thymectomy prevents the development of lymphadenopathy and the other aspects of the disorder, whereas regrafting restores the same functions (Theofilopoulos *et al*, 1981; Steinberg *et al*, 1980).

Normal adult thymus has been shown to constitute about 3% double-negative T cells (similar to the observation in this study) and in a 15-day old fetus, 90% of the thymocytes are double-negative (Ceredig *et al*, 1983; Kisielow *et al*, 1984; Mathieson and Fowlkes, 1984). Budd *et al* (1987) recently reported an inconsistent increase in the double-negative T cells in the thymus of older *lpr* mice. The percentage of these cells was 11 ± 7%, in contrast to our study, in which we observed a consistently higher number of double-negative cells (41.8 ± 6.2%). Although the reason for this discrepancy is not clear, it could be due to the fact that in the former study, C57BL/6 *lpr* mice were used and differences have been reported, for example, in CBA/H and C57BL/6 strains, in the subpopulations of double-negative T cells in the thymus (Ceredig et al, 1987). Also, Morse *et al* (1982) have earlier reported that the abnormal T cells present in the LN were also detected in the thymus of two of the *lpr*-bearing strains, SJL and MRL, but not in other *lpr*-bearing strains such as C57BL/6. In addition, age and sex of the mice used and variation in the severity of the autoimmune disease in individual mice may also have contributed to the inconsistent increase in the double-negative T cell population in the thymus. It should be noted that, while removing the thymus from old *lpr* mice, extreme care was taken to avoid harvesting lymph nodes present in close proximity to the thymus. Thus,

it was unlikely that the increase in the double-negative T cell population in the present study was caused by LN T cells contaminating the thymocyte cell preparation. The second possibility was that the double-negative LN cells were actively infiltrating the thymus. Although this possibility cannot be ruled out, it is less likely, because such an infiltration could lead to thymic enlargement. In contrast, the thymus in old *lpr* mice is atrophied.

In the present study, it was observed that 8- to 10-wk-old *lpr* mice had two subpopulations of J11d$^+$ T cells, the first population expressing high density Thy-1 Ag (Thy-1 high) and the second population expressing low density Thy-1 Ag (Thy-1 low). In contrast, 20- to 24-wk *lpr* mice had only one population of J11d$^+$ Thy-1 high cells. Although it is difficult to speculate on the two subpopulations of J11d$^+$ T cells, it is interesting to note that Nakano *et al* (1987) recently identified two subpopulations of double-negative thymocytes, one bearing Thy-1 low and the other one bearing Thy-1 high, and found that Thy-1 low double-negative cells were the intrathymic T progenitor cells. It is interesting, therefore to investigate further whether the J11d$^+$ Thy-1 low and J11d$^+$ Thy-1 high double-negative cells found initially in the LN of *lpr* mice correspond to the Thy-1 high and Thy-1 low subpopulations of the double-negative thymocytes. In the present study, it was also observed that the majority of the LN T cells of +/+ mice or 3-wk-old *lpr* mice demonstrated Thy-1 low when compared with the majority of +/+ or *lpr* thymocytes which expressed Thy-1 high. Interestingly, however, with the onset of lymphadenopathy and J11d$^+$ T cells in the periphery, the LN cells of old *lpr* mice expressed Thy-1 high similar to the thymocytes. These data suggested either that the LN T cells of *lpr* mice, with age, switch from expressing Thy-1 low to Thy-1 high or, more likely, that the double negative Thy-1$^+$ cells in older *lpr* mice were migrating from the thymus before complete maturation and therefore expressed Thy-1 high similar to the thymocytes. It should be noted that these findings contradict those of an earlier study in which it was observed that the LN cells of old C57BL/6 *lpr* mice expressed Thy-1 low Ag with use of a mAb, AT83 (Budd *et al*, 1985). Further studies are essential to delineate whether the differences found in the two studies are due to mouse strain differences or due to the use of different mAb, inasmuch as

monoclonal antibodies directed against Thy-1 Ag have been shown earlier to have differential binding reactivities and T cell-activating properties (Gunter *et al*, 1984; Ledbetter and Herzenberg, 1979).

Recently, several phenotypic, functional and molecular genetic analyses of the double-negative cells have provided evidence to suggest that the peripheral abnormal T cells of *lpr* mice are similar to the cells of the double-negative thymocyte subpopulation: 1) Using the combination of phorbol ester and calcium ionophore, Katagiri *et al* (1987), studied the proliferation, IL-2-expression and secretion by the double-negative T cells of *lpr* mice and found them to be similar to the responsiveness of the double-negative thymocytes found in the 16-day fetus. 2) The double-negative T cells of *lpr* mice bear similarities to the early double-negative thymocytes with regard to the TCR gene expression (Mountz *et al*, 1986; Budd *et al*, 1985). Furthermore, a novel population of double-negative thymocytes bearing $\alpha\beta$ TCR and expressing predominantly the products of a single Vβ gene family (Vβ8) has recently been characterized and suggested to be similar to the double-negative cells of *lpr* mice (Fowlkes *et al*, 1987). 3) Cultured double-negative T cells from the *lpr* LN or from the normal thymus demonstrate cytotoxicity toward a wide variety of targets (Budd *et al*, 1986). 4) The double-negative thymocytes contain J11d$^+$ and J11d$^-$ subpopulations (Crispe and Bevan, 1987), similar to the observation in this study, with the double-negative cells from the *lpr* mice. Furthermore, it has been demonstrated that the double-negative J11d$^+$ thymocytes which act as progenitor T cells, express CD3 $\gamma\delta$ type TCR but not the CD3 $\alpha\beta$ type (Crispe *et al*, 1987). In contrast, the double-negative J11d$^-$ thymocytes expressed CD3 $\alpha\beta$ T cell receptor (Ceredig *et al*, 1987; Crispe *et al*, 1987). It should be noted that the peripheral T cells of *lpr* mice have been shown to rearrange and express the α and β T cell receptor genes as well as the γ gene (Davidson *et al*, 1986; Nemazee *et al*, 1985; Mountz *et al*, 1986; Miescher *et al*, 1987). Therefore, it would be interesting to investigate whether the J11d$^+$ and J11d$^-$ double-negative T cell subpopulations present in the periphery of *lpr* mice similarly express a distinct set of TCR genes.

Recent studies on the T cell lineages in the *lpr* thymus have suggested that the massive enlargement of *lpr* LN may result from abnormal intrathymic differentiation rather than abnormal proliferation of T cells in the LN (Budd *et al*, 1987). The double-negative Pgp-1$^+$ B220$^+$ thymocytes in *lpr* mice were suggested to migrate to the LN unchanged before differentiation (Budd *et al*, 1987). Alternatively, in *lpr* mice the abnormal T cell precursors may migrate first to the periphery and subsequently proliferate to give rise to the double-negative T cells. This possibility is less likely because 98% of the *lpr* LN DN T cells are in G0/G1 phase of the cell cycle (Budd *et al*, 1985), and there is no evidence of rapidly expanding precursor cells (Budd *et al*, 1987). The third possibility is that the abnormal *lpr* DN T cells may result from the failure of intrathymic elimination of undifferentiated T cells, inasmuch as it is known that, daily, a majority of the normal thymocytes die intrathymically (Scollay *et al*, 1984). The possibility that, in *lpr* mice, the DN thymocytes fail to differentiate further and, instead, migrate abnormally to the periphery is a new perspective to explain the cause of lymphadenopathy and needs further investigation.

Summary

MRL-*lpr/lpr* (*lpr*) mice spontaneously develop massive lymphadenopathy resulting from the expansion of a unique population of Thy-1$^+$ cells which are CD4$^-$ and CD8$^-$ (DN) and the nature of which is not clear. The antibody J11d has been shown to define a differentiation antigen found on immature thymocytes but not on mature and functional peripheral CD4$^+$ or CD8$^+$ T cells. To analyze the possible relationship between the *lpr* double-negative T cells and the thymocytes, the simultaneous expression of J11d and Thy 1 Ag on the DN *lpr* lymph node cells was investigated using a two-color immunofluorescent staining technique. It was observed that *lpr* mice at 3 to 4 weeks of age, before the onset of lymphadenopathy, did not have significant numbers ($<4\%$) of J11d$^+$ T cells in the periphery, similar to the number found

in the control MRL-+/+ mice. However, with increasing age of approximately 8-10 weeks and coinciding with the appearance of lymphadenopathy, a significant number (~ 35%) of J11d$^+$ Thy-1$^+$ cells started appearing in the periphery of *lpr* mice and was maintained until the mice died at 20-24 weeks of age. The J11d$^+$ T cells in the *lpr* mice belonged to the abnormal DN T cell pool inasmuch as J11d$^+$ CD4$^+$ or J11d$^+$ CD8$^+$ cells were absent in the lymph nodes of 20-wk-old *lpr* mice. Furthermore, 20-wk-old *lpr* mice demonstrated increased numbers (~ 41%) of double-negative T cells in the thymus, a significant proportion of which were J11d$^+$. In contrast, the 20-wk-old +/+ mice or 4-wk-old *lpr* mice had only 4% double- negative T cells in the thymus. The present study suggests that a significant number of peripheral double-negative T cells of *lpr* mice bear the immature thymic differentiation Ag J11d. The possibility that the accumulation of double-negative T cells results from abnormal peripheralization of double-negative J11d$^+$ thymocytes, before complete differentiation into CD4$^+$ or CD8$^+$ T cells, is discussed.

Chapter 7. Autoreactive T Cell Clones Isolated from Normal and Autoimmune-susceptible Mice Exhibit Lymphokine Secretory and Functional Properties of Both T_H1 and T_H2 Cells

Introduction

Recent studies using murine T helper (T_H) cell clones have suggested the existence of two major mutually exclusive subpopulations of T_H cells. One subset called T_H1 produces IL-2 and IFN-γ but not IL-4 after mitogenic or antigen-specific stimulation. In contrast, the second subset called T_H2 produces IL-4 and IL-5 but not IL-2 when activated (reviewed by Mosmann and Coffman, 1989). Also, T_H1 subset is believed to play a major role in inducing cell-mediated immune responses such as delayed-type hypersensitivity reaction, while T_H2 cells may play a major role in humoral immunity by providing help to B cells (Mosmann and

Coffman, 1989). Furthermore, T_H1 cells use IL-2 and T_H2 cells use IL-4 as autocrine growth factors *in vitro* . However, following antigenic stimulation, both types of clones respond to the proliferative effects of both IL-2 and IL-4 (Fernandez-Botran *et al*, 1988).

Autoreactive T cells are T_H cells which respond to self-Ia antigens (reviewed by Weksler *et al*, 1981). These cells have been shown to perform several important immunoregulatory functions (Weksler *et al*, 1981). $CD4^+$ autoreactive T cell clones isolated from normal mice have been shown to induce other naive $CD4^+$ T cells to proliferate and help in the differentiation of cytotoxic T lymphocyte (CTL) precursors, as well as induce both proliferation and differentiation of B cells (Nagarkatti *et al*, 1985a, 1985b; Udhayakumar *et al*, 1988; Nagarkatti *et al*, 1989; Finnegan *et al*, 1984; Sano *et al*, 1987). Recently, in our laboratory, an autoreactive T cell clone was isolated from autoimmune MRL-*lpr/lpr* mice and found to exhibit similar helper functions. In the present study, whether these autoreactive T_H clones belong to T_H1 or T_H2 subset was investigated. Interestingly, it was observed that the autoreactive T_H clones exhibited the lymphokine secretory properties of both T_H1 and T_H2, inasmuch as they produced IL-2, IFN-γ and IL-4. Furthermore, the autoreactive T cell clones could activate both B cells and macrophages.

Materials and Methods

Mice

MRL-*lpr/lpr* and MRL-+/+ mice originally procured from the Jackson Laboratory, Bar Harbor, ME were bred in our animal facilities (Seth *et al*, 1988). DBA/2, C3H and Balb/c mice were purchased from the National Cancer Institute, Bethesda, MD. B10.K, B10.RKD1, B10.MBR, A.TFR1, B10.RKB and B10.A(5R) strains were generously provided by Dr. Chella David (Mayo Clinic, Rochester, MN). The strain B10.BASR1 was kindly provided by Dr. D. C. Shreffler (Washington University School of Medicine, St. Louis, MO).

Cell lines used in the bioassays

The IL-2/IL-4 responsive T cell line, HT-2 was kindly provided by Dr. Ellen S. Vitetta, University of Texas Southwestern Medical School, Dallas, TX. This cell line does not respond to GM-CSF (unpublished data). HT-2 cells were maintained in RPMI complete medium with 5 U/ml of rIL-2. The IFN-γ-sensitive B lymphoma cell line, WEHI-279 was kindly provided by Dr. C. Sidman, Jackson Lab., Bar Harbour, ME. This cell line was maintained in complete RPMI medium. D10.G4, a T_H2 type of T cell clone was obtained from the American Type Culture Collection, Rockville, MD, and maintained by stimulating with conalbumin and H-2^k stimulator spleen cells (Kaye *et al*, 1983). P815 tumor cell line was maintained by *in vitro* culture in complete RPMI medium.

Antibodies, lymphokines and reagents

The monoclonal antibodies 11B11 (anti-IL-4), R4.6A2 (anti-IFN-γ), 14.4.4 (anti-IE^d), GK-1.5 (anti-CD4), 3.155 (anti-CD8), 7D4 (anti-IL-2R) and J11d (anti-immature T cells and B cells) were obtained from the American Type Culture Collection, Rockville, MD, were grown *in vitro* or *in vivo* as ascites and used as concentrated supernatant after precipitation with 50% ammonium sulfate and dialysis as described elsewhere (Udhayakumar *et al*, 1988;Nagarkatti *et al*, 1989). The antibodies S4B6 (anti-IL-2) and 6B2 (anti-B220) were generous gifts from Drs. R.L. Coffman and T.R. Mosmann, DNAX Research Institute, Palo Alto, CA. Recombinant IL-2 and IL-4 were purchased from Genzyme, Boston, MA and used as described elsewhere (Nagarkatti *et al*, 1989). Reference murine IFN-γ was kindly provided by the National Institute of Health, Bethesda, MD.

Autoreactive T cell lines

An autoreactive T cell clone from normal DBA/2 mice designated AutoD1.4 was established as described elsewhere (Udhayakumar *et al*, 1988; Nagarkatti *et al*, 1989). Briefly, $CD4^+$ T cells were stimulated with syngeneic splenic adherant cells (SAC) enriched for macrophages and dendritic cells in primary and secondary cultures. Subsequent cultures were carried out using 10% purified human T cell growth factor (TCGF, Cellular Products, Inc., Buffalo, NY) and syngeneic SAC. The cell line was cloned by seeding the cells at 0.3 cells per well and the clones isolated were further characterized. AutoD1.4 is a $CD4^+$ T cell clone which responds to syngeneic IE^d molecules as characterized and reported elsewhere (Udhayakumar *et al*, 1988; Nagarkatti *et al*, 1989). A similar autoreactive T cell clone was isolated from 4 month old MRL-*lpr/lpr* mice. Since 4 month old MRL-*lpr/lpr* mice have a large number of $CD4^-CD8^-$ (double-negative) T cells in the periphery, the $CD4^+$ T cells were purified from the lymph nodes

by positive-selection using panning techniques. Briefly, lymph node cells were incubated with anti-CD4 mAbs followed by panning on petriplates coated with anti-rat IgG. The adherent cells were removed by forceful pipeting and used in cultures. The autoreactive T cell clone isolated was further subcloned and designated AutoK1.4. Genetic mapping was carried out by incubating $5x10^5$ cells with irradiated spleen cells ($6x10^5$) from various strains of mice for 48 hours and the proliferation was measured by ^{3}H-thymidine incorporation assay (Udhayakumar et al, 1988; Nagarkatti *et al*, 1989). Both AutoD1.4 and AutoK1.4 clones were maintained by bi-weekly stimulation of $5x10^5$ T cells with $1x10^6$ syngeneic SAC in the presence of 5 U/ml of recombinant (r) IL-2 in RPMI 1640 medium supplemented with 2mM L-glutamine, $5x10^{-5}$ M 2-mercaptoethanol, 40μg/ml gentamicin sulfate, 10mM Hepes and 10% fetal bovine serum (GIBCO Laoratories, Grand Island, NY). Both clones are $CD3^+$, $CD4^+$, $Thy1.2^+$, Ia^- and have maintained a stable phenotype and functional properties for over a year since their establishment.

Bioassays for IL-2, IL-4 and IFN-γ

Autoreactive T cell clones ($2x10^6$/ml) were stimulated with Con A (2μg/ml) or with irradiated syngeneic SAC ($2x10^6$/ml) and 24 hours later, the culture supernatants (CS) were harvested and tested for the presence of various interleukins. To measure IL-2/IL-4, $5x10^3$ HT-2 cells were incubated with the CS obtained from autoreactive T cell lines in the presence or absence of anti-IL-2, anti-IL-2R or anti-IL-4 antibodies. In every experiment, appropriate controls were included consisting of HT-2 cells stimulated with rIL-2 or rIL-4 in the presence or absence of anti-IL-2R or anti-IL-4 antibodies. The IL-4 activity was also measured by its capacity to induce hyper-Ia expression on B cells (Nagarkatti *et al*, 1989). Briefly, purified B cells ($5x10^5$/well) were cultured with 0.1ml of CS or medium (control) in 96 well plates. After 24 hours, the B cells were stained for Ia expression by incubating B cells in cold with 14.4.4

(anti-IE^d) or with normal mouse IgG (control) followed by FITC conjugated anti-mouse IgG $(Fab')_2$ antibodies (Cappel Laboratories, Inc., Cochranville, AA) and the cells were next analyzed on a flow cytometer (Epics V, Coulter Electronics, Hialeah, FL) as described elsewhere (Nagarkatti *et al*, 1989). IFN-γ was detected in the culture supernatants by using the IFN-γ sensitive B lymphoma cell line, WEHI-279 as described by Reynolds *et al* (1987). The WEHI-279 cells ($5x10^3$/well) were cultured with 0.1ml of culture supernatants in the presence or absence of anti-IFN-γ antibodies. After 24 hours, the cells were pulsed with ^{3}H-thymidine and the radioactivity was determined 18 hours later using a liquid scintillation counter as described elsewhere (Udhayakumar *et al*, 1988; Nagarkatti *et al*, 1989).

T-B cell interaction

B cells were purified from the spleen by first removing the splenic adherent cells following incubation on plastic petriplates followed by depletion of T cells using a cocktail of mAbs (anti-Thy1.2, anti-CD4 and anti-CD8) plus rabbit complement as described elsewhere (Udhayakumar *et al*, 1988). This treatment resulted in a preparation that consisted of >95% Ig^+ cells as judged by analysis on a cell sorter. To study the T-B interaction, varying numbers of purified B cells were cultured with irradiated autoreactive T cell clones, AutoD1.4 or AutoK1.4 for 48 hours in 0.2 ml medium, in 96 well flat-bottom tissue-culture plates. The cultures were pulsed with 2 μCi of ^{3}H-thymidine during the last 18 hours, harvested and the radioactivity measured using a liquid scintillation counter.

T-T interaction

The T-T interaction was studied as described in detail elsewhere (Nagarkatti *et al*, 1985b; Udhayakumar *et al*, 1988). Briefly, $CD4^+$ T cells from DBA/2 mice were prepared by passing the spleen cells over nylon wool columns, followed by depleting the contaminating B cells and accessory cells by treatment with 1:10 dilution of anti-Ia, J11d, anti-B220 and anti-CD8 mAbs plus complement. Since 6 month old MRL-*lpr/lpr* mice have large numbers of double-negative T cells, the $CD4^+$ T cells from MRL-*lpr/lpr* mice were purified by the panning method as described earlier in the methodology dealing with the establishment of autoreactive T cell lines. The purity of the $CD4^+$ T cells was usually >98% as determined by flow cytometric analysis. The purified $CD4^+$ T cells were mixed with varying numbers of irradiated autoreactive T cell clones. After 48 hours, the proliferative responses of the $CD4^+$ T cells were measured by ^{3}H-thymidine incorporation assay as described above.

Activation of macrophages and cytostasis of tumor cells

To study the macrophage-mediated cytostasis of tumor cells, normal resident macrophages were isolated from peritoneal exudate cells (PEC) of DBA/2 mice by repeatedly washing the peritoneal cavity using cold PBS. The PEC were plated onto plastic plates and the adherent cells removed. The adherent macrophages ($5x10^5$/well) were next incubated with 0.1 ml of culture supernatants of activated autoreactive T cell clones in the presence or absence of anti-IFN-γ mAb in 96 well plates at 37°C for 48 hours. The cells were treated with 20μg/ml of mitomycin-C, washed thrice and incubated with $5x10^4$ P815 cells. The cultures were pulsed 8 hours later with 2 μCi of ^{3}H-thymidine, harvested 18 hours thereafter and the radioactivity incorporated was determined using a liquid scintillation counter.

Results

Characterization of an autoreactive T cell clone from MRL-lpr/lpr mice

Several autoreactive T cell clones from normal unimmunized mice have been previously isolated in our laboratory (Nagarkatti *et al*, 1985a, 1985b; Udhayakumar et al, 1988; Nagarkatti *et al*, 1989). Using similar culture conditions, an autoreactive T cell line from the MRL-*lpr/lpr* strain was isolated and a cloned cell line (AutoK1.4) was analyzed by genetic mapping for specific reactivity. The clone autoK1.4 responded by proliferation to H-2^k stimulator cells from B10.K, MRL-+/+, MRL-*lpr/lpr* and C3H mice (Table 7.1). In contrast, the clone did not respond to allo-Ia antigens of b or d haplotype nor to K^k or D^k-encoded antigens. The clone did not respond in the presence of I-Eα^k [B10.A(5R)] but demonstrated a good response in the presence of I-A^k and I-Eβ^k (B10.RKB). Further studies using B10.BASR1 strain which expresses only I-A^k, clearly demonstrated that autoK1.4 responded to I-A^k molecules. These data were further confirmed using mAb blocking studies in which it was observed that the proliferative response of autoK1.4 clone when stimulated with syngeneic spleen cells was inhibited by anti-IA^k but not by anti-IE^k mAb (data not shown). In the above studies significant variability in the responsiveness of autoK1.4 T cells was observed, when stimulated with spleen cells from different strains of mice. This was probably due to the variation in the number of Ia^+ cells, the density of Ia expression or amount of IL-1 produced by the stimulators cells.

Table 7.1. AutoK1.4 responds to syngeneic I-A^k molecules as determined by genetic mapping[a]

Stimulator[b] cells	MHC						Cell proliferation (Δ cpm)[c]
	K	I - A		I - E		D	
		α	β	α	β		
BIO.K	k	k	k	k	k	k	17,351
BIO.RKDI	k	k	k	k	k	d	21,485
BIO.MBR	b	k	k	k	k	q	4,575
A.TFR1	s	k	k	k	k	f	6,499
BIO.RKB	k	k	k	b	k	b	7,709
BIO.A(5R)	b	b	b	k	b	d	-1,145
B10.BASR1	k	k	k	-	-	s	7,049
MRL-+/+	k	k	k	k	k	k	3,965
MRL-*lpr/lpr*	k	k	k	k	k	k	16,372
C3H	k	k	k	k	k	k	8,593
C57BL/6	b	b	b	—	—	b	-1,204
Balb/c	d	d	d	d	d	d	333
DBA/2	d	d	d	d	d	d	-512

[a] AutoK1.4 clone was used at $2x10^4$ cells/well. The results represent data from a single experiment except for B10.BASR1. The autoK1.4 cells incubated in medium alone incorporated 2,325cpm and in the second experiment using B10.BASR1, the autoK1.4 cells incorporated 1,812cpm.

[b] Spleen cells from different strains were irradiated and used at $6x10^5$/ml. These cells incorporated approximately 1,500cpm.

[c] Cell proliferation was measured by ^{3}H-thymidine uptake 48 hours after culture. The Δcpm was calculated by subtracting the control cpm obtained using the cloned cells incubated in medium alone and stimulator cells incubated alone from the experimental cpm.

Induction of T-B and T-T interaction by autoreactive T cell clones

Earlier studies demonstrated that autoreactive T cell clones can polyclonally activate naive, resting B cells and furthermore induce a unique T-T interaction in which naive $CD4^+$ T cells would respond directly to autoreactive T cell clones in the absence of accessory cells (Nagarkatti *et al*, 1985a, 1985b; Udhayakumar *et al*, 1988; Nagarkatti *et al*, 1989). In the current study it was therefore investigated whether this property was restricted to clones isolated from normal DBA/2 mice or whether similar characteristics were demonstrable from the autoreactive T cell clone isolated from autoimmune-susceptible MRL-*lpr/lpr* mice. The data shown in Table 7.2 suggested that autoK1.4 had similar properties as AutoD1.4 inasmuch as it could also polyclonally activate B cells and induce naive $CD4^+$ T cells to proliferate. Also, the T-T interaction induced by autoK1.4 was inhibited by anti-CD4 but not by anti-Ia mAb (data not shown), similar to earlier studies on other autoreactive clones (Nagarkatti *et al*, 1985b; Udhayakumar *et al*, 1988). It has been shown that MRL-*lpr/lpr* mice develop severe lymphadenopathy and autoimmune disease at ~2 months of age characterized by the accumulation of $CD4^-CD8^-$ T cells (Wofsy *et al*, 1984). Therefore, the T-B and T-T interaction in these mice was investigated before (1 month old) or after the onset (6 month old) of autoimmunity. Interestingly, $CD4^+$ T cells purified from 6 month old MRL-*lpr/lpr* mice failed to respond to the autoreactive T cell clone, while, similar cells from 1 month old mice exhibited strong responsiveness. The B cells from 1 or 6 month old MRL-*lpr/lpr* mice, in contrast, responded in a similar fashion when stimulated with the autoK1.4 clone.

Table 7.2. Autoreactive T cell clones can induce T-B and T-T cell interactions.

Expt.[a]	Responders[b]		Stimulators[c]	Cell Proliferation[d] (cpm ± S.E.)
1	$CD4^+$	DBA/2	—	4,351 ± 511
	$CD4^+$	DBA/2	AutoD1.4	45,462 ± 5,671
	B cells	DBA/2	—	3,605 ± 276
	B cells	DBA/2	AutoD1.4	34,803 ± 2,767
	—		AutoD1.4	2,660 ± 389
2.	$CD4^+$ T	MRL-*lpr/lpr* (1 month old)	—	14,854 ± 960
	$CD4^+$ T	MRL-*lpr/lpr* (1 month old)	AutoK1.4	34,249 ± 2,228
	$CD4^+$ T	MRL-*lpr/lpr* (6 month old)	—	4,227 ± 471
	$CD4^+$ T	MRL-*lpr/lpr* (6 month old)	AutoK1.4	4,528 ± 130
	—		AutoK1.4	372 ± 159
3.	B cells	MRL-*lpr/lpr* (1 month old)	—	15,283 ± 780
	B cells	MRL-*lpr/lpr* (1 month old)	AutoK1.4	33,736 ± 1,090
	B cells	MRL-*lpr/lpr* (6 month old)	—	7,692 ± 103
	B cells	MRL-*lpr/lpr* (6 month old)	AutoK1.4	23,871 ± 655
	—		AutoK1.4	422 ± 19

[a] The data presented are representative of multiple experiments.

[b] $CD4^+$ T cells were used at a concentration of $2\text{-}4 \times 10^5$ cells/well and B cells were used at 2×10^5 cells/well.

[c] The stimulator AutoD1.4 or AutoK1.4 cells were irradiated at 2000 rads and used at a concentration of 5×10^4 cells/well. Irradiated autoD1.4 or autoK1.4 cells when incubated with irradiated $CD4^+$ T cells or B cells usually incorporated 2,000cpm.

[d] The T-B interaction was studied after 48 hours and T-T interaction after 72 hours incubation. The cultures were pulsed with ^{3}H-thymidine during the last 18 hours.

Autoreactive T cell clones produce IL-2 and IL-4

The type of lymphokine produced by the autoreactive T cell clones was next investigated. The autoreactive T cell clones, AutoD1.4 and AutoK1.4 were stimulated with irradiated syngeneic SAC or with Con A and 24 hours later, the supernatants were collected and tested for their capacity to induce HT-2 cell proliferation in the absence or presence of 7D4 (anti-IL-2R mAb) or 11.B.11 (anti-IL-4 mAb). The data shown in Table 7.3 suggested that the autoreactive T cells upon activation produced growth factors for the HT-2 cells which was partially inhibited by anti-IL-2R or anti-IL-4 mAbs. The anti-IL-2R or anti-IL-4 mAbs at the dilution used inhibited specifically only the rIL-2 or rIL-4 mediated proliferation of HT-2 cells respectively. Also, D10.G4 which is a T_H2 type of T cell clone, following Con A activation, produced only IL-4 but not IL-2 in confirmation with other studies (Kaye *et al*, 1983). The proliferation of HT-2 cells induced by culture supernatants from activated autoreactive T cell clones was also partially inhibited by anti-IL-2 (S4B6) antibodies (data not shown).

Table 7.3. Detection of IL-2 and IL-4 in the culture supernatants of activated autoreactive T cell clones.

Expt. No.[a]	Supernatant/ Interleukin[b]	mAb added to cultures[c]	HT-2 cell proliferation (cpmx10^{-3})[d]
1	AutoD1.4	—	14,807 ± 698
		anti-IL-2R	9,766 ± 973 (35)[e]
2	AutoK1.4	—	13,317 ± 151
		anti-IL-2R	7,610 ± 362 (43)
3	AutoD1.4	—	17,710 ± 3251
		anti-IL-4	5,457 ± 499 (70)
4	AutoK1.4	—	14,201 ± 158
		anti-IL-4	4,606 ± 269 (68)
5	AutoD1.4	—	14,438 ± 1012
		anti-IL-2R	8,238 ± 634 (43)
		anti-IL-4	7,652 ± 516 (48)
6	AutoK1.4	—	12,002 ± 524
		anti-IL-2R	8,306 ± 104 (31)
		anti-IL-4	4,012 ± 230 (67)
7	D10.G4	—	10,300 ± 788
		anti-IL-2R	13,394 ± 392 (0)
		anti-IL-4	6,364 ± 440 (39)
8	rIL-2	—	28,778 ± 737
		anti-IL-2R	15,503 ± 1,610 (47)
		anti-IL-4	31,618 ± 1,126 (0)
9	rIL-4	—	14,651 ± 328
		anti-IL-2R	16,573 ± 270 (0)
		anti-IL-4	3,737 ± 597 (75)

[a] The data presented are representative of multiple experiments.

[b] Autoreactive T cell clones, AutoD1.4 or AutoK1.4 were cultured with ConA (in expts. 1-4) or with syngeneic irradiated SAC cells (in expts. 5 and 6) for 24 hours. Antigen-specific D10.G4 cells were cultured with Con A for 24 hours. The culture supernatants were next harvested and tested for the presence of interleukins. Recombinant IL-2 and IL-4 were added at a final concentration of 5 U/ml and 25 U/ml, respectively.

[c] Anti-IL-2R antibodies (7D4) and anti-IL-4 antibodies (11.B.11) were added at 1:10 and 1:250 final dilution, respectively.

[d] HT-2 cells ($5x10^3$/well) in 0.05ml of complete medium were cultured with 0.05ml of appropriate dilutions of the mAb and 0.1ml of culture supernatant from activated T cells or the recombinant interleukins. The plates were incubated at 37°C for 24 hours, pulsed with 2μCi of ^{3}H-thymidine and harvested 18 hours later.

[e] Figures in parentheses show percent suppression.

The fact that the culture supernatants from autoreactive T cells contained IL-2 was also confirmed by using an IL-2 dependent tumor specific cytotoxic T cell clone, C9, developed in our laboratory. This T cell clone responds only to IL-2 but not to IL-4 and it was observed that the culture supernatants from activated AutoD1.4 or AutoK1.4 induced proliferation of this cell line which was inhibited by anti-IL-2R (data not shown). The presence of IL-4 was also confirmed by its capacity to cause hyper-Ia induction on B cells. When B cells were incubated with culture supernatants from AutoD1.4 or AutoK1.4 clones for 18-24 hours, there was an increase in the density of Ia expression as measured by flow cytometry (Fig.7.1). This increase was similar to the hyper-Ia induction by rIL-4 and furthermore, was inhibited following addition of anti-IL-4 antibodies during culture.

Production of IFN-γ by autoreactive T cell clones

WEHI-279, an IFN-γ-sensitive B cell lymphoma line was used to detect the presence of IFN-γ in the culture supernatants of activated autoreactive T cells. The data shown in Table 7.4 suggested that the autoreactive T cells produced a factor which could inhibit the proliferation of WEHI-279 and mAbs to IFN-γ could reverse this inhibition significantly. As a control, the reference IFN-γ could similarly inhibit the proliferation of WEHI-279 which was also reversible by mAbs to IFN-γ. Together, these data suggested that both the autoreactive T cell clones produced IFN-γ.

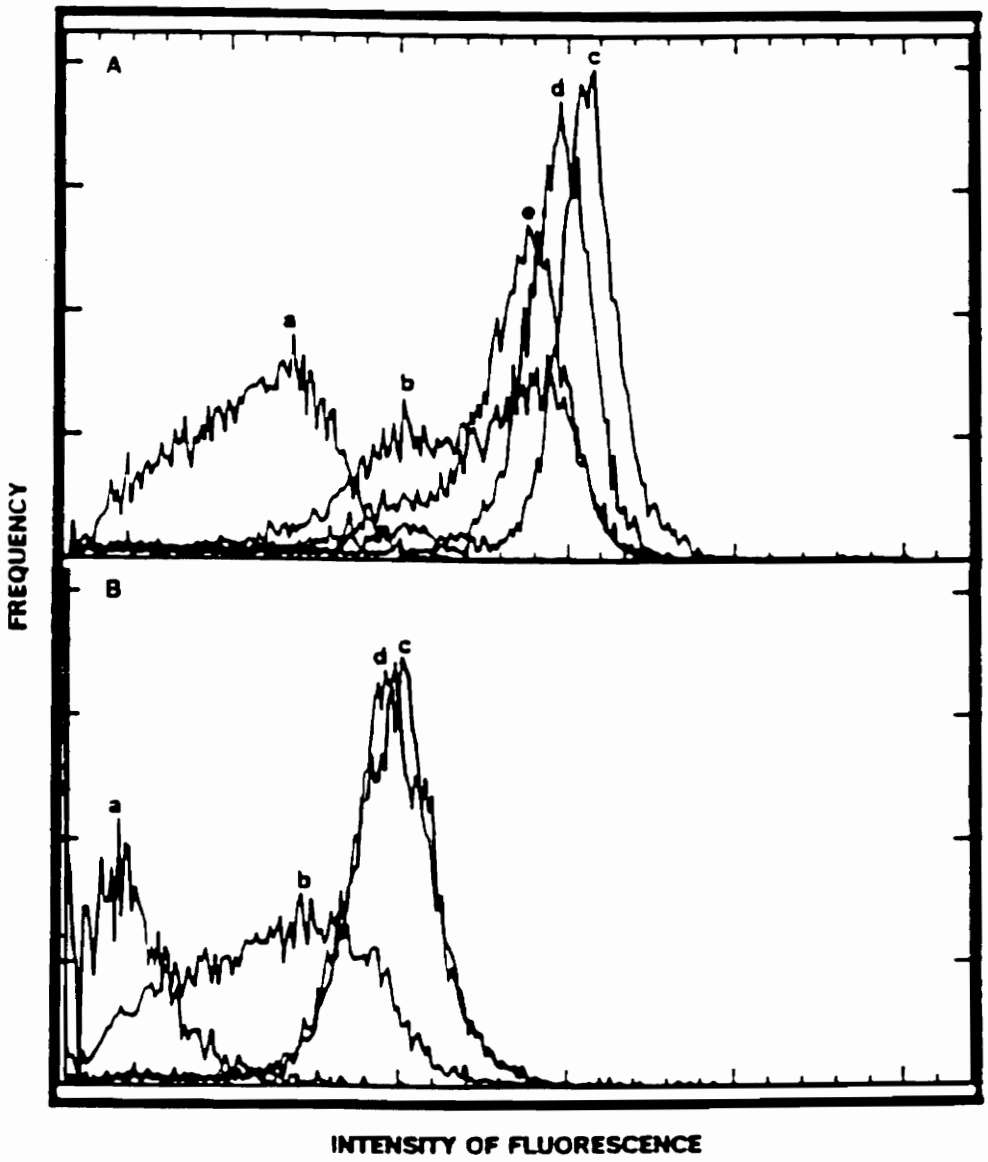

Figure 7.1. Enhanced Ia antigen expression on B cells following culture with the supernatants from activated autoreactive T cell clones, AutoK1.4 (panel A) or AutoD1.4 (panel B): The B cells were incubated with medium alone (b), rIL-4 (c) or with culture supernatants from autoreactive T cell clones (d). In panel A, the B cells were incubated with culture supernatant from autoreactive T_H cell clone plus anti-IL-4 antibodies (e). The B cells were stained with anti-IE^d antibodies followed by FITC-conjugated anti-mouse IgG (b,c,d,e) or with normal mouse IgG followed by the FITC-conjugated second step reagent as a negative control (a).

Table 7.4. Detection of IFN-γ in the supernatants of activated autoreactive T cells.

Expt. No.	Culture supernatant[a]	Antibody added[b]	Proliferation of WEHI-279 (c.p.m. ± S.E.)[c]
1	—	—	23,479 ± 600
	AutoK1.4	—	1,055 ± 30
	AutoK1.4	anti-IFN-γ	17,748 ± 177
	IFN-γ	—	1,145 ± 100
	IFN-γ	anti-IFN-γ	23,457 ± 1,931
	—	anti-IFN-γ	25,229 ± 1,090
2	—	—	19,566 ± 431
	AutoD1.4	—	7,741 ± 508
	—	anti-IFN-γ	11,427 ± 503

[a] Autoreactive T cells were activated using Con A, as described in Table 7.1. NIH reference murine IFN-γ was used at a final concentration of 10 IU/ml.

[b] Anti-IFN-γ mAb (R4.6A2) was used at 1:4 final dilution of the hybridoma supernatant.

[c] WEHI-279 cells ($5x10^3$/well) were cultured for 24 hours in the presence of the culture supernatant and in the presence or absence of mAb to IFN-γ. The cultures were next pulsed with 2μCi of ^{3}H-thymidine and harvested 18 hours later.

Activation of tumoristatic properties of macrophages

T_H1 cells have been shown to produce IFN-γ and activate macrophages to inhibit tumor cell growth. To further substantiate whether our autoreactive T cell clones could activate the macrophages, culture supernatants from activated autoD1.4 and autoK1.4 clones were tested for their capacity to induce tumoristatic activity. The data shown in Table 7.5 clearly demonstrated that normal macrophages cultured in the presence of culture supernatants from activated autoD1.4 or autoK1.4 cells significantly inhibited the proliferation of P815 tumor cells similar to the action of IFN-γ. This effect was reversible in the presence of mAbs to IFN-γ thereby suggesting that the macrophage-activation was caused by IFN-γ.

Activated autoreactive T cell clones respond only to rIL-2 but not to rIL-4:

Following antigen-specific activation, both T_H1 and T_H2 clones have been shown to proliferate in response to exogenous IL-2 and IL-4 (Fernandez-Botran *et al*, 1988). To test this responsiveness, autoreactive T cells were cultured with irradiated syngeneic spleen cells for 48 hours and were next incubated with rIL-2 or rIL-4. The data shown in Table 7.6 demonstrated that both AutoD1.4 and AutoK1.4 responded only to exogenous IL-2 but not IL-4.

Table 7.5. Culture supernatants from activated autoreactive T cell clones activate the tumoristatic potential of the macrophages.

Peritoneal macrophages[a]	Culture supernatant/IFN-γ[b]	Anti-IFN-γ mAb[c]	P815 tumor cell proliferation (c.p.m. ± S.E.)[d]
—	—	—	182,450 ± 5,056
+	—	—	180,896 ± 2,479
+	IFN-γ	—	61,784 ± 1,559
+	IFN-γ	+	154,259 ± 1,389
+	AutoK1.4	—	84,041 ± 650
+	AutoK1.4	+	162,454 ± 1,532
+	AutoD1.4	—	94,758 ± 1,522
+	AutoD1.4	+	175,245 ± 1,322

[a] Peritoneal macrophages from DBA/2 mice were used at $2x10^5$ cells/well. Following activation with IFN-γ or culture supernatants from activated autoreactive T cell clones, the macrophages were treated with mitomycin-C, washed and used. The macrophages incubated alone incorporated less than 1,000 cpm.

[b] IFN-γ was used at 10 IU/ml. The culture supernatants from autoreactive T cell clones were collected 24 hours after stimulation with conA.

[c] Anti-IFN-γ mAbs were used at 1:10 final dilution

[d] P815 tumor cells ($5x10^4$) were incubated for 24 hours and during the last 18 hours pulsed with ^{3}H-thymidine.

Table 7.6. Autoreactive T cell clones respond to IL-2 but not to IL-4.

Responder clone[a]	Interleukin	Cell Proliferation[b] (cpm ± S.E.)
AutoK1.4	-	7,130 ± 363
	rIL-2 (25U/ml)	34,388 ±2,014
	rIL-4 (50U/ml)	8,259 ± 267
AutoD1.4	-	2,493 ± 2,014
	rIL-2 (25U/ml)	11,767 ± 806
	rIL-2 (50U/ml)	20,446 ± 4,725
	rIL-4 (50U/ml)	2,679 ± 46
	rIL-4 (100U/ml)	2,946 ± 72

[a] Autoreactive T cell clones were activated with irradiated syngeneic spleen cells for 48 hours, harvested and viable cells purified by centrifuging over ficoll-hypaque. The cells ($5x10^4$/well) were next incubated with medium or with IL-2 or IL-4.

[b] Cell proliferation was measured by pulsing the plates with ^{3}H-thymidine after 24 hours incubation and harvested 18 hours later.

Discussion

In the present study it was observed that autoreactive T cell clones isolated from normal DBA/2 mice or autoimmune-susceptible MRL-*lpr/lpr* mice produced IL-2, IL-4 and IFN-γ, thereby exhibiting the lymphokine secretory properties of both T_H1 and T_H2 subsets. The clones were derived by limiting dilution at <0.3 cells/well and also subcloned. Furthermore, the clones were stable in their phenotypic and functional properties for over 2 years since their establishment (Udhayakumar *et al*, 1988). The presence of IL-2, IL-4 and IFN-γ in the supernatants of activated autoreactive T cells was confirmed by two independent bioassays. The IL-2 was demonstrable using HT-2 cells whose proliferation was inhibited in the presence of anti-IL-2R and anti-IL-2 antibodies and also using an IL-2 but not IL-4 dependent cytotoxic T cell line recently cloned in our laboratory. The presence of IL-4 was detected using HT-2 cells and further confirmed by studying the hyper-Ia induction on B cells. The IFN-γ was demonstrable using an IFN-γ sensitive B lymphoma line and by the activation of tumoristatic properties of macrophages. Furthermore, functionally both autoreactive T cell clones could activate or induce proliferation of naive B cells, $CD4^+$ T cells and macrophages.

Earlier studies have demonstrated that autoreactive T cell clones isolated from normal DBA/2 mice can induce the proliferation of syngeneic naive $CD4^+$ T cells (Nagarkatti *et al*, 1985b; Udhayakumar *et al*, 1988). This response was independent of MHC molecules and did not require antigen-presenting accessory cells. The T-T interaction was regulated by IL-2 and IL-4, and when the autoreactive T cell clone was $V\beta8^+$, the T-T interaction was inhibited in the presence of anti-Vβ8 mAbs (Nagarkatti *et al*, 1985b; Udhayakumar *et al*, 1988 and unpublished data). Based on these observations, it was speculated that the $CD4^+$ T cells were responding directly to the idiotypic determinants on the autoreactive T cells. Similar autoreactive T cell induced T-T network interactions have been described by others in recent years (Sano *et al*, 1987; Kennedy *et al*, 1986; Suzuki *et al*, 1986). Although the functional significance of the T-T

interaction is not clear, Suzuki *et al* (1986) demonstrated that the autoreactive T cell clones could initiate a chain of regulatory cell interactions leading to either the stimulation or inhibition of the response. Sano *et al* (1987) further demonstrated that an autoreactive T cell clone when administered *in vivo* could induce an MHC-restricted minimal regulatory circuit. This clone activated an MHC-restricted T suppressor cell which in turn suppressed heterogenous T helper cells provided that the latter had the same MHC-restricted specificity as that possessed by the autoreactive T cell clone.

MRL-*lpr/lpr* mice develop severe lymphadenopathy characterized by the accumulation of a large number of double-negative T cells (Wofsy *et al*, 1984). Such cells have been shown not to respond to polyclonal T cell activators such as ConA (Altman *et al*, 1981). Recently, it was demonstrated that a significant proportion of these cells express J11d, a marker seen only on immature thymocytes but not on mature peripheral T cells, thereby suggesting that the double-negative cells closely resemble the $CD4^-CD8^-$ thymocytes (Seth *et al*, 1988). It was also observed that the double-negative cells do not respond to syngeneic Ia molecules nor do they participate in the T-T interaction induced by the autoreactive T cell clones (unpublished data). Lymph node cells from young MRL-*lpr/lpr* mice have been shown to spontaneously proliferate when cultured *in vitro* and respond to self-Ia antigens (Cohen *et al*, 1986; Weston *et al*, 1988). Furthermore, the cultured cells have been shown to be $CD4^+$, induce syngeneic B cell proliferation and differentiation and to produce IL-2, IL-3 and IFN-γ (Cohen *et al*, 1986; Weston *et al*, 1988). However, lymph node T cell responsiveness to the self-Ia antigens decreases in aged MRL-*lpr/lpr* mice (Hom and Talal, 1982). Whether such a decrease is due to low numbers (~8%) of $CD4^+$ T cells and large numbers (~80%) of unresponsive double-negative T cells found in aged mice (Seth *et al*, 1988) or whether it is due to an intrinsic defect in the $CD4^+$ responding T cells has not been resolved. Our data on isolation of autoreactive T cell clones from aged mice suggested that 4-6 month old MRL-*lpr/lpr* mice may still have T cells capable of responding to self-Ia antigens and such cells may be able to polyclonally activate B cells to produce autoantibodies.

It was interesting to note that the clone that we isolated from MRL-*lpr/lpr* mice could also initiate a similar T-T interaction when the responding $CD4^+$ T cells were used from 1 month old mice before the onset of the disease. Interestingly, 6 month old mice with severe lymphadenopathy and autoimmune disease failed to demonstrate a T-T interaction. It is not clear at this juncture whether this defect is due to a decrease in numbers of $CD4^+$ T cells capable of responding to the autoreactive T cells or whether it is due to the decreased responsiveness to the IL-2 and IL-4 produced by the autoreactive T cells. Further studies are in progress to address these possibilities. However, a lack of T-T network suggests the failure to activate T suppressor cells (Sano *et al*, 1987) and this may lead to unregulated production of auto-antibodies in MRL-*lpr/lpr* mice. Recently the T-T interaction was shown to be greatly diminished in aged mice (Udhayakumar *et al*, 1988) which also exhibit increased susceptibility to autoimmunity.

It has been demonstrated that T_H2 clones can provide excellent help to B cells due to their capacity to secrete IL-4 and IL-5 (Mosmann and Coffman, 1989). The role of T_H1 cells in providing help to B cells is uncertain. Boom *et al* (1988) reported that T_H2 but not T_H1 clones specific for rabbit γ-globulin could induce polyclonal proliferation and Ig production in resting B cells in the presence of rabbit anti-mouse Ig antibodies. In contrast to the B cell activation, Stout and Bottomly (1989) demonstrated that T_H1 but not T_H2 clones could activate the macrophages to exhibit tumoristatic activity. In this context it is interesting to note that the autoreactive T cell clones provide help to B cells as well as activate the macrophages.

Since the autoreactive T cell clones could activate B cells and macrophages and also induce T-T interaction, we investigated the type of lymphokines generated by such clones. All murine long-term T_H clones have been shown to secrete either IL-2 and IFN-γ (T_H1 cells) or IL-4 and IL-5 (T_H2 cells) (Mosmann and Coffman, 1989). It was interesting in this context to note that both the autoreactive clones produced IL-2, IL-4 and IFN-γ thereby exhibiting the properties of both T_H1 and T_H2 cells. This observation appeared to be somewhat similar to that

demontrated with some human T_H1 clones which were found to secrete IL-2, IL-4 and IFN-γ (Umetsu *et al*, 1988). IFN-γ has been shown to inhibit IL-4-mediated B cell-activation (Rabin *et al*, 1986). If this was true, how do the autoreactive T cell clones activate B cells to proliferate and differentiate ? One possibility is that the IFN-γ produced by the autoreactive T cells may not be sufficient to inhibit the B cell activation and thus the outcome of B cell stimulation may depend on the relative amounts of IL-2, IL-4 and IFN-γ secreted by the autoreactive T cells. Our data are similar to those from a human T_H clone which inspite of producing IL-2, IL-4 and IFN-γ could efficiently induce Ig synthesis by the B cells (Umetsu *et al*, 1988). Lastly, it is also possible that the type and amounts of lymphokine secreted by the autoreactive T cells may vary depending on the accessory cells involved in autoreactive T cell activation such as macrophages (Nagarkatti *et al*, 1985a), dendritic cells (Nussenzweig and Steinman, 1980) or B cells (Nagarkatti *et al*, 1989).

Recently Gajewski *et al* (1989) reported that when rIL-2 was used as a growth factor for T cells, the majority of the clones derived were of the T_H2 type, whereas when rIL-2 plus rIFN-γ was used, T_H1 clones were isolated. Using either procedure, some clones were obtained that produced IL-2, IL-4 and IFN-γ. Whether such clones represent autoreactive T cells remains an interesting possibility which needs further elucidation. It should be noted that the TCGF employed in this study to isolate the autoreactive T cell clones has only IL-2 and no detectable IL-4 or IFN-γ activity and inspite of this autoreactive T cells having mixed lymphokine-secreting properties of T_H1 and T_H2 cells were consistently isolated.

T_H1 and T_H2 cells have been shown to use IL-2 or IL-4, respectively, as autocrine growth factors *in vitro*. However, following antigenic stimulation, both types of clones respond to the proliferative effects of IL-2 and IL-4 (Fernandez-Botran, 1988). In this context it was interesting to note that the autoreactive T cell clones responded only to IL-2 but not IL-4, following antigen specific activation. These data suggested that the autoreactive T cells although capable of secreting IL-4 do not utilize the IL-4 and use only IL-2 for growth. Whether this is due to

preferential expression of IL-2R over IL-4R needs to be investigated. Further studies on the kinetics and quantitation of the lymphokines produced by autoreactive T cells may explain their unique capacity to help, suppress and contrasuppress (Clayberger *et al*, 1984; Kotani *et al*, 1986; Quintans *et al*, 1986) an on-going immune response *in vitro*. In summary, the current study suggests that self-Ia reactive T cell clones isolated from unprimed mice exhibit properties that are distinct from those of antigen-specific murine T_H1 or T_H2 cells. Whether this property is exhibited by all autoreactive T cells or only a fraction of such cells remains to be resolved by studying a large number of long-term autoreactive T cell clones. Such studies are currently in progress in our laboratory.

Summary

Recent studies have suggested the existence of two mutually exclusive subpopulations of T helper (T_H) cells in the murine immune system, called T_H1 which produces IL-2 and IFN-γ but not IL-4 and T_H2 which secretes IL-4 and IL-5 but not IL-2. Also, functionally, T_H1 cells generally activate the macrophages and mediate delayed-type hypersensitivity whereas T_H2 cells provide help efficiently to B cells. In the present study, the lymphokine secretory properties of two well-characterized autoreactive (self-Ia reactive) T cell clones isolated from normal DBA/2 mice and autoimmune-susceptible MRL-*lpr/lpr* mice was investigated. It was observed that both the autoreactive T cell clones following activation, produced IL-2, IL-4 and IFN-γ. They induced hyper-Ia expression and cell proliferation in syngeneic B cells as well as activated the macrophages to exhibit tumoristatic properties. Both clones could also induce T-T network interaction in which syngeneic naive $CD4^+$ T cells responded directly to stimulation with autoreactive T cell clones. The T-T interaction was demonstrable in 1 month old MRL-*lpr/lpr* mice prior to the onset of the autoimmune disease but not in 6 month old mice

having lymphadenopathy and autoimmune disease. Unlike T_H1 and T_H2 cells which upon antigenic stimulation respond to exogenous IL-2 and IL-4, the autoreactive T cell clones responded only to IL-2 but not to IL-4. The current study suggests the existence of a unique subset of immunoregulatory $CD4^+$ T_H cells having the lymphokine secretory and functional properties of both the murine T_H1 and T_H2 subsets.

Conclusions

The results of this study indicate that aging has manifold effects on the immune system of normal as well as autoimmune mice. Using several autoreactive T cell clones, various aspects of the immune response were studied in normal DBA/2 mice. The T-T and T-B interactions studied were found to be defective in aged mice as compared to the responses of the young mice. The T-T interaction in young but not aged mice was enhanced by the exogenous addition of rIL-4 but not rIL-2. This decreased T-T interaction was also correlated with a decreased SMLR in aged mice, which was studied using purified $CD4^+$ T cells from young and old mice as responders and SAC as stimulators.

In the T-B interaction, the B cells from aged mice were found to have an intrinsic defect resulting in a decreased response to stimulation by autoreactive T cells. The B cells from aged mice were found to be defective in progressing from the G_1 phase of the cell cycle to the S phase. Thus these B cells, when stimulated with the autoreactive T cell clone, could increase in size and express increased amounts of Ia antigens but could not incorporate tritiated thymidine to any significant levels. The exogenous addition of rIL-4 had a similar effect of increase in size and Ia expression by B cells in both young and aged DBA/2 mice.

As mentioned earlier, a defective SMLR was also observed in aged DBA/2 mice. This was studied by stimulating purified $CD4^+$ T cells from young and aged mice with SAC from the same age groups. The T cells from aged mice were found to have an intrinsic defect in the ability to respond to syngeneic stimulators. The stimulatory capacity of cells from aged mice was also studied by stimulating $CD4^+$ T cells from young DBA/2 mice with either whole spleen cells, SAC or B cells from aged mice. A decreased proliferation was observed only when SAC were used as stimulators. Upon studying the mechanism of this defective stimulatory capacity, it was found to be due to a decrease in IL-1 synthesis rather than an enhanced synthesis of inhibitory factors such as prostaglandins.

From the above results, we concluded that aged mice show a defect in cell-cell interactions which are essential in the maintenance of a normal immune response. This defect is probably the major factor responsible for the enhanced susceptibility of these aged but otherwise normal mice to various diseases and the decreased ability to produce an immune response to various infectious agents.

In the autoimmune MRL-*lpr/lpr* mice, as the mice age, a marked autoantibody production and a drastic increase in lymph node size is noted. The onset of these changes is around 2 months of age and the mice usually die by 6 months of age. The lymph node enlargement is known to be due to an accumulation of abnormal $CD4^-$ $CD8^-$ T cells. In this study, these abnormal double-negative T cells were analyzed for cell surface marker expression and the source of origin. In addition, the lymphokine secretory properties of an autoreactive T cell clone isolated from *lpr* mice was also studied.

Phenotypic analyses revealed that about 37% of the double-negative T cells from the LN of MRL-*lpr* mice express the marker J11d, which is an antigen usually present on immature T cells and B cells but not on mature or peripheral T cells. The expression of the J11d marker increased with increase in age of the mice, beginning at 2 months of age, coinciding with the onset of lymphadenopathy and being maintained until death of the mice around 5-6 months

of age. This correlated with an increase of double-negative T cells in the thymus, a significant number of which also expressed the marker J11d. These results suggested that in aged *lpr* mice, there is a defect in T cell differentiation in the thymus leading to the production and migration of a large number of abnormal T cells.

A study of the lymphokine secretion patterns of autoreactive T cell clones isolated from normal DBA/2 mice and autoimmune MRL-*lpr* mice revealed that both clones, Auto D1.4 and Auto K1.4, respectively, secreted IL-2, IL-4 and IFN-γ. Both clones were also able to activate $CD4^+$ T cells and B cells to proliferate. However, $CD4^+$ T cells isolated from 6 month old *lpr* mice showed a defect in response when compared to the proliferation by $CD4^+$ T cells from 1 month old MRL-*lpr/lpr* mice or the control MRL-+/+ mice. B cells from both 1 and 6 month old mice responded equally well to stimulation with the autoreactive T cell clone. The two autoreactive T cell clones were also able to cause the induction of tumoristatic activity in macrophages from normal DBA/2 mice. These findings led to the conclusion that the autoreactive T cell clones isolated from normal DBA/2 mice and autoimmune MRL-*lpr* mice exhibit the properties of both T_H1 and T_H2 types of T helper cells. The immunological dysfunction seen in aged mice may therefore result from decreased functions of these unique immunoregulatory cells.

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Abbreviations

Ag	antigen
Ab	antibody
Con A	concanavalin A
DN	double-negative
γIFN	gamma interferon
Ig	immunoglobulin
IL	interleukin
LN	lymph node
MHC	major histocompatibility complex
PHA	phytohemagglutinin
SMLR	syngeneic mixed lymphocyte reaction

T_H	helper T cell
T_S	suppressor T cell

Vita

ARUNA SETH

Department of Biology

Microbiology and Immunology Division

Derring Hall, Virginia Tech

Blacksburg, Virginia 24061

(703) 231-5029

Personal Information:

Date of birth: October 21, 1963, New Delhi, India.

Citizenship: Indian.

Educational Background:

Virginia Polytechnic Institute and State University. 3/87 to 12/90. Ph. D degree

Department of Biology, Division of Microbiology and Immunology.

Dr. Prakash S. Nagarkatti, Advisor.

Research: Age-associated alterations in the immune system of normal and autoimmune-susceptible mice.

University of Delhi. 9/84 to 6/86. M. Sc. Degree (Microbiology)

University of Delhi. 7/80 to 6/83. B. Sc. Degree (General Science)

Professional Experience:

Junior Research Fellowship:

All India Institute of Medical Sciences, New Delhi, India. 8/86 to 3/87.

Project: Experimental vaccine against *Pseudomonas aeruginosa* infections

Research Assistantships: VPI & SU, Department of Biology.

Summer, 1987. (Dr. P. S. Nagarkatti).

Summer, 1988. (Dr. P. S. Nagarkatti).

Summer, 1989. (Dr. P. S. Nagarkatti).

Summer, 1990. (Dr. P. S. Nagarkatti).

Fall, 1990. (Dr. P. S. Nagarkatti).

Graduate Teaching Assistantships: VPI & SU, Department of Biology.

General Biology Laboratory (Spring Quarter, 1987).

General Biology Laboratory (Fall Quarter, 1987).

General Microbiology Laboratory (Winter Quarter, 1988).

General Microbiology Laboratory (Spring Quarter, 1988).

General Microbiology Laboratory (Fall Semester, 1988).

General Microbiology Laboratory (Spring Semester, 1989).

General Microbiology Laboratory (Fall Semester, 1989).

General Microbiology Laboratory (Spring Semester, 1990).

General Biology Laboratory (1st Summer Session, 1990).

Awards:

American Foundation for Aging Research (AFAR):

Fellowship award of $ 500.00.

Department of Biology:

Tuition Waiver (Fall Semester, 1988).

Tuition Waiver (Fall Semester, 1989).

Award of $ 500 to match AFAR fellowship.

Publications:

1. Seth, A., R. H. Pyle, M. Nagarkatti and P. S. Nagarkatti. Expression of the J11d Marker on $CD4^+$ $CD8^+$ peripheral T lymphocytes of MRL-lpr/lpr mice. 1988. **Journal of Immunology** *141* : 1120-1125.

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Presentations at National/Regional Meetings and Symposia:

1. Nagarkatti, P. S., A. Seth and M. Nagarkatti. Expression of the J11d marker on $CD4^+$ $CD8^+$ peripheral T lymphocytes of MRL-lpr/lpr mice. Poster presented at the National Meeting of the American Association of Immunologists (AAI) held in Las Vegas, May 1988.

2. Seth, A., P. S. Nagarkatti, M. Nagarkatti, V. Udhayakumar and B. Subbarao. Impaired autoreactive T cell- induced T cell- T cell interaction in aged mice. Presented at a mini-symposium at the National AAI meeting, May 1988.

3. Subbarao, B., M. Natarajan, A. Seth, M. Nagarkatti and P. S. Nagarkatti. A specific defect in the ability of B lymphocytes from old mice to proliferate in response to stimulation with and autoreactive T cell clone Poster presented at the National AAI meeting, May 1988.

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8. Seth, A., M. Nagarkatti and P. S. Nagarkatti. Macrophages but not B cells from aged mice are defective in stimulating autoreactive T cells *in vitro*. Presented as a poster at the 6th Annual Graduate Research Symposium at Virginia Tech., October 1989.

9. Seth, A., V. Udhayakumar, B. Subbarao, M. Nagarkatti and P. S. Nagarkatti. Impaired autoreactive T cell responses in aged mice. Poster presented at the National AAI meeting at New Orleans, March 1989.

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Presentations at International Meetings and Symposia:

1. Subbarao, B., V. Udhayakumar, M. Natarajan, R. Cross, A. Seth, M. Nagarkatti and P. S. Nagarkatti. Abnormalities of intrinsic B and T lymphocyte function in old mice. Presented at the 5th International Symposium on Infections in the immunocomprised host held in the Netherlands in June, 1988.

2. Nagarkatti, P. S., A. Seth, V. N. Kakkanaiah and M. Nagarkatti. $CD4^+$ autoreactive T cell clones having the properties of both T_H1 and T_H2 type of T helper cells. Presented at the 7th International Congress of Immunology (a special workshop) at Berlin, July 30 - August 5, 1989.

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Professional Memberships:

American Association of Immunologists (Student member since 1987).

Anna Seth