AGE-RELATED CHANGES IN MURINE T CELL FUNCTION

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AGE-RELATED CHANGES IN MURINE T CELL FUNCTION

Leeftijdsafhankelijke veranderingen in het functioneren van muize T cellen

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AAN MIJN OUDERS

CONTENTS

CHAPTER 1

INTRODUCTION

1.1. GENERAL REMARKS ABOUT THE IMMUNE SYSTEM

The immunological apparatus is indispensable in order to survive many kinds of infections. The immune system recognizes the presence of foreign material or modified substances of the body itself and triggers an elimination reaction. Each entity that induces an immune reaction is called an antigen. The immune system is based upon specific recognition of an antigen and selective elimination. The immune systems consists of many morphologically and functionally different cell types that are all derived from a pluripotent hemopoietic stem cell in the bone marrow (Fig. 1). From this cell the precursor cells of the lymphoid, myeloid, thrombocytic and erythroid lineages are formed. The lymphoid compartment contains at least two major cell types: B cells and T cells.

The B cells mature and differentiate to cells that can produce antibodies (immunoglobulins). Antibodies, assisted by complement and phagocytic cells can eliminate extracellular pathogens from the body fluids.

The T cells mature and differentiate for a great part in the thymus. They finally differentiate into at least four different cell types: helper T cells (Th), suppressor T cells (Ts), cytotoxic T cells (Tc), and delayed type hypersensitivity (DTH) T cells (Tdth) or Th1 (1). Whereas Tc and Tdth represent effector functions, Th and Ts are regulatory cells. The helper and suppressive activities are considered as an essential component of the immune system to keep the course and size of an immune response under control.

The myeloid lineages can be split into three sublines called macrophages, dendritic cells and polymorphonuclear leukocytes. The function of both macrophages and dendritic cells in the immune response is to present antigen

to B and T cells. The dendritic cells have no extensive phagocytic properties and therefore present small antigens without extensively degrading them (2,3). Macrophages, in contrast, do phagocytose large corpuscular material and degrade this into smaller antigenic fragments (2,3). The polymorphonuclear leukocytes can be divided into three subtypes, the neutrophylic, eosinophylic and basophylic leukocytes. The function of these cells, especially the neutrophylic leukocytes, will be discussed later with regard to their contribution in DTH reactions.

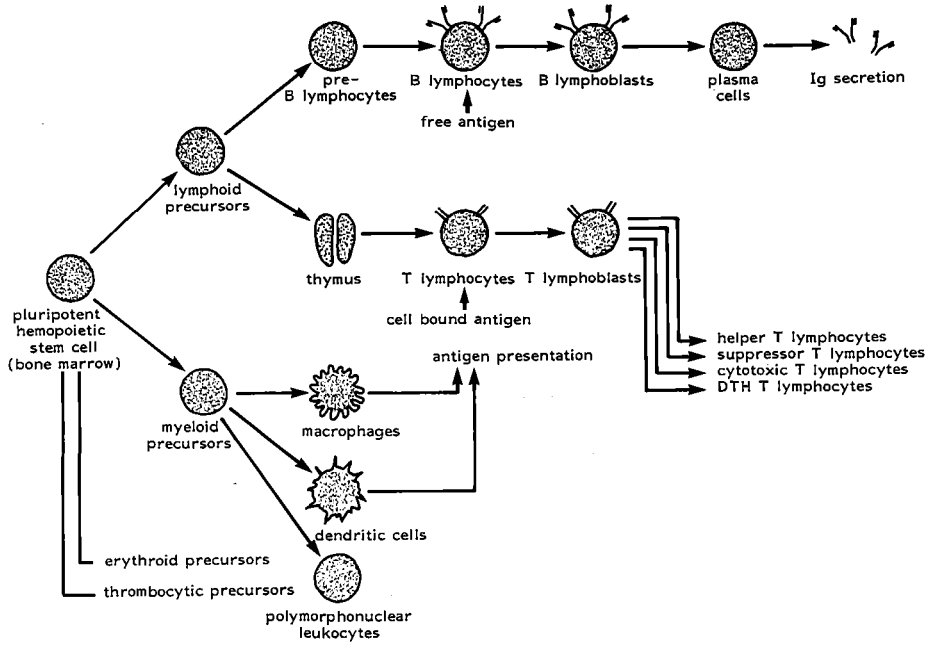

Fig.1 *Schematic representation of the cells involved in an immune response. All cells are derived from pluripotent stem cells. They give rise to lymphoid, myeloid, erythroid and thrombocytic precursors. The lymphoid precursor has the potential to differentiate into either T or B cells depending on the microenvironment to which it "homes", that is thymus or bone marrow/fetal liver. The B cells differentiate to cells that can produce antibodies (immunoglobulins). The T cells differentiate into at least four different cell types: helper T lymphocytes, suppressor T lymphocytes, cytotoxic T lymphocytes or DTH T lymphocytes. The myeloid precursors can differentiate to macrophages, dendritic cells or polymorphonuclear cells.*

1.1.1. ANTIGEN RECOGNITION

The recognition of antigen by B and T cells shows elements of similarity and difference. Both T and B cells use a specific receptor. However, whereas the B cell receptor for antigen (i.e. immunoglobulin) can bind to the antigen alone, the T cells recognize antigen only in the context of products of the major histocompatibility complex (MHC)(4,5). Moreover, T cell responses are controlled or guided by products of the MHC. The murine MHC is called the H-2 complex (6) and consists of a set of linked genes encoding four classes of molecules, of which the class I (K, D and L) and class II (I-A and I-E) cell surface molecules known to be relevant in this context.

Class I molecules consist of a heavy and a light chain glycoprotein (Fig. 2). The heavy chain consists of an intracellular, a transmembrane and an extracellular fragment, of which the latter carries two highly polymorphic domains (α_1 and α_2). The light chain (β_2-microglobulin) is noncovalently attached to the heavy chain. Class I molecules are found on the cell surface of all nucleated cells (7). These molecules are important for instance during a viral infection. In such cases, the viral antigen is presented on the cell surface of infected cells in association with the MHC class I molecules and recognized by Tc. As a result the infected cell will be killed by the Tc. This process will take place only if the viral antigen is presented in the context of self class I molecules.

Class II molecules consist of two noncovalently bound glycoproteins, two α chains and two β chains. The β chain (β_1 and β_2) carries the polymorphic region (Fig. 2). Class II molecules are expressed on the surface of B cells, endothelial cells, Langerhans cells, monocytes/macrophages, dendritic cells and activated T cells (8-15). The class II molecules also have a function in presenting antigen by antigen presenting cells (APC). The APC activate Th and thereby initiate an antigen specific immune response. Also the DTH reaction against bacterial (e.g. Listeria monocytogenes) and xenogeneic antigens is restricted by H-2I coded antigens (16). The interactionbetween APC and Th is thus restricted by class II MHC molecules.

Besides the class II MHC molecules, also the lymphokine interleukin-1 (IL-1) plays an important role in T cell activation (17,18). Historically, IL-1

has been proposed to be a product of macrophages. However, in recent years it appeared that many cell types are able to produce IL-1 (19-25). It is assumed that cell contact in a class II restricted interaction between activated T cells and macrophages induces the release of IL-1 during an immune response. IL-1 affects a variety of target cells involved in an immune response e.g. polymorphonuclear leukocytes, B cells (reviewed in 26), and T cells (27-29). In addition, IL-1 acts directly on T cells, e.g. to induce the release of other lymphokines, like IL-2, or the responsiveness to IL-2 (29,30).

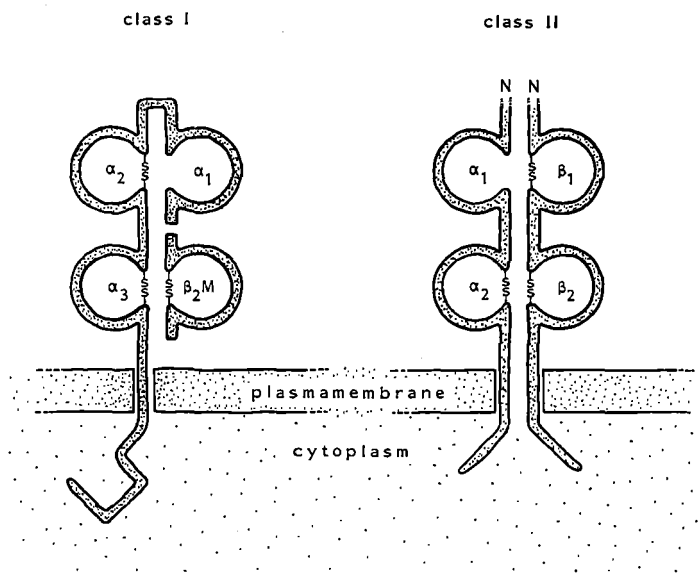

Fig.2 *Schematic organisation of class I and class II molecules. The MHC-encoded chain of class I molecules has three globular domains (termed α1, α2 and α3). The β2-microglobulin (β2m) is a small globular peptide. A short hydrophobic section of the MHC-encoded component lies within the cytoplasm. The class II molecules consist of two non-identical peptides (α and β) that are non-covalently bound and lie partly within the cytoplasm. Both chains have two globular domains.*

1.1.2. RECEPTORS AND CLUSTERS OF DIFFERENTIATION INVOLVED IN CELLULAR INTERACTION

Since the introduction by Köhler and Milstein (31), the technique of producing monoclonal antibodies (Mab) has been applied to generate highly specific reagents against a large variety of antigenic determinants, including those on the cell surface of leukocytes. Subsets of T lymphocytes can be distinguished through different cell surface determinants called clusters of differentiation (CD) and through the expression of a variety of receptors. The development of Mab reactive with such antigens now permits the isolation and specific staining of subpopulations of T cells. Furthermore, by monitoring the effects of these Mab in in vivo and in vitro assays, the role of CD and receptors in cellular interactions during an immune response can be studied. In Table 1 an overview is given of the major functional T cell structures. The functional properties of a number of relevant determinants will be discussed below.

1.1.3. T CELL ACTIVATION

T cells recognize antigen via the T cell receptor (TCR)(32,33). The TCR is composed of a disulfide α/β heterodimer noncovalently associated with CD3, a multipolypeptide cell membrane complex (34). This is true for 95–98% of all peripheral blood lymphocytes. Recently, T cell clones were generated with a $CD3^+$ phenotype and lacking the α and β chain. A new T cell receptor was reported, which consisted of a TCR γ/δ (35). Cells expressing TCR γ/δ can be found in 2–5% of the periphery (36). For murine T cells TCR γ/δ (37) has also been reported. During the recognition of antigen by the TCR, the avidity of the interaction between the T cell and the APC is increased by binding of CD4 and CD8 determinants from the T cell to the monomorphic regions of class II and class I MHC molecules on the surface of the APC, respectively (38,39). Therefore, T cells expressing CD4 molecules are regarded as class II–MHC restricted T cells (Fig. 3). T cells expressing CD8 molecules are known as class I–MHC restricted T cells. The expression of CD4 and CD8 molecules is considered to be restricted to functionally distinct populations

Table 1: Major receptors and clusters of differentiation (CD) on T cells involved in cellular interaction

Receptor/CD	cellular distribution	function
TcRαβ	95–98% peripheral T cells	clonotypic receptor for antigen, associated with CD3. MHC–restricted recognition.
$TCR_{\gamma}\delta$	2–5% peripheral T cells	clonotypic receptor for antigen, associated with CD3. MHC non restricted recognition.
IL–2 receptor		receptor for IL–2.
– p55	activated T cells	
– p75	resting and activated T cells	
IL–1 receptor	T cells, B cells	receptor for IL–1.
CD2	all T cells	receptor for SRBC. alternative receptor for lymphocyte activation.
CD3	all peripheral T cells	associated with TCR. essential for TCR expression and antigen mediated T cell activation.
CD4	50–70% peripheral T cells	involved in class II specific and restricted responses.
CD8	20–30% peripheral T cells	involved in class I specific and restricted responses.
LFA–1	all leukocytes	associated with cell adhesion. involved in Tc–mediated lysis, T cell proliferation.

of T lymphocytes, i.e. Th and Tc. Recently, however, $CD4^+$ T cells with cytotoxic functions and $CD8^+$ T cells with helper function have been reported (40–43).

Besides adhesion function of the class II and I molecules these molecules are also recognized by the TCR. From work with $CD4^+$ T cell lines, which express the TCR α/β, it is concluded that the Vβ region (variable β chain region) of the TCR has a low affinity for self MHC, whereas the Vα region has a high affinity for non–self MHC.

Once an antigen is appropriately presented, its interaction with TCR leads to perturbation of the TCR–CD3 complex (44) (Fig. 4). As a result phosphodiesterase (PDE) catalyzes the hydrolysis of phosphatidylinositol biphosphate (PIP_2) to inositol triphosphate (IP_3) and diaglycerol (DAG). These products are also known as second messengers. IP_3 is released from the membrane in the cytoplasm where it mobilizes Ca^{2+}. This Ca^{2+} release causes an increase of $[Ca^{2+}]$; DAG activates protein kinase C (PKC) and increases $[Ca^{2+}]$ (45–48). Antigen–dependent T cell activation can be mimicked by stimulation with phorbol myristate acetate (PMA), which directly activates PKC (49), and calcium ionophores (i.e. ionomycin), which directly

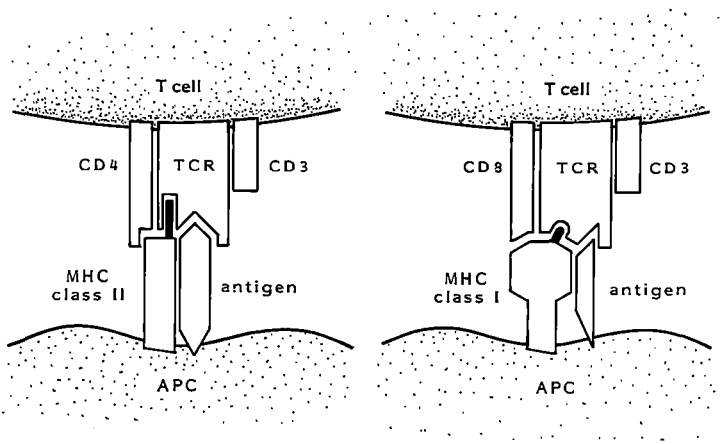

Fig.3 *Schematic representation of antigen recognition by T cells. MHC class II restricted T cells possessing the CD4 molecule recognize MHC class II in combination with antigen. MHC class I restricted cells possess a CD8 molecule and recognize MHC class I in combination with antigen. Activation signal is transmitted into the T cell via CD3, which is associated with the TCR.*

induces increase of $[Ca^{2+}]_i$ (50). The synergistic cooperation of PMA and ionomycin therefore induces T cell activation. The activation events lead to the initiation of a second stage event, in which previously untranscribed genes become expressed (51). The products of these genes are considered to function in T cell activation and proliferation: for instance the production of IL-2 and the (inducible) IL-2 receptor (reviewed in 52).

IL-2 is an important lymphokine for T cells and delivers various signals by interacting with the IL-2 receptor (IL-2R) (53-55). Furthermore, the IL-2/IL-2R system appears to be an important tool in the developmental control and amplification of the T cell mediated immune system. The IL-2R can also be expressed on non T cells, including B cells (56), large granular lymphocytes (57) and non-lymphoid cells (58). Therefore, numerous biological activities have been ascribed to IL-2.

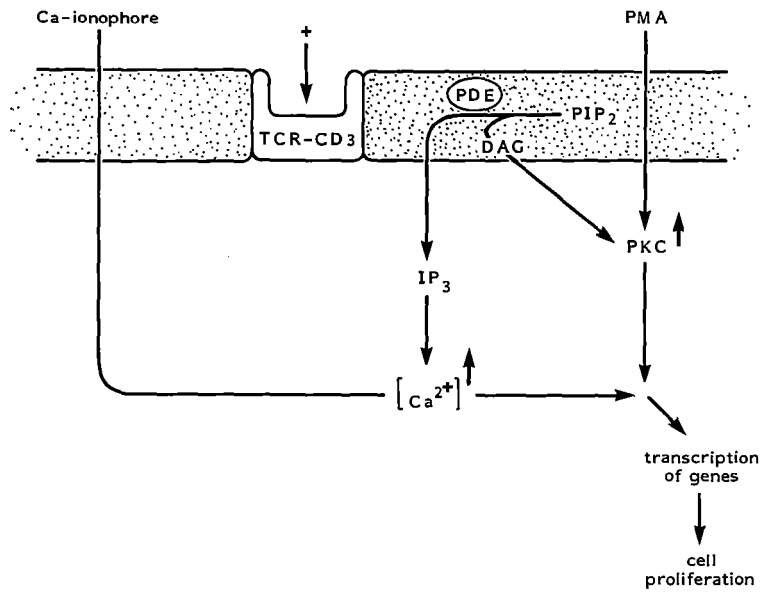

Fig.4 *Schematic representation of T cell activation. Upon appropriate presentation of an antigen perturbation of TCR-CD3 takes place. As a result phosphodiesterase (PDE) catalyzes the hydrolysis of phosphatidylinositol biphosphate (PIP_2) to inositol triphosphate (IP_3) and diaglycerol (DAG). IP_3 mobilizes $Ca2^+$ and DAG activates protein kinase C (PKC). Antigen-dependent T cell activation can be mimicked by stimulation with phorbol 12-myristate 13-acetate.*

1.1.4. IMMUNE RESPONSES

Upon contact with an antigen, a variety of immune reactions can develop depending on the route of administration, the dose of antigen and the type of antigen (59,60). Foreign antigens like virus fragments or bacteria are recognized by T cells in association with self MHC-encoded proteins, the so called MHC restriction phenomenon. It is well established that under physiological conditions, in which the body is intruded with viruses or bacteria, $CD4^+$ T cells are class II restricted, whereas $CD8^+$ T cells are class I restricted. T lymphocytes can also respond to polymorphic differences on MHC molecules. It appears that class I and class II MHC determinants have polymorphic sites, which may serve as alloantigenic sites. For class I the α_1 and α_2 domains and the carbohydrate unit attached to the α_2 domain are known, whereas for class II the β chain contains the alloantigenic sites. This type of response is better known as allogeneic response. In addition, a striking feature of the T cell repertoire is the high frequency of cells specific for allo-determinants encoded for by genes of the MHC (61). The MHC-restriction of an allogeneic response remains conjectural (62,63).

Since this thesis deals with T cell immunity, especially DTH reactions and MLR, these two types of immune reactions will be discussed in more detail.

1.1.4.1. DELAYED TYPE HYPERSENSITIVITY

Delayed type hypersensitivity (DTH) can be defined as an immunologically specific inflammatory reaction which is maximal at 24 to 48 hours after elicitation. In mice it shows a characteristic histological appearance of infiltration of granulocytes and mononuclear cells and of increase of vascular permeability and edema (64). DTH reactivity has been most widely studied in species such as guinea pig, rat and mouse, employing antigens diverging from bacteria, viruses, fungi, protozoa and xenogeneic erythrocytes to allogeneic histocompatibility antigens (65,66). In man, DTH related phenomena are believed to play a role during the course of diseases such as tuberculosis, sarcoidosis, leprosy, listeriosis, leishmania and Chagas'

disease.

DTH reactions in mice can be transferred with lymphoid cells from a sensitized donor to a naive syngeneic recipient (67). The DTH in mice can be manifested in vivo in the footpad (68). Instead of the footpad the antigen can also be applicated to the ear (69). As a result of the inflammatory reaction in the footpad an increase in foot thickness is observed. The increase in foot thickness can be considered as a measure for the DTH reaction.

In DTH an early and a late component can be observed. Studies on the early and late component of the DTH reaction were performed using picrylchloride as antigen. The increase in foot thickness is a time-dependent process, which shows a peak at 2-4 hours after injection (early component), and triggers the release of the vasoactive amine serotonin from mast cells (70). Serotonin causes an increased local vascular permeability and is accompanied by intravascular accumulation of leukocytes at the reaction site. The late classical component of DTH appears at 24 hours after challenge (71). This component is accompanied by extravascular infiltrates of leukocytes and results in skin swelling. The occurrence of the late component of DTH depends on the occurrence of the preceeding early component. Whereas the early component is not genetically restricted and can be transferred across H-2 barriers, the late component is H-2 restricted.

It is generally accepted that recognition of Sheep Red Blood Cells (SRBC) and minor H-antigens by DTH effector cells is restricted by H-2 coded molecules (72-74). Transfer of DTH to SRBC is mediated by Lyt-1^+ T cells and is class II restricted (72). In classical DTH responses it is thought that $CD4^+$ T cells upon presentation of antigen in the context of MHC molecules by an APC develop into Tdth inducer cells. In response to the second encounter with the (locally injected) antigen, presented in the context of MHC molecules, these cells develop into Tdth effector cells, which attract the nonspecific bone marrow derived macrophages and granulocytes that give the DTH inflammation (Fig. 5). For transfer of DTH to most antigens, class II determinants function as restriction elements (75-77), although class I H-2 compatibility appears sometimes necessary for transfer of DTH to minor H-antigens (73) and viral antigens (78). As for alloantigens DTH reactions to SRBC are transferred by Lyt-1^+ T cells as determined using T cell lines

(79,80). It appeared that these cell lines could express DTH reactivity across a H-2 barrier. Therefore these investigators concluded that H-2 specific DTH effector cells are unrestricted. On the other hand Bianchi et al. (81) showed that anti-class I DTH responses are class II restricted, whereas anti-class II DTH responses are class I restricted. Based on these findings, H-2 specific DTH effector cells are considered to be H-2 restricted.

The appearance of the early and late component of the DTH reaction is described for test systems, in which mice are immunized and subsequently challenged with the antigen locally. Another possibility to measure DTH reactivity is the use of "local passive transfer". In this case mice are immunized and after some days the spleen or lymph nodes are removed. The cells from these organs are then transferred to young syngeneic naive recipients in the footpad or ear. Using such a local passive transfer many antigen-specific, MHC-restricted T cell lines and clones have been described to transfer DTH. However, most of these clones have not been reported to transfer DTH systemically (82-84). This failure was probably due to the lack of the early component. With the use of the local passive transfer the ability to mediate a DTH reaction is solely restricted to the capacity of Tdth cells (late component) to trigger an infiltrate consisting of granulocytes and mononuclear cells and by increased vascular permeability and edema.

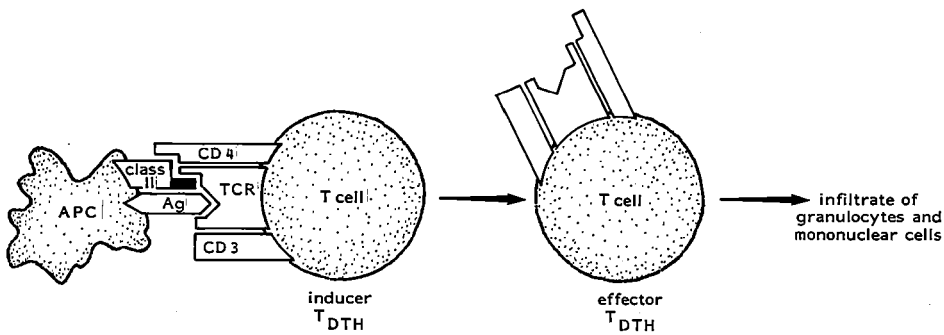

Fig.5 *Schematic representation of a classical DTH response. $CD4^+$ T cells develop upon presentation of antigen in the context of class II molecules into inducer Tdth cells. After reencounter with the antigen these develop into effector Tdth cells, which attract the nonspecific bone marrow derived macrophages and granulocytes that give the DTH inflammation.*

1.1.4.2. MIXED LEUKOCYTE REACTION

The mixed leukocyte reaction (MLR) is an immune response, in which immunological "defense" reactions to alloantigens are studied in vitro. The MLR is a complex interaction of cells of different phenotypes. With MLR elicited by unseparated T cells, it is well accepted that the stimulus for MLR is provided by Ia^+ cells (85), either by macrophages (86) or dendritic cells (87). In general, in an MLR, it is the $CD4^+$ T cell that reacts to allo class II determinants, whereas $CD8^+$ T cells respond predominantly to allo class I molecules. Furthermore, the $CD4^+$ T cell subset provides the proliferative signals by producing IL-2. The proliferation of other cells, i.e. the $CD8^+$ T cells, is secondary to this signal, as they need to be propagated by helper signals such as IL-2. The MHC restriction of this type of response remains elusive (88).

1.2. AGING

As every other organ system the immune system is subject to aging. One of the consequences of aging in higher animals is the deterioration of the immune system. Because of the role of the immune system in maintaining homeostasis, such a deterioration is said to predispose animals to infectious diseases, malignancy and auto-immune diseases (89,90). Age-associated changes in immune reactivity have been demonstrated for most of the responses. Results of these studies show that various immunological activities are altered, i.e., certain immunological activities decrease, others show no change, and still others increase. The polymorphic effects of aging on immune response indexes underscore the complexity of the mechanisms involved in aging of the immune system. In the subsequent paragraphs age-related effects on the antigen presenting cell system and the B cell system will be discussed briefly, whereas the T cell system will be discussed in more detail.

1.2.1. ANTIGEN PRESENTING CELLS

When antigens enter the body, they are caught by antigen presenting cells (APC). These APC subsequently activate Th and an antigen specific immune response is initiated. The investigations on the antigen presenting functions of monocytes, neutrophils and phagocytic cells in relation to aging is of relatively recent date and the results are rather contradictory. Cell adherence in general is not changed (91). Ingestation of particulate antigens, however, has been reported to decrease (92) or to remain unchanged in aged humans (93). Animal studies, although less frequent, present a similar confusing picture. Fc-mediated ingestion of opsonized antigens or immune complexes show contradictory results (94-96). Clearance of micro-organisms from the circulation in general is decreased in mouse (97,98).

At the level of antigen presentation by macrophages some controversial results were found. According to one group, age-related defects in the capacity of macrophages of old animals occur (99). It appeared that removal of adherent cells from senescent F344 rat spleen cells restored the decreased mitogen responsiveness. In another study, however, it was reported that adherent cells from young animals were not able to restore the response of spleen cells from aged animals (100). This suggests that the immunological defect in old age is not due to changes in the APC system.

One of the products of macrophages during an immune response is IL-1. IL-1 is regarded as a pleiotropic lymphokine that affects T cells as well as many other cell types. In aging studies some controversial results were found at the level of IL-1 production. One group found a decreased capacity of IL-1 production by macrophages from old animals (101), whereas in another report no change in the IL-1 production ability of macrophages was observed (102). Thus, during aging defects in antigen processing and presention are observed. However, the immunological defect in old age could only partly be explained by these changes.

1.2.2. B CELL SYSTEM

B cells differentiate into cells that can produce antibodies (immunoglobulins). Antibodies, assisted by complement and phagocytic cells can eliminate foreign material as viruses, bacteria and other microorganisms from the body fluids.

In 1929, Thompson and Kettel (103) showed that the titer of natural haemagglutinins in human subjects decreased with age. Makinodan and coworkers (104) were the first to approach immunological aging experimentally. They showed that old mice responded with only 10% of the number of plaque forming cells after challenge with SRBC when compared with young mice. Later studies confirmed these observations for a number of different antigens. Thus, the responsiveness of B cells as measured by antibody formation is affected to some extent by aging. The major defect in this type of response appeared to be associated with the primary response, since the secondary responses appeared to be much less affected (105–110).

A remarkable phenomenon during aging is the appearance of monoclonal gammapathies (MG) (111). MG are the result of proliferation disorders of individual B-cell clones. MG may be divided into a transient and a persistent state. Persistent MGs can be of benign (BMG) or malignant (MMG) nature. BMG may be considered as a characteristic of the senescent immune system. A hypothesis has been put forward to explain the BMG phenomenon (111). It is assumed that a selective age-related defect in the T cell compartment occurs allowing excess proliferation of some B cell clones.

1.2.3. T CELL SYSTEM

In contrast to the relatively slight changes in the APC and B cell system during aging, a substantial loss of effector function is observed for the T cell system as measured by T cell-mediated immunity. With increasing age, a decreased cytotoxic response of spleen cells from old mice to alloantigens (112–114) and to modified-self (115,116) has been reported. In the regulation of cellular immunity Th have an essential role: they are involved in

the induction of DTH reactions, in which foreign material is eliminated, in the induction of allogeneic proliferation as measured in a MLR, and they mediate diverse immunomodulatory effects by production of lymphokines. Th also function in the expansion of Tc.

With the use of mitogenic stimuli antigen-non-specific responses can be induced. It is shown in numerous reports that the ability of T cells from old animals to respond to such stimuli is decreased compared to the ability of cells from young animals (117-120). Mitogen responses are in vitro induced responses, which reflect the immune responsiveness of the animal tested. A more direct way to assess T cell function is the DTH response. The DTH reaction, an in vivo induced effector function of the T cell system, showed a clear age-related decline in mice (121,122). This is shown for the TNP response of young and old BALB/c mice and for the contact sensitivity response of young and old humans.

The effector T cell function can also be measured in vitro. This can be done with a MLR. Also for the MLR an age-related decline has been observed (123-125). As described previously, IL-2 plays an important role in the magnitude of the MLR. It appeared from several studies that the IL-2 production ability in old mice is impaired (117,125,126). This decline in IL-2 production is assumed to play a role in the age-related decrease of the MLR.

1.2.4. POSSIBLE EXPLANATIONS FOR THE AGE-RELATED DECLINE IN THE T CELL SYSTEM

Comparison of the three systems described above, shows that in particular effector T cell functions appear to decline with age, whereas functions of B cells and the APC system are affected to a much lesser extent (Fig. 6). Therefore, the decline in effector T cell function will be further analyzed.

In order to explain these changes one can think of a variety of possibilities:

- a reduced number of cells, which is capable to respond (Fig. 7.1)
- an imbalance in the enhancing (Fig. 7.2) and suppressive (Fig. 7.3) activities of regulatory T cells.

- a defect at the level of antigen recognition (Fig. 7.4)
- a defect at the level of the formation of second messengers (Fig. 7.5)
- a reduction in the IL-2 production ability (Fig. 7.6)
- a defect at the level of IL-2 receptor expression (Fig. 7.7)
- combination of the above mentioned possibilities.

These possibilities (Figs. 7.1–7.7) will be discussed in the next three sections. In the first section the first possibility (Fig. 7.1), the so called quantitative changes, in the second section the activities of regulatory T cells (Figs. 7.2 and 7.3); and in the third section the remaining aspects (Figs. 7.4–7.7), the so called qualitative changes will be discussed.

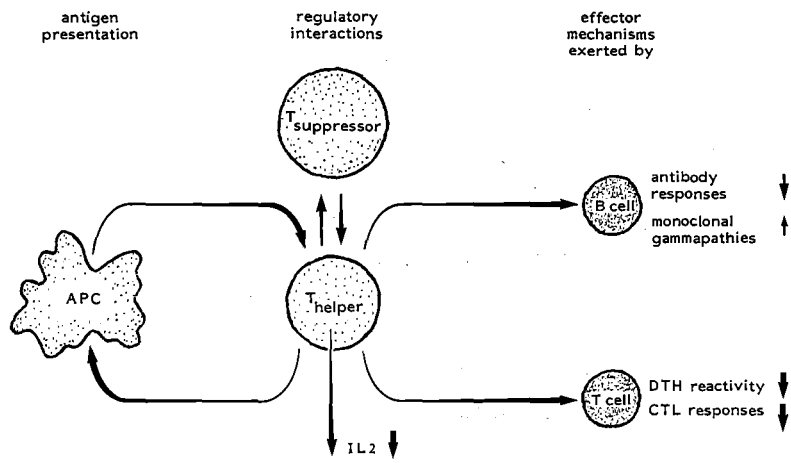

Fig.6 Schematic representation of the age-related changes in immune responsiveness. At the level of regulatory interactions an age-related decline in IL-2 production is observed. At the level of effector functions the B cell responses are affected to some extent, whereas the T cell responses show a clearcut decrease.

1.2.4.1. QUANTITATIVE CHANGES

Quantitative changes can be considered as changes in the number of T cells capable to respond to an antigen (precursor frequencies). Precursor frequencies are determined in a limiting dilution assay (LDA). LDA analysis permits the estimation of precursor frequency when the responder cells are the only limiting factor in the culture. Decreases have been reported for precursor frequencies during aging. A reduced frequency of alloreactive CTL precursors was observed in aged mice (127–129). Moreover, a decreased specificity of the response was observed: Tc activated in vivo by immunization of old mice with allogeneic cells were more likely to kill third party and isologous cells than those from young mice (114). In another report, however, no evidence for impaired specificity of Tc was observed (127).

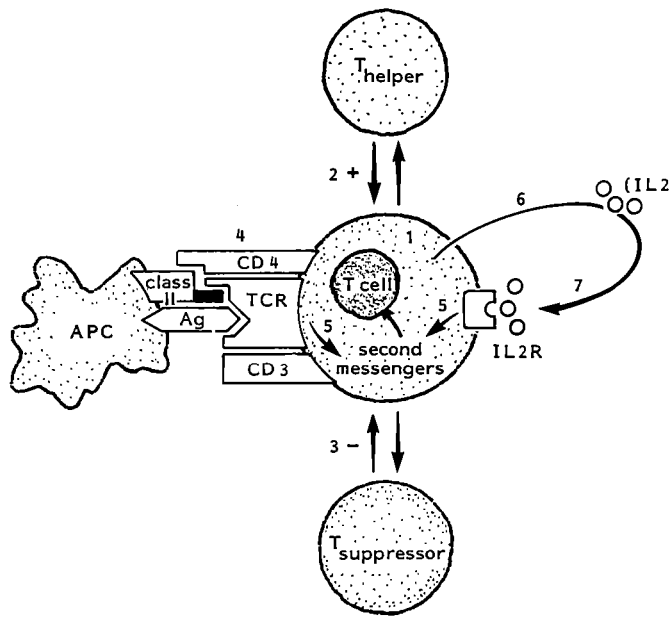

Fig. 7 Schematic representation of possible age-related changes at various levels. The CD4$^+$ T cell might be affected during aging at the level of precursor frequency (Fig. 7.1) or of suppressive (Fig. 7.2) or helper (Fig. 7.3) influences. CD4$^+$ T cells might also be affected in the expression of CD4 (Fig. 7.4), in the formation of second messengers (Fig. 7.5), in the production of IL-2 (Fig. 7.6) or in the IL-2 receptor (IL-2R) expression (Fig. 7.7).

Upon mitogenic stimulation the number of responder cells was also found to be decreased as measured by proliferation and IL-2 production (130-132). Thus, a reduced precursor frequency is observed for a number of T cell-mediated immune reactions. Since no changes or at most only a moderate decrease in the total number of T cells in spleen and lymph nodes was observed (133) the reduced precursor frequency seems to be the major quantitative contribution to the impaired immune reactivity.

1.2.4.2. REGULATORY IMBALANCE

Besides changes in precursor frequencies changes in the imbalance of regulatory cells may also attribute to the decline in immune responsiveness. The rate and magnitude of the immune responses are subject to regulation by helper and suppressive activities exerted by T cells. An imbalance of these activities can contribute to the impaired immune functions of old animals. Functionally, a decrease in helper activities has been reported (132,134,135). For suppressive activities exerted by T cells a decrease (136-138) as well as an increase (139-141) has been observed. Although controversial, these results nevertheless indicate that a shift in regulatory activities might occur with increasing age.

1.2.4.3. QUALITATIVE CHANGES

The outcome of an immune response is not only influenced by precursor frequencies or regulatory activities, but also by the effect generated per cell. Age-related changes on this level, such as alterations of the activation process may also contribute significantly to the decline in immune responsiveness.

T cells recognize antigen with the TCR. The TCR is responsible for the transduction of the activating signal to the interior of the cell and for initiating the cellular response to the antigen. In the interior of the cell a second stage of events will take place, through which new gene systems

become expressed. For instance, the genes encoding the lymphokine IL-2 and those encoding the inducible IL-2 receptor (IL-2R). Both the production of IL-2 and the expression of the IL-2R determine to a great extent the magnitude and duration of the response. With age considerably fewer IL-2 receptors are detected on antigen/mitogen activated blast cells from old animals as judged by the ability of these cells to bind IL-2 (134). At the initial stage of T cell activation signals are transduced to the interior of the cell. This process is among other biochemical events characterized by the uptake of Ca^{2+}-ions. In humans, a decrease in ionized calcium uptake in T cells of old individuals stimulated by mitogen was observed (142). This suggests that in cells from old individuals the process of signal transduction is also affected.

In conclusion, not only quantitative and regulatory but also qualitative defects may cause either independently or in combination the observed decline in T cell responsiveness during aging.

1.3. INTRODUCTION TO THE EXPERIMENTAL WORK

The aim of the studies presented here was to obtain a more detailed and integrated picture of the age-related changes in cellular immunity.

The age-related changes of cellular immunity were studied by in vivo induction of DTH responses to a variety of antigens (**Chapters 2 and 3**). The results show that the capacity to elicit a DTH responses to SRBC, non H-2 and H-2 antigens declines during aging. This decline is observed in two mouse strains tested. Because the underlying causes of impaired DTH responsiveness are not easily studied in vivo, in vitro induction of DTH responses to H-2 antigens was studied (**Chapter 3**). It appeared that the $CD4^+$ T cells give rise to the DTH reactive T cells and that the capacity of old mice to generate DTH reactive T cells in vitro is decreased. Since it was also shown that the IL-2 production ability of the mouse strains tested is impaired with increasing age (**Chapter 2**), the effect of the addition of exogenous IL-2 was studied. The decline in the capacity of cells from old mice to generate DTH reactive cells in vitro, however, could not be restored by addition of exogenous IL-2.

More insight in possible causes for this age-related decrease of in vitro DTH responsiveness were obtained by studies on precursor frequency analysis of alloreactive $CD4^+$ and $CD8^+$ T cells in an MLR (Chapter 4). Especially, studies on the separate T cell populations were necessary, first since the in vitro induced DTH response is mediated by $CD4^+$ T cells, secondly because possible suppressive effects exerted by $CD8^+$ T cells on $CD4^+$ T cells should be excluded. The results indicate that the alloreactive precursor frequency of both $CD4^+$ and $CD8^+$ T cells declines with increasing age. The observed decline in the frequencies can, however, not completely explain the reduced proliferative capacity observed in the MLR. It may be that there exists a defect at the level of signal transduction into the interior of the cell. Therefore, studies were performed, aimed at the process of signal transduction of $CD4^+$ and $CD8^+$ T cells. With the use of PMA and ionomycin, that bypass the TCR, the cells were activated and studied for some functional aspects like proliferative capacity, IL-2 production ability and IL-2 receptor expression (Chapter 5). It appeared that this type of activation in contrast to allogeneic stimulation resulted in comparable IL-2 production by $CD4^+$ T cells from young and old mice. In contrast the proliferative responses of $CD4^+$ T cells from old mice were lower than those from young mice, whereas the proliferative responses of $CD8^+$ T cells from old mice were only slightly lower than those from young mice. Furthermore, the reduced capacity of $CD4^+$ T cells to proliferate was not reflected by a reduced IL-2 receptor (IL-2R) expression. The IL-2R expression on $CD8^+$ T cells was reduced on cells from old mice compared to cells from young mice. Therefore, it is most likely that the impaired immune responsiveness by T cells from aged mice is partly but not completely due to a reduced signal transduction process.

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CHAPTER 2

DETERIORATION OF CELLULAR IMMUNITY DURING AGING AS MEASURED BY DTH AND IL-2 PRODUCTION ABILITY

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SUMMARY

The effect of aging on the delayed type hypersensitivity (DTH) to sheep red blood cells (SRBC) in vivo and the interleukin 2 (IL-2) production capacity in vitro by spleen cells from young (17 weeks) and old (125 and 155 weeks) CBA/Rij and C57BL/Ka mice were investigated. For both CBA/Rij and C57BL/Ka mice an age-related decline in the DTH response to SRBC and the IL-2 production capacity was observed. Both parameters are mediated by Thy-1+,Lyt-2- spleen cells. For both mouse strains the proportion of Thy-1,Lyt-2- spleen cells declined less strongly with aging than the DTH reactivity and the IL-2 production capacity. Therefore it was concluded that not only a quantitative but also a qualitative decrease of T cell function occurs during senescence. It was also investigated whether the proportion of Thy-1+,Lyt-2- peripheral blood lymphocytes can be used as a predictive value with regard to the decline of DTH with aging of the corresponding mouse. This was indeed found to be the case in CBA/Rij mice, but not in C57BL/Ka mice.

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INTRODUCTION

As the individual ages, the immune system loses its effectiveness (1). A decreased response to newly introduced antigens and an increased frequency of lymphoproliferative disorders are regularly found. Furthermore, in aged individuals the susceptibility to infections is increased (2). There is ample evidence that the deterioration of the immune system is related to a decreased function of T cells (3,4). This decline in immune function appears to be mainly due to impairment of T helper cell activity (5–7).

The helper T cells play an important role in the induction of B cell responses, cytotoxic T cell responses, and responses of monocytes and macrophages in delayed type hypersensitivity (DTH) reactions. The helper T cells can be characterized on the basis of their surface markers. They have Thy–1 surface markers but lack Lyt–2 membrane antigens (8). The function of the regulatory forces can only indirectly be demonstrated.

The changes in the ability to induce B cell responses and, to a lesser extent, cytotoxic T cell responses in aging individuals have been described (9–12). DTH responses represent the capacity of the immune system to cope with various types of infections of intracellular microorganisms, such as *Mycobacterium lepraemurium* (13) and *Mycobacterium tuberculosis* (14). The elicitation of DTH requires a T cell dependent activation of mast cells to initiate a cascade of events (15). This type of immune response has gained relatively little attention with respect to its progression in aging. It has been shown that the DTH response to a panel of five antigens was severely impaired in individuals over 70 years of age. This reduced DTH responsiveness was shown to be correlated to increased mortality (16).

In order to investigate the age–related deterioration of DTH in detail we simultaneously determined in individual mice of different ages the ability to mount a DTH reaction to sheep red blood cells (SRBC) as well as the capacity to produce interleukin 2 (IL–2), which promotes growth of activated T cells. In addition, the proportion of T cells with the Thy–1 and Lyt–2 phenotype was determined using cells of the same suspension. The peripheral blood cells (PBL) with the Thy–1 and Lyt–2 surface markers were also enumerated. The aim was to investigate the predictive value of the frequency of Thy–1^+,Lyt–

2^- PBL with regard to the DTH responsiveness of the individuals.

In the present study we have used two mouse strains, CBA/Rij and C57BL/Ka, with a highly different age-related pathology. C57BL/Ka mice are more sensitive to age-related immune disorder than CBA/Rij mice. C57BL/Ka mice show a relatively high frequency of pathological lesions of the immune system with age (17); CBA/Rij mice, on the other hand, show an extremely low frequency of lymphoreticular pathological lesions. These age-related changes observed in C57BL/Ka mice may well be due to changes within the T cell system (18).

MATERIALS AND METHODS

Mice. C57BL/KaLwRij female and CBA/BrARij male mice (abbreviated in the text as C57BL/Ka and CBA/Rij, respectively) were bred and raised in the TNO-REP Institutes, Rijswijk, The Netherlands, under SPF conditions. Aged mice were obtained from cohorts of mice that were barrier maintained and regularly screened for age-related pathology (19).

Antigens. Sheep red blood cells were collected and stored in Alsever's solution at 4°C. Washed three times before use, they were diluted to the appropriate concentration in Hank's balanced salt solution (HBSS).

Peripheral blood cells. Before and after priming, about ten drops of blood were collected from the tail vein of each mouse in 0.25 ml of a 2% solution of Thromboliquine (Organon Teknika, Oss, The Netherlands) in HBSS. Peripheral blood leukocytes were separated on a one-step density gradient composed of 12.7% bovine serum albumin (BSA) (35% w/v in HBSS), 64% v/v Ficoll (9% w/v in H_2O) and 12.7% v/v Isopaque (440 mg/ml; Nyegaard, Oslo), diluted with HBBS (9.8%) (14). After centrifugation (15 min; 2500 g; 20°C) the white cells were collected, washed twice in 3.5 ml HBSS and resuspended in a volume of about 0.2 ml HBSS containing 0.1% w/v of BSA and 0.1% sodium azide.

Spleen cell suspension. All mice were anesthetized with ether before sacrifice. After killing, spleens were removed and placed in Hanks' Eagle's medium (Gibco Europe) supplemented with 15 mM Hepes (H+H). They were

squeezed through a nylongauze filter to provide single-cell suspensions. Viable cell counts were based on the trypan blue dye exclusion. Nucleated cells were counted with an Electrozone Celloscope counter.

Assay for adoptive DTH transfer. C57BL/Ka and CBA/Rij mice of different ages were intravenously (i.v.) immunized with $2x10^5$ and $1x10^6$ SRBC, respectively. After 5 or 4 days for C57BL/Ka and CBA/Rij mice, respectively, spleens were removed, prepared for single-cell suspensions, and tested for DTH reactivity. From dose-response and kinetic experiments in young animals, these conditions appeared to be optimal. Spleen cells from each donor mouse were transferred subcutaneously (s.c.) into the left hind footpad of five syngeneic naive recipients (local passive transfer). Each recipient received $5x10^6$ viable nucleated spleen cells plus 10^8 SRBC in a volume of 0.05 ml HBBS. The DTH reaction was measured as the relative increase in thickness of the contra-lateral noninjected hind foot. The swelling due to the DTH reaction was measured at 18, 24, 48 and 72 h after transfer. The increase in foot thickness of the recipient mice in all experiments was corrected for the nonspecific swelling caused by a challenge dose of 10^8 SRBC only. Nonspecific foot swelling caused by transfer of immunized spleen cells was always less than that observed after transfer of the challenge dose of SRBC only.

IL-2 assay. Spleen cells were suspended at $15x10^6$ viable cells per milliliter of complete RPMI medium (RPMI 1640 supplemented with 10% fetal calf serum (FCS), L-glutamine (2 mM), Hepes (20 mM), penicillin (100 IU/ml), streptomycin (0.1 mg/ml), $NaHCO_3$ (24 mM), and 2-mercaptoethanol ($5x10^{-5}$ M). One milliliter of the suspension was incubated with Con A (5 µg/ml concanavalin A) for 24 h at 37°C, 5% CO_2 in culture plates (Costar 3524). Supernatants were harvested, filtered through 0.45 µm-pore filters, and stored at -20°C until the time of testing. IL-2 activity was determined using a murine IL-2 dependent cytotoxic T cell line (CTLL-2). This cell line was maintained in complete RPMI and IL-2 (15 IU/ml). Forty-eight hours before use, IL-2 was no longer added to medium to decrease background incorporation values in the assay. Quantitation of IL-2 was done according to a modified procedure from Gillis et al. (20). Briefly, 0.1 ml of a suspension containing $5x10^4$ cells/ml complete RPMI was cultured in 96-well microplates (Greiner, Nurtingen, FRG). Serial dilutions of the samples (0.1 ml) were added

and the plates were incubated for 20 h at 37°C, 5% CO_2. Per well, 0.075 mCi of [14C]thymidine was used to measure proliferation in a 4-h pulse assay. The cultures were harvested on glass-fiber strips and counted in a liquid scintillation counter. The incorporation of [14C] thymidine by CTLL-2 cells incubated with various dilutions of each sample tested was compared to the titration curve of the response with a reference batch of IL-2 (1725.5 IU/ml).

Quantitation of a T-cell subpopulation in spleen and peripheral blood. Separate aliquots of spleen and peripheral blood cell suspensions (maximum $1x10^6$ cells) were stained with fluorescein isothiocyanate (FITC)-labeled rat anti-mouse monoclonal antibodies directed to Thy-1 (clone 59 AD 2.2) and Lyt-2 (53-6.7) (21). The appropriately diluted FITC conjugates (0.050 ml) were added to 0.050 ml of the cell suspension and incubated for 45 min. on ice. Subsequently the cells were washed and analyzed according to standard flow cytofluorometric procedures with a FACS-II cell sorter equipped with logarithmic amplifiers (22). Dead cells and nonlymphoid cells were gated out on the basis of their forward and perpendicular light scatter signals.

RESULTS

Experimental Approach.

The in vivo DTH reactivity to SRBC and the in vitro IL-2 production ability by spleen cells were determined in parallel from the same cell suspension. In addition, from the spleen cells and the peripheral blood cells, collected before and after priming, the Thy-1^+,Lyt-2^- T cell subpopulation was determined using immunofluorescent staining.

Age-Related Decline in DTH Responsiveness.

The specific swelling due to the local passive DTH response showed a steep increase, with a maximum at 24 h after transfer. For young C57BL/Ka donor mice (17 weeks old) 41% specific foot swelling was measured at 24 h after transfer, which decreased to 12% at 72 h after transfer. For young CBA/Rij donor mice (17 weeks old) 27% specific foot swelling was measured at 24 h after transfer, which decreased to 10% at 72 h after transfer. For

CBA/Rij mice as well as for C57BL/Ka mice, differences were observed in the magnitude of the DTH reaction in the two different age groups studied (17 and 125 or 155 weeks). The time course of the DTH responses by spleen cells was the same for young and old mice. C57BL/Ka donor mice 125 weeks of age showed a maximum specific DTH response of 12% at 24 h, which decreased to 5% at 72 h after transfer. For CBA/Rij donor mice of 155 weeks of age the same pattern was observed. At 24 h after transfer 10% specific swelling was observed and at 72 h 4% specific swelling (Fig. 1). The

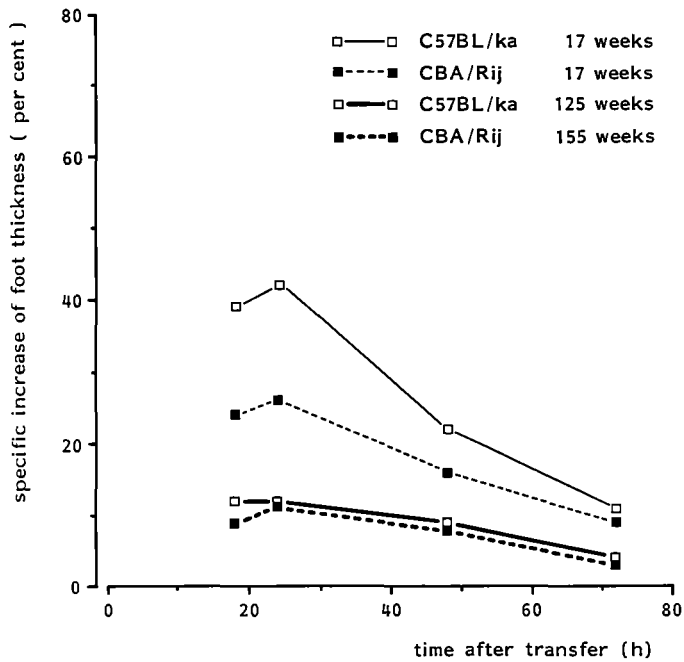

Fig. 1 Kinetics of DTH in young and old C57BL/Ka and CBA/Rij mice. C57BL/Ka mice: 17 and 125 weeks old. CBA/Rij mice: 17 and 155 weeks old.

maximum DTH response for all individual donors of different ages was observed at 24 h after transfer. Therefore only data of the DTH reactivity at 24 h after transfer are shown.

In four groups of C57BL/Ka donor mice and five groups of CBA/Rij donor mice of different ages, DTH responsiveness was determined in a passive transfer system. A direct relationship was observed between the age of the mice and the magnitude of the DTH response in the recipients. Experiments in mice of both C57BL/Ka and CBA/Rij strains showed a decreased DTH reactivity by spleen cells from old donors as compared to spleen cells from young mice (Fig. 2).

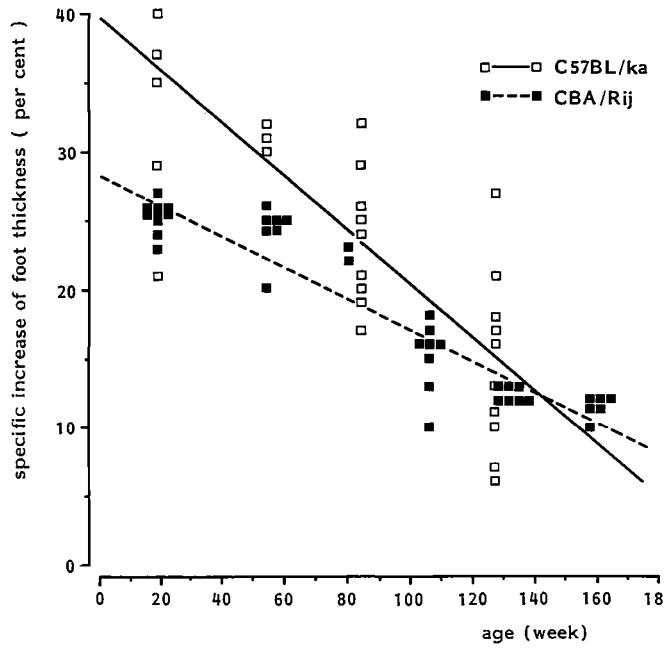

Fig. 2 Relationship between DTH response at 24 h after transfer and age of C57BL/Ka donor mice and CBA/Rij donor mice. C57BL/Ka: $n = 46$, $r = -0.86$, $P < 0.00001$. CBA/Rij: $n = 39$, $r = -0.94$, $P < 0.00001$.

Age-Related Decline in IL-2 Production Capacity.

The highest IL-2 production capacity was observed after in vitro stimulation of young mouse spleen cells with Con A. An age-related decrease of the in vitro IL-2 production ability was noticed for spleen cells of both C57BL/Ka and CBA/Rij mice (Fig. 3). A remarkable difference was observed between C57BL/Ka and CBA/Rij mice in the levels of IL-2 production under the conditions employed. Spleen cells from young CBA/Rij mice produced up to 30 times more IL-2 than young C57BL/Ka mice. This difference became smaller with increasing age.

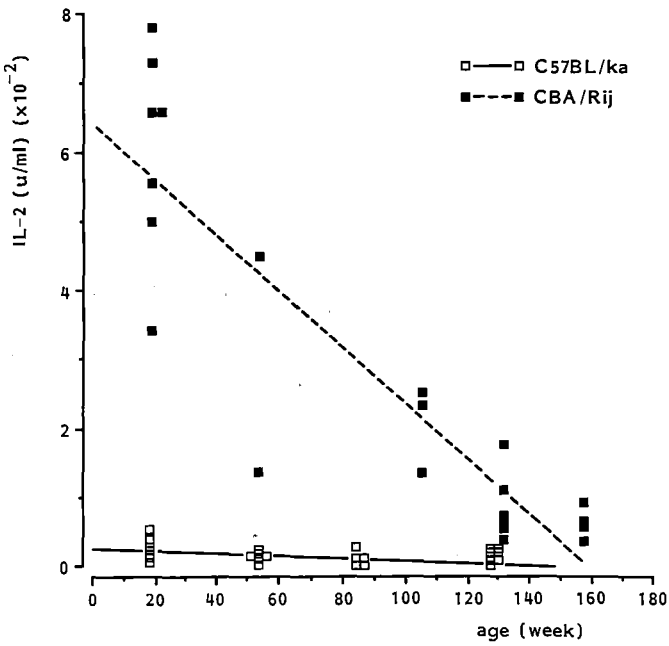

Fig. 3 Relationship between IL-2 production capacity and age of C57BL/Ka donor mice and CBA/Rij donor mice. C57BL/Ka: n = 29, r = -0.60, P = 0.0006. CBA/Rij: n = 22, r = -0.90, P < 0.00001.

Relationship between DTH Responses, IL-2 Production Capacity, and Frequency of Thy-1$^+$,Lyt-2$^-$ Cells.

The proportion of Thy-1$^+$,Lyt-2$^-$ cells was determined in the same spleen

cell suspension of which DTH responsiveness and IL-2 production ability were determined. An age-related decline in the proportion of Thy-1^+,Lyt-2^- spleen cells was observed for both strains investigated (Fig. 4). In young mice the proportion of Thy-1^+,Lyt-2^- spleen cells was 23 ± 3% for CBA/Rij mice and 21 ± 3% for C57BL/Ka mice. In aged CBA/Rij mice (155 weeks) this proportion had decreased to 13 ± 2% and in aged C57BL/Ka mice (125 weeks) to 10 ± 4%.

Since the proportion of Thy-1^+,Lyt-2^- cells diminished simultaneously with the function during aging, we also investigated whether a relationship existed between these parameters. A direct relationship between DTH reactivity and the frequency of Thy-1^+,Lyt-2^- spleen cells was found for aging C57BL/Ka and CBA/Rij mice (Fig. 5). The decreased number of Thy-1^+, Lyt-2^- cells correlated with the reduced DTH responsiveness. A direct

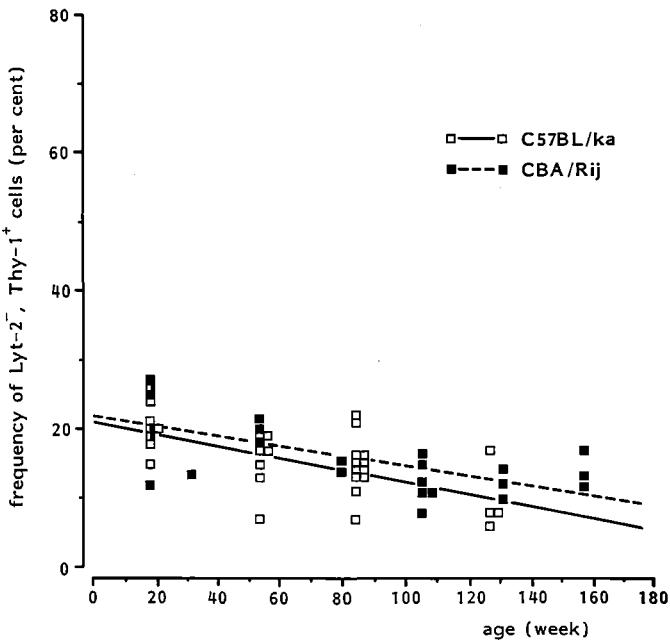

Fig. 4 Relationship between the frequency of Thy-1+,Lyt-2- spleen cells and the age of C57BL/Ka donor mice and CBA/Rij donor mice. C57BL/Ka: n = 30, r = -0.62, P = 0.0003. CBA/Rij: n = 23, r = -0.70, P = 0.0002.

relationship was also found between the proportion of Thy-1^+,Lyt-2^- peripheral blood cells after priming and the DTH reactivity in both strains investigated (Fig. 6). The reduced DTH responsiveness correlated with a decreased number of Thy-1^+,Lyt-2^- PBL. However, before immunization this relationship was only found for aging CBA/Rij mice and not for aging C57BL/Ka mice (Fig. 7). Furthermore, for CBA/Rij mice a direct relationship was found between the IL-2 production capacity and the proportion of Thy-1^+,Lyt-2^- spleen cells (Fig. 8). The age-related decrease of the number of

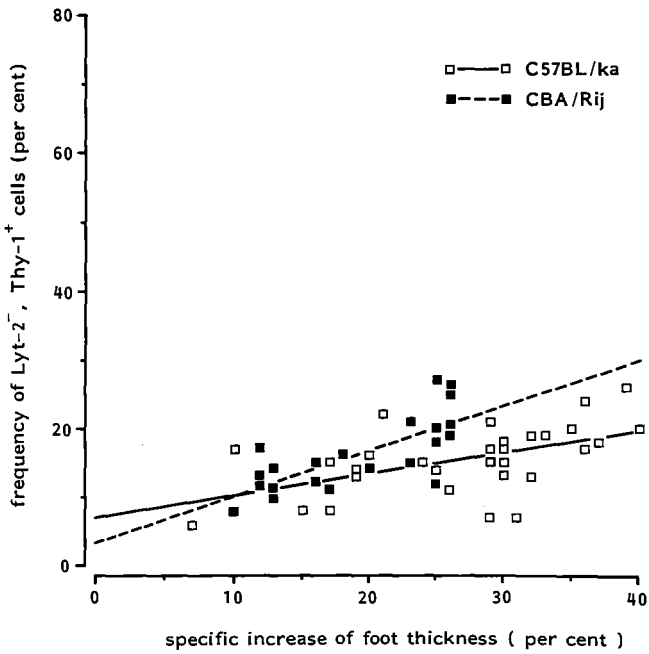

Fig. 5 Relationship between DTH response at 24 h after transfer and the frequency of Thy-1+,Lyt-2- spleen cells in aging C57BL/Ka mice and CBA/Rij mice. C57BL/Ka: n = 30, r = 0.54, P = 0.002. CBA/Rij: n=23, r = 0.75, P < 0.00001.

Thy-1$^+$,Lyt-2$^-$ cells again correlated with a lower IL-2 production. In contrast, in aging C57BL/Ka mice the frequency of Thy-1$^+$,Lyt-2$^-$ cells did not significantly influence the low IL-2 production ability. As DTH responsiveness and IL-2 production capacity are functions of regulatory T cells, we also investigated whether both aspects were related. For both mouse strains a relationship between DTH reactivity and IL-2 production was observed (Fig. 9). For aging C57BL/Ka mice, however, this relation was less strong than for aging CBA/Rij mice (C57BL/Ka: r=0.64, P=0.00002; CBA/Rij: r=0.87, P<0.00001).

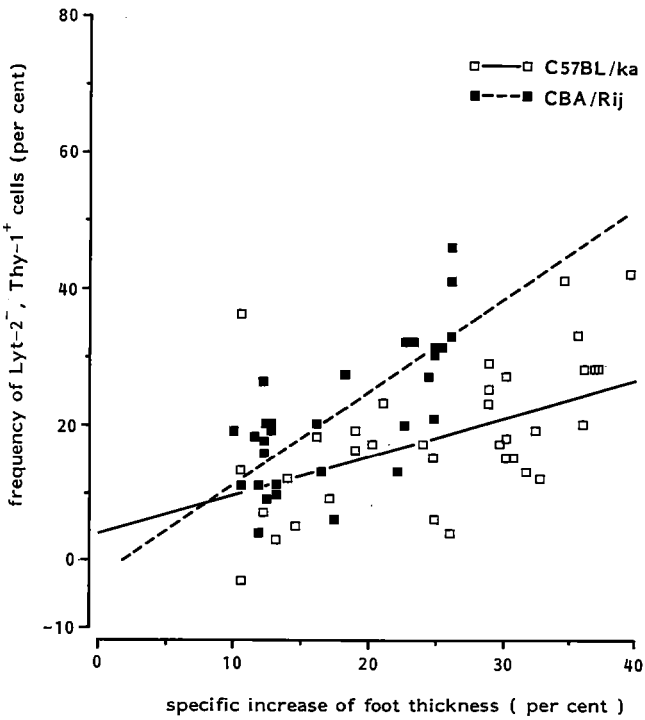

Fig. 6 Relationship between DTH response at 24 h after transfer and the frequency of Thy-1+,Lyt-2- PBL after immunization in aging C57BL/Ka mice and CBA/Rij mice. C57BL/Ka: n = 37, r = 0.51, P = 0.001. CBA/Rij: n = 31, r = 0.75, P < 0.00001.

The measurements reported here demonstrate a significant age-related decline in 24 h DTH reactivity for both mouse strains. For old CBA/Rij mice (155 weeks) the DTH reactivity decreased to about 37% of the level of young mice and for old C57BL/Ka mice (125 weeks) the response declined to 29% of

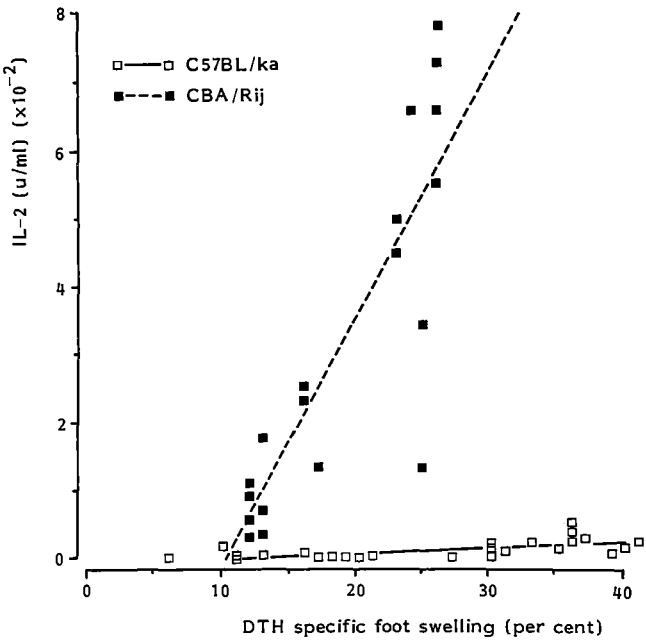

Fig. 9 Relationship between DTH response at 24 h after transfer and IL-2 production capacity in aging C57BL/Ka mice and CBA/Rij mice. C57BL/Ka: n = 30, r = 0.64, P = 0.00002. CBA/Rij: n = 22, r = 0.87, P < 0.00001.

that of young animals. This age-related impairment in anti-SRBC DTH responses is in agreement with the observation reported by Baumgartner et al. (24), who showed a 35% decline in DTH to 2,4-dinitrofluorobenzene in old BALB/c mice (96 weeks old) using the ear swelling test for DTH.

An age-related decrease was also observed for the ability to produce IL-2, a growth factor for T cells. The two mouse strains investigated showed a remarkable difference in IL-2 production capacity under the conditions

employed. At the age of 16 weeks, 30 times more IL-2 is produced by CBA/Rij spleen cells than by C57BL/Ka spleen cells under the same conditions. At an age of 120 weeks CBA/Rij spleen cells produce up to fourfold more IL-2 than C57BL/Ka spleen cells. The observed low levels of IL-2 produced by C57BL/Ka mouse spleen cells most probably are not due to excessive adsorption of IL-2 by the cells cultured since this phenomenon was not observed when exogenous IL-2 was added (data not shown). There is evidence that a decreased IL-2 production capacity during aging is due to a reduced IL-2 synthesis (25,26).

Since IL-1 is needed to trigger IL-2 production (27,28), a reduced IL-1 production capacity might account for the observed age-related impairment of IL-2 production. As reported by Inamizu et al. (29), the IL-1 production capacity reduces with increasing age to about 35% of the capacity of young individuals. Still IL-1 levels produced by spleen cells of old mice are sufficient to support the production of IL-2 (30,31), suggesting that the decline in IL-2 production with age is at least in part a primary defect that contributes to the impairment of T cell proliferation and therefore to the decrease of T-cell-dependent immunological functions as DTH.

Our analysis of T cell subpopulations was based on only two T cell markers (Thy-1 and Lyt-2). It may be possible that an analogous analysis with anti-L3T4 antibodies (32) may reveal subpopulations within the $Thy\text{-}1^{+},Lyt\text{-}2^{-}$ compartment. It has been shown that there exists a subpopulation of $Thy\text{-}1^{-}$ $L3T4^{+}$ cells in the spleen (32). From this it can be assumed that L3T4 alone is not a suitable marker to determine age-related changes in the regulatory T cell receptoire.

The IL-2 production capacity and the DTH reactivity are mediated by $Thy\text{-}1^{+},Lyt\text{-}2^{-}$ spleen cells. For both mouse strains investigated the proportion of $Thy\text{-}1^{+},Lyt\text{-}2^{-}$ spleen cells declined less strongly with aging than the IL-2 production and DTH reactivity. This suggests that probably not only a quantitative but also a qualitative decrease of T cell function occurs during senescence. This idea of a qualitative decrease of T cells with aging is supported by findings of Witskowski and Micklem (31), who showed that a large proportion of T lymphocytes of old CBA/Rij mice (144 weeks) have a decreased membrane potential. It has been demonstrated by Birx et al. (33)

that the membrane potential plays an important role in the initial events of T cell activation. Therefore, it has been proposed that the decline of T cell function in aging mice is related to the depolarized state of T cells (31).

A direct relationship was found between the DTH responsiveness and the IL-2 production ability in aging C57BL/Ka and CBA/Rij mice. The decreased IL-2 production most probably causes an impaired proliferation of T cells involved in the DTH to SRBC. This idea is consistent with data reported by Thoman and Weigle (34). They showed that IL-2 could reconstitute cell-mediated lympholysis responses in aged C57BL/Ka mice. In addition, it has been shown by others (35-37) that IL-2 administration in vivo augments T cell immune responses, which opens prospects for the therapy of immunodeficient states and malignancy.

We investigated whether the proportion of Thy-1^+,Lyt-2^- PBL can be used as a predictive value with regard to DTH responsiveness of the corresponding mouse. This was done before and after priming. For both mouse strains a direct relationship was found between the proportion Thy-1^+,Lyt-2^- PBL after priming and DTH responsiveness. However, before priming a direct relationship was only observed for CBA/Rij mice and not for C57BL/Ka mice. This implies that in aging CBA/Rij mice PBL can be used for monitoring of the capacity to mediate DTH, regardless of immunization.

For C57BL/Ka and CBA/Rij mouse strains the development of age-related immunodeficiency follows a similar general pattern. The proportion of Lyt-2^+ PBL (38) and Lyt-2^+ spleen cells (39, and this study) does not change very much during aging. In contrast, the proportion of Thy-1^+,Lyt-2^- spleen cells declines simultaneously with the ability to mount a DTH reaction. Young mice of both strains have similar proportions of Thy-1^+,Lyt-2^- spleen cells, which decrease similarly with age. C57BL/Ka mice are relatively high responders in a DTH reaction, but produce low amounts of IL-2 (this study) and interferon-γ (γ-IFN) in vitro (39). These findings suggest that a low IL-2 production capacity does not prelude high DTH responses. However, it cannot be excluded that the in vitro situation does not reflect the actual in vivo situation. In contrast, CBA/Rij mice produce high levels of γ-IFN (39) and IL-2 (this study) but are low responders in a DTH reaction. Further studies are required in order to gain insight whether the observed differences contribute

to the variation in susceptibility of CBA/Rij and C57BL/Ka mice to immunopathological lesions.

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CHAPTER 3

IMPAIRED DTH REACTIVITY FROM OLD MICE IS DUE TO DECREASED FUNCTIONAL CAPACITY OF $CD4^+$ T CELLS

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Wim J.A. Boersma* and Jan Rozing

SUMMARY

The efficiency of the immune system declines with age. This has been reported frequently for DTH. We confirmed this for the response to SRBC. The present paper extends these findings to DTH to H-2 and non-H-2 alloantigens and responses to modified self. Since the decline may be a consequence of the impaired IL-2 production observed in old mice, this was studied in an in vitro model. Cells generated in an allogeneic mixed leukocyte reaction were capable to mediate DTH in young naive syngeneic recipients, if injected together with the antigen. Using this in vitro system it was shown that cells from old CBA/Rij mice induced considerably lower DTH responses than cells from young CBA/Rij mice. By depletion of the effector cells (T cells) for CD4+ T cells or for CD8+ T cells, it was demonstrated that the effector phase was mediated by CD4+ T cells. Lower responses with cells from old mice were not due to increased suppressor cell activity, since removal of the CD8+ cells at the onset of the cultures did not influence the results. CD4+ cells from old mice were less effective in the generation of DTH effector T cells than CD4+ cells from young mice. This difference could not be attributed to a defective IL-2 production entirely, since the addition of IL-2 to the in vitro cultures did not improve the subsequent DTH response. Our data suggest that in aged mice CD4+ cells, effective in DTH, like the CD4+ IL-2 producing T cells, cannot be activated effectively resulting in a low level of secretion of mediators accounting for the characteristic DTH inflammation.

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INTRODUCTION

The delayed type hypersensitivity (DTH) reaction is an inflammatory response which reaches its maximum at 24 hours and is mediated by T lymphocytes (1,2). The T cells involved in DTH can be distinguished in T cells which induce the DTH response and T cells which secrete factors to activate granulocytes and mononuclear cells (effector T cells)(3). During the induction phase of the DTH reaction IL-2, a lymphokine, is involved (4). The effector phase of the Jones-Mote type DTH which is elicited by a challenge in the footpad of previously immunized mice is characterized by an infiltrate, consisting of granulocytes and mononuclear cells and by increased vascular permeability and edema (5).

The phenotype of the subset of T cells that mediate DTH has been disputed. It has been demonstrated that the DTH effector T cell population, like the helper T cell population, bears the Lyt-1 antigen and not the Lyt-2 antigen (1). Other studies have shown that DTH to alloantigens (6) or influenza virus (7) can be transferred by both Lyt-1^+ and Lyt-2^+ cells. Furthermore, DTH to non H-2 alloantigens (8) and viral antigens (9) can be H-2K or H-2D restricted, which suggests that Lyt-2^+ cells are involved. Analysis at the clonal level has shown that, in most cases, T cell clones causing local swelling upon transfer with antigen into footpads of mice were Lyt-1^+,Lyt-2^-. This is true for a wide variety of antigens (10-14).

One of the consequences of aging in higher animals is the functional decline of the immune system. Aged animals display a spectrum of immunological deficiencies involving functions of both T and B cells. Almost every function of lymphocytes has been shown to be diminished in aged animals (15-19), including DTH to sheep red blood cells (SRBC)(20) and dinitrophenyl (DNP) (21). It has also been reported that during senescence the suppressive activity of Lyt-2^+ cells increases (22-24).

Causes for a declined DTH reactivity might be at the level of the induction phase, e.g. due to an impaired antigen processing or production of IL-2 (17-19) or at the level of the effector phase. Alternatively, low DTH responses may be due to an increased activity or number of suppressor cells. The underlying causes of this impaired immune responsiveness are not easily

studied in vivo. Therefore the ability of responder cells from mice of different ages to generate DTH reactive T cells was studied in vitro.

The results indicate that compared to young mice the capacity of old mice to generate DTH effector T cells in vitro is decreased. This decline cannot be restored by addition of exogenous IL-2 to the in vitro cultures.

MATERIALS AND METHODS

Mice. C57BL/KaLwRij (H-2^b), C57BL/LiARij (H-2^b), CBA/BrARij (H-2^q), C3Hf/LwRij (H-2^k) and BALB/c (H-2^d) male mice (abbreviated in the text as C57BL/Ka, C57BL/Rij, CBA/Rij, C3H and BALB/c, respectively) were bred and raised in the TNO-REP Institutes, Rijswijk, The Netherlands, under SPF conditions. Aged mice were obtained from cohorts of mice that were barrier maintained and regularly screened for age-related pathology (25).

Antigens. Irradiated (25 Gy) spleen cells from young (20 weeks of age), male BALB/c, C3H, C57BL/Rij and CBA/Rij mice were used as antigens. Before irradiation, spleen cells from CBA/Rij mice were used for TNBS-modification (26).

DTH induction in vivo. C57BL/Ka and CBA/Rij mice of different ages were immunized intravenously (i.v.) with antigen (10^7 viable nucleated irradiated spleen cells). After 5 days, spleens were removed and subsequently prepared for single-cell suspensions and tested for DTH reactivity. From dose-response and kinetic experiments in young animals, these conditions appeared to be optimal. Spleen cells from each donor were transferred subcutaneously (s.c.) to the left hind footpad of five syngeneic naive recipients (local passive transfer). Each recipient received $5x10^6$ viable nucleated spleen cells together with antigen (10^7 of the relevant allogeneic spleen cells) in a volume of 50 µl HBSS.

Preparation of cells. Spleen cells from young or old mice (120 weeks of age), were washed twice with culture medium (RPMI 1640 supplemented with 5% fetal calf serum (FCS) (Seralab, West Sussex, UK), L-glutamine (2mM), Hepes (20 mM), penicillin (100 IU/ml), streptomycin (100 µg/ml) and 2-mercaptoethanol ($5x10^{-5}$M)). When indicated, spleen cells were separated by

"panning". Viable cell counts were based on trypan blue dye exclusion.

Panning. Petridishes (Greiner, Nurtingen, FRG) were coated (24 h, 4°C) with 100 µgr purified rat-anti-mouse-kappa-light chain monoclonal antibody (Mab; 187-1) in 10 ml 50 mM $NaHCO_3$, pH=9.6. Petridishes were washed twice with PBS and incubated for 30 min with PBS containing 1% FCS. B cells were depleted by "panning" as described by Wysocki et al. (27). Spleen cells, resuspended in PBS containing 1% FCS were added to the washed petridishes (maximum of $30x10^6$/dish) and allowed to adhere for 90 min at 4°C. The non-adherent cells were collected and these cells are further referred to as T cells. The T cells were, if necessary, further depleted of $L3T4^+$ or $Lyt-2^+$ cells by an indirect panning technique. T cells were incubated (30 min, 4°C) with anti-Lyt-2 (clone 53-6.7(28)) or with anti-L3T4 (clone GK1.5 (29)), washed and then transferred to petridishes coated with 250 µg affinity-purified goat-anti-rat-Ig. The non-adherent cells were collected. These cells are referred to as $CD4^+$ (Lyt-2-depleted) and $CD8^+$ (L3T4-depleted) T cells, respectively. In order to determine the purity of the cell populations after panning, the cells were analyzed with a FACS II as described previously (20).

DTH induction in vitro. Responder cells were stimulated with antigen. The cells were cultured at 37°C in a 5% CO_2 humidified atmosphere in culture flasks (75 cm^2, Greiner) with $25x10^6$ responders and $25x10^6$ irradiated stimulators in 10 ml culture medium. Unless otherwise stated, cells were harvested after 6 days of culture. Cells were washed twice, counted and together with antigen s.c. transferred to the left hind foot of five young syngeneic naive recipients.

Measurement of DTH reactivity. The DTH reaction was measured as the relative increase in thickness of the injected hind foot of the recipient mice as compared to the thickness of the contralateral noninjected hind foot. The increase of foot thickness was measured at 24 hours after transfer. In all experiments the increase in foot thickness of the recipient mice was corrected for the non-specific swelling caused by a challenge dose of antigen only. The corrected increase of foot thickness measured after induction of DTH responses was referred to as specific increase of foot thickness.

FACS analysis. FITC (Nordic Immunological Laboratories, Tilburg, The Netherlands) was conjugated to purified anti-Lyt-2 Mab. Purified anti-L3T4

Mab was biotinylated (Sigma) and used in combination with Avidin-FITC (AvF)(Sigma). The reagents were diluted in HBSS containing 0.1% bovine serum albumin (BSA)(Sigma) and 0.1% sodiumazide. For analysis, $5x10^5$ cells were incubated with conjugated Mab for 30 min at 4°C, washed and resuspended when necessary in AvF. The samples were then incubated for 30 min. at 4°C and washed. The cells were analyzed with a FACS II (Becton Dickinson, Sunnyvale, Ca.) equipped with logarithmic amplifiers. After FITC staining and prior to FACS analysis, propidium iodide (1.5 µg/ml) (at which viable cells exclude the dye, but dead or dying cells fail to exclude the dye) was added to enhance live-dead discrimination.

RESULTS

Age-related decline of in vivo DTH responsiveness.

Previously, we demonstrated that the DTH to SRBC declines with age (20). In order to analyze impaired DTH in vitro another antigen was used, i.e. alloantigens which are well known to induce in vitro proliferation. Therefore, we first studied the DTH of young and old CBA/Rij and C57BL/Ka mice to BALB/c spleen cells in vivo.

Figure 1 shows that young mice of both strains can generate DTH reactivity to such antigens and that the DTH response elicited by spleen cells from old mice is lower. Young CBA/Rij and C57BL/Ka mice responded with 20.1±3.2% and 25.3±4.1% specific increase of foot thicknesss, while old CBA/Rij mice (100-120 weeks of age) and old C57BL/Ka (85-95 weeks of age) mice responded with 6.0±1.8% and 10.5±2.3% specific increase of foot thickness, respectively. Thus in both mouse strains an age-related decline in DTH responsiveness was observed.

We investigated also the ability of old C57BL/Ka and CBA/Rij mice to elicit in vivo a DTH response to non-H-2 alloantigens and TNP. In this situation a similar decrease of DTH responsiveness was found (Figs. 2 and 3).

In order to analyze the underlying cause of this diminished DTH responsiveness, the induction of DTH reactivity was further studied in vitro. Since both mouse strains showed a comparable age-related decline in DTH

responsiveness to H-2 and non-H-2 alloantigens, we concentrated on CBA/Rij mice for the following experiments.

In vitro activation of DTH reactive T cells.

As shown above, alloantigens are strong inducers of DTH. Therefore, we anticipated that T cells activated during an allogeneic mixed lymphocyte reaction are well able to induce DTH by passive transfer and that an age-related decline should be detectable at the in vitro level. To this end, we first optimized the conditions for the in vitro induction of DTH effector T cells. The culture period after which cells from young and old mice appeared to be optimally activated, was determined. This was done with spleen cells from young and old CBA/Rij mice stimulated with irradiated (25 Gy) BALB/c

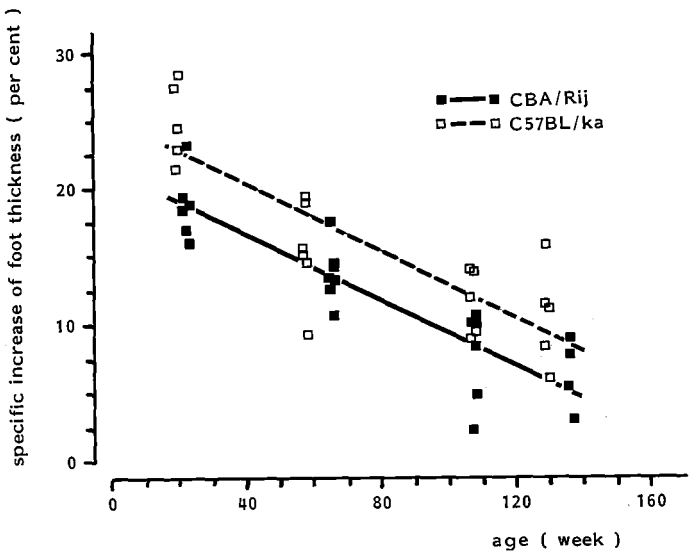

Fig.1 *Relationship between DTH responsiveness to irradiated (25 Gy) BALB/c spleen cells and age of CBA/Rij and C57BL/Ka mice. CBA/Rij: n = 23, r = −0.91, p < 0.00001; C57BL/Ka: n = 22, r = −0.83, p < 0.00001.*

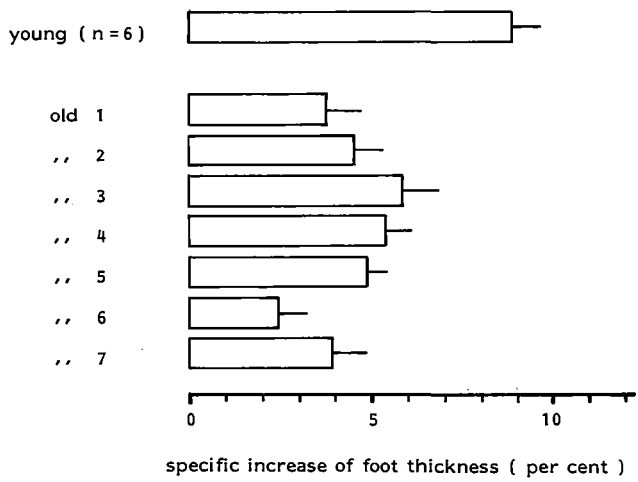

Fig.2 *DTH responsiveness of young (20 weeks, n=6) and old individual (85–95 weeks, n=7) C57BL/Ka mice to irradiated (25 Gy) C57BL/Rij spleen cells. Mann–Whitney test: P<0.002.*

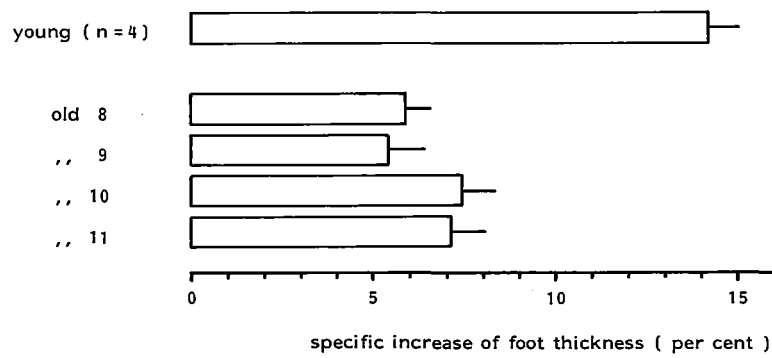

Fig.3 *DTH responsiveness of young (20 weeks, n=4) and old individual (100–120 weeks, n=4) CBA/Rij mice to irradiated (25 Gy) CBA/Rij–TNP spleen cells. Mann–Whitney test: P<0.005.*

spleen cells. Figure 4 shows that a culture period of 6 days led to a maximum DTH response for young as well as old mice. After transfer of 10^6 cells together with alloantigenic spleen cells, cells from young mice induced

31.0±2.8% specific increase of foot thickness, whereas the maximum increase of foot thickness by cells from old mice was 20.5±2.4%. Based on these data, in subsequent experiments a culture period of 6 days was used.

CD4$^+$ T cells mediate DTH.

Subsequently, we investigated which in vitro activated T cells were responsible for mediating the DTH reaction. This was done as follows: T cells (≥80% Thy-1$^+$, ≤10% Ig$^+$) were cultured together with the stimulators. After 6 days we separated the harvested T cells into CD4$^+$ and CD8$^+$ T cells by panning. For both subsets the purity varied between 80-90%. The three cell

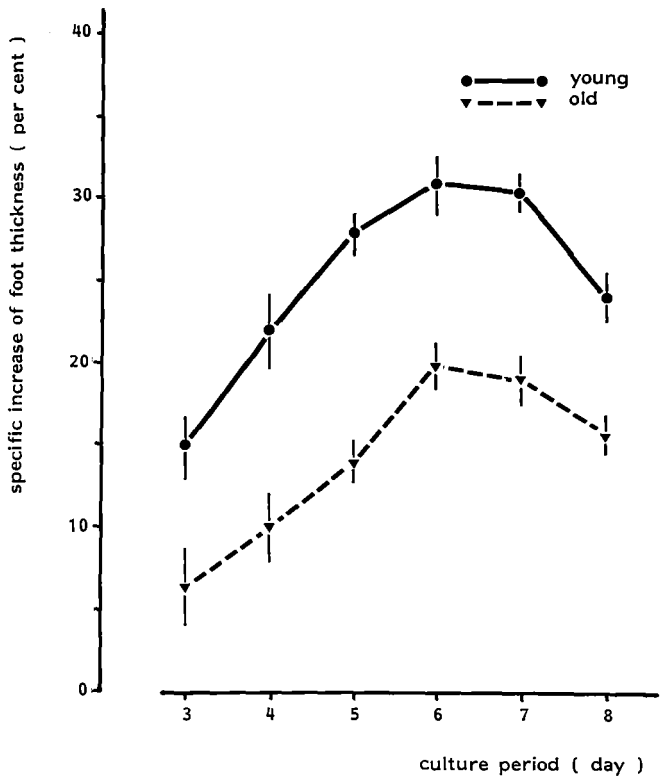

Fig.4 *Kinetics of in vitro induced DTH responsiveness of CBA/Rij mice to irradiated (25 Gy) BALB/c spleen cells. Responder cells (spleen cells) were derived from young (20 weeks, n=4) and old (120 weeks, n=4) CBA/Rij mice. 10^6 responder cells/recipient were used to measure DTH reactivity.*

populations (total T cells, $CD4^+$ T cells, $CD8^+$ T cells) were tested for the ability to elicit a DTH reaction. As shown in Figure 5, the DTH response evoked by T cells derived from young mice (32.0 ± 2.0%) specific increase of foot thickness) was higher than the DTH response evoked by T cells from old mice (16.0±2.0%). Furthermore, it can be seen that in both young and old mice

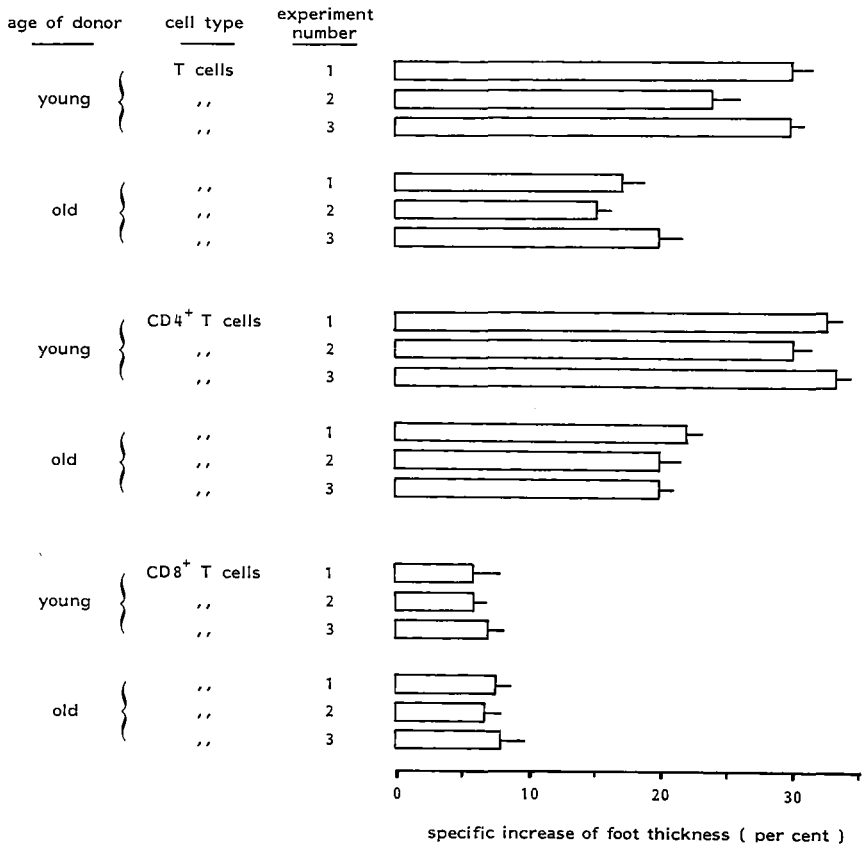

Fig.5 *DTH responsiveness of CBA/Rij responder cells to irradiated (25 Gy) BALB/c spleen cells. Responder cells were obtained from young (20 weeks, n=5) and old (120 weeks, n=5) mice, cultured together with the stimulator cells in vitro for 6 days and then depleted for Ig+ cells (T cells), Ig+,Lyt-2+ cells (CD4+ T cells) or Ig+, L3T4+ T cells (CD8+ T cells). 5 x 10^5 responder cells/recipient were used to measure DTH reactivity. Mann-Whitney test: P<0.03 (T cells); P<0.03 (CD4+ T cells); P>0.09 (CD8+ T cells).*

the $CD4^+$ cells accounted for the DTH reaction. The contribution of the $CD8^+$ T cells appeared to be marginal.

As expected, the DTH responsiveness elicited by $CD4^+$ T cells from old mice (20.0±1.6% specific increase of foot thickness) was decreased compared to that by young $CD4^+$ cells (32.0±1.7%).

Age-related decrease of the capacity to give rise to DTH.

Because the activity of $CD8^+$ T suppressor cells is suggested to increase with age, this might be a reason for the age-related decline in DTH

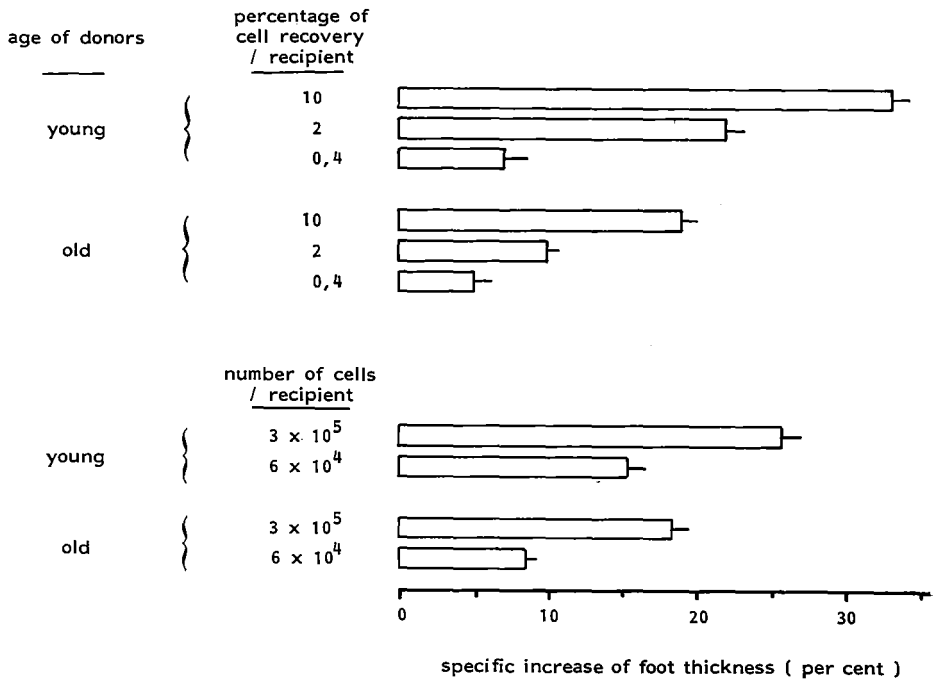

Fig.6 DTH responsiveness of CBA/Rij responder cells to irradiated (25 Gy) BALB/c spleen cells. Responder cells were CD4+ cells from young (20 weeks, n=5) and old (120 weeks, n=5) mice, which were cultured together with the stimulator cells for 6 days in vitro. DTH reactivity to a third party antigen (25 Gy irradiated C3H spleen cells) was 7.0 ± 1.2% for young and old mice.

responsiveness. In order to rule out the influence of suppressive activity by $CD8^+$ T cells, we performed experiments in which Lyt-2-depleted T cells were used as responder cells. Thus, $CD4^+$ cells (≥80% L3T4+, ≤10% Ig+) were stimulated in vitro and tested for their capacity to give rise to DTH. A fixed percentage of the output as well as a constant number of viable cells was used to measure DTH responsiveness. The results are shown in Figure 6. The DTH response elicited by ten percent of the cells from young mice appeared to be 36.3±1.8% specific increase of foot thickness, whereas this was 19.5±2.2% for old mice.

The measured DTH response was antigen-specific since transfer of the cells together with an irrelevant antigen resulted in 7.0±1.2% specific increase of foot thickness for young and old mice. Apparently, the declined DTH responses induced in vitro were not due to the presence of $CD8^+$ suppressor T cells. Differences between cells from young and old mice may still be related exclusively to the number of cells that had proliferated in vitro. Therefore, we also did experiments in which the same number of effector cells ($3x10^5$) was transferred. When $CD4^+$ T cells were derived from young mice we observed 27.6±2.0% specific increase of foot thickness and with $CD4^+$ T cells derived from old mice 18.0±1.7% was observed. Thus the difference in DTH responsiveness between young and old mice was smaller in the case of a fixed number of effector cells.

Exogenous IL-2 does not improve the impaired DTH response in old mice.

As suggested above poor DTH responses induced in vitro may in part be due to a low degree of proliferation as a consequence of an impaired production of IL-2. In order to investigate this possibility, we studied the effect of supplementation with IL-2 on the DTH responsiveness.

Purified $CD4^+$ T cells were cultured together with stimulator cells and supplemented with exogenous IL-2. Addition of IL-2 increased the total cell number for young (110% of the input) and for old (80% of the input) mice as compared to the cultures without exogenous IL-2 (young: 90% of the input; old: 60% of the input). Addition of IL-2 to the cultures improved the DTH responsiveness by cells from old mice (Table 1). This improvement was probably due to the transfer of a higher number of cells, since the transfer

of a constant number of cells resulted in 18.0±1.6% specific increase of foot thickness, independent of whether or not IL-2 had been present during culture.

The cells that were tested for DTH responsiveness were also analyzed with the FACS. Cells from young and old mice were $L3T4^+$ (>99%) (Fig.7) and $Lyt\text{-}2^-$ (data not shown). The histograms further show that the $CD4^+$ cells from young and old mice differed in percentage of blastlike cells. For young mice this figure was 60% and for old mice just 30% of the total viable cells. Addition of IL-2 did not influence these percentages of blastlike cells.

Table 1: IL-2 does not improve impaired DTH responsiveness.

age of donor (wks)	cells transferred	DTH response (% specific increase of foot thickness ± S.E.)	
		-IL-2	+IL-2
20	10%of recovery	36.3±1.8	35.2±2.0
120	"	19.5±2.2	23.1±1.4
20	$3x10^5$	27.6±2.0	27.3±1.2
120	"	18.0±1.7	18.0±1.6

CD4+ T cells from young and old mice were cultured with irradiated (25 Gy) BALB/c spleen cells in the absence or presence of exogenous IL-2 (50 U/ml). After 6 days of culture the cells were harvested and the DTH responsiveness was determined. Either a fixed percentage of the recovery (10%) or a constant number of cells ($3x10^5$) was used as transfer dose to induce together with irradiated (25 Gy) BALB/c spleen cells a DTH response. The DTH responsiveness was determined as the specific increase of foot thickness. The DTH response to a third party alloantigen (C3H) appeared to be 7.8±1.1% (for young and old mice)(data not shown).

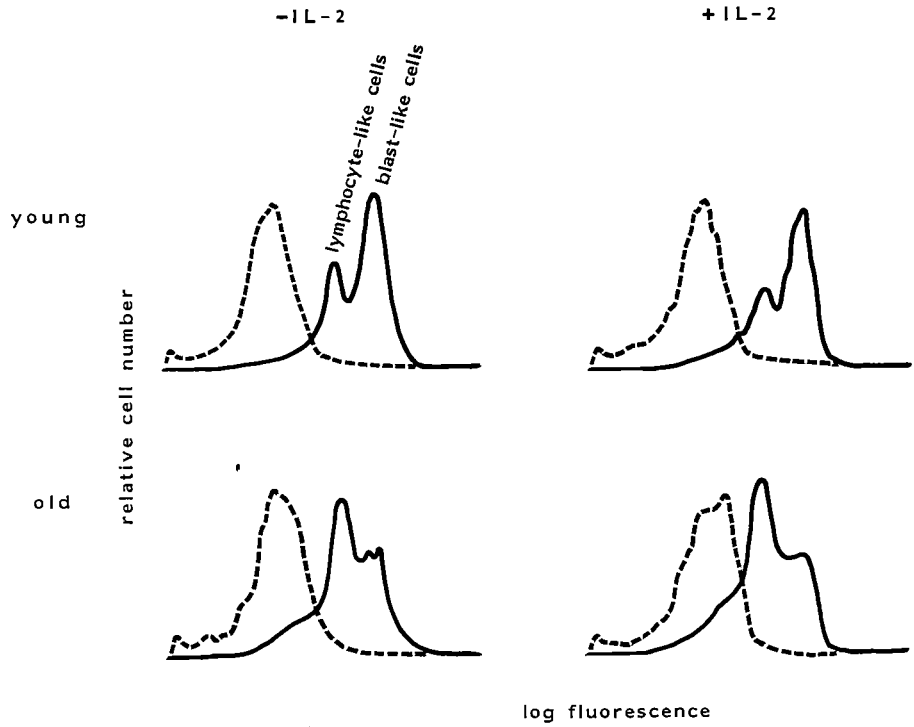

Fig.7 *FACS analysis of cultures of CBA/Rij responder cells and irradiated (25 Gy) BALB/c spleen cells. Aliquots of cells from experiments presented in figure 6 and table 1 were stained with anti-L3T4+ and analyzed with a FACS II. Histograms show that all cells are L3T4+. Furthermore, on the basis of green fluorescence intensity, cells can be distinguished in lymphocyte-like and blast-like cells. All cells were negative for Lyt-2-FITC staining (data not shown).*

DISCUSSION

The efficiency of the immune system declines with age. This has been reported frequently for DTH. We confirmed this for the response to SRBC

(20). The present paper extends these findings to DTH to H-2 and non-H-2 alloantigens and responses to modified self. The underlying cause is not easily studied in vivo. Therefore an in vitro model was required. We developed such a model for DTH to alloantigens. We found that cells from young and old mice after culture in vitro together with alloantigenic spleen cells showed an maximum response on day 6 of culture. This observation fits well with the work of Barrington (30). According to the latter study ten times less cells were needed to mediate DTH responses when using cultured T cells than in the case of in vivo induction of DTH.

With this in vitro approach we studied various aspects of the age-related decline of DTH. We found that the contribution of $CD8^+$ cells to the DTH reactivity is marginal and non-antigen-specific. This observation is in agreement with data from other groups: they reported that DTH reactivity is mediated by $Lyt-1^+$ or $L3T4^+$ (murine homologue for CD4) T cells (1,7,11,14). Cher et al. (31) reported that a $CD8^+$ class I MHC restricted Tc clone could not transfer DTH. In contrast, Von Boehmer et al. (32) found that some, but not all $CD8^+$ class I MHC restricted Tc clones did transfer DTH reactivity by local administration. In these experiments only the small IL-2 producing $CD8^+$ T cells were able to transfer DTH. Probably, these Tc clones represent a small percentage of the in vivo circulating immune reactive lymphocytes, since IL-2 production is almost entirely restricted to the $CD4^+$ population (33).

The age-related decline of in vitro induced DTH reactivity was not due to an age-related increase in $CD8^+$ suppressor T cell activity, because results remained unchanged if Lyt-2-depleted T cells were cultured. Thus, the capacity of the population of $CD4^+$ cells to generate DTH reactivity decreased during aging. Another explanation for our findings is an inefficient proliferation due to a lack of IL-2, as reported by several groups (17-19). However, although addition of IL-2 improved the proliferative activity, there was no effect on the capacity to generate DTH. Several possible explanations for this age-related impaired immune responsiveness can be put forward.

One possibility might be the reported decline in frequencies of alloreactive $CD4^+$ T cells (34). There is, however, a complicating factor. It is reported by Cher and Mosmann (31) that two types of helper T cell clones exist, one of which is capable of inducing DTH reactions. These two helper T

cell types can be distinguished on the basis of the different patterns of lymphokine secretion. The helper T cell clones that induce DTH reactions synthesize IL-2 and γ-IFN. The other subset is characterized by the production of IL-4 and does not mediate DTH. Since the two T helper cell types bear the same phenotype, is it not clear whether the decline in precursor frequency of alloreactive $CD4^+$ T cells is equally spread over these two helper T cell types.

Secondly, there might be a defect in one of the signals required for the activation of T cells. IL-1 is an important signal in this respect and produced by a variety of cells (35). The decreased DTH responsiveness might therefore be due to a reduced IL-1 production ability of for example macrophages. Some controversial results about the IL-1 production capacity by macrophages of aged animals are reported. According to some investigators there is a decrease (36,37), while according to another report there is no change in the IL-1 production (38). In the cultures, however, also macrophages from the stimulator cell population occurred. These stimulator cells were obtained from young donors. Therefore, it seems not very likely that a defect in IL-1 production ability accounts for the decreased DTH responsiveness during aging.

Furthermore, there might be a defect at the level of triggering of the T cell receptor (TCR). It is known that the activation of T lymphocytes is mediated by the TCR and the CD3 complex. It has been proposed that the CD8 and CD4 molecules bind to monomorphic regions of the MHC proteins on the target cell, CD4 to class II and CD8 to class I. The function of the CD4 determinant then is to serve as a cell surface adhesion molecule which augments the T cell receptor-antigen interaction (39,40). One can imagine that in $CD4^+$ cells from old mice the activation process mediated by the T cell receptor or the interaction of the CD4 determinant with class II molecules is impaired, e.g. by decreased glycosylation. The net result would be an impaired activation process. The lower percentage of blastlike $CD4^+$ cells in cultures from old donor mice in comparison to those from young donor mice supports this idea.

Finally, there might be a defect at the level of the effector T cell. Employing a read-out system of young naive syngeneic recipients differences

in DTH reactivity are most likely due to changes in the effect generated by effector T cells. It can be imagined that DTH effector T cells derived from old mice are impaired in the secretion of factors which activate macrophages. More insight into this possibility might be obtained with the use of panels of T cell clones obtained from old mice.

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CHAPTER 4

IMPAIRED ALLOGENEIC RESPONSES IN AGED MICE ARE DUE TO A DECREASED FREQUENCY OF ALLOREACTIVE $CD4^+$ AND $CD8^+$ T CELLS.

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SUMMARY

T cell responses were studied in old CBA/Rij mice. It was found that the proliferative response of total T cells to irradiated BALB/c spleen cells was impaired in old mice. Since it was unclear whether these low responses were due to an increased suppressor cell activity, we also studied CD4+ T cells and CD8+ T cells separately. In the absence of exogenous IL-2, proliferative responses of CD4+ T cells from old mice were low compared to responses from CD4+ T cells from young mice. In addition, IL-2 production by CD4+ T cells in response to alloantigens was lower in old mice than in young mice. Although impaired IL-2 production contributes to the low responses in old mice, the addition of IL-2 to in vitro cultures only partly restored the proliferative responses. Besides a great interindividual variability in the incidence of CD8+ T cells in old mice, responses by CD8+ T cells in the presence of exogenous IL-2 were always lower in old mice than in young mice. Therefore we studied whether these low responses were due to decreased frequencies of antigen-specific T cells in old mice. By using purified T cell subpopulations we found that the mean frequency in CD4+ T cells from old mice (1/1900) was 3 times lower than the frequency in young mice. The frequency in CD8+ T cells from old mice (1/800) was 8 times lower than the frequency in young mice. The lower frequency cannot, however, fully explain the low level of proliferation and IL-2 production by CD4+ T cells.

INTRODUCTION

The age-related decline of immune responses is well established (1-3). This has also been demonstrated in vivo for the delayed type hypersensitivity (DTH) response of C57BL/Ka and CBA/Rij mice (4) to sheep red blood cells. Furthermore, an impaired immune responsiveness during senescence was found for the DTH response to allogeneic spleen cells in vivo, and subsequently for the capacity of cells from old mice to generate alloreactive DTH cells in vitro (Chapter 3). The induction of alloreactive DTH cells in vitro requires similar conditions as the mixed leukocyte reaction (MLR). Besides cytotoxic T cells ($CD8^+$) also DTH reactive T cells ($CD4^+$) are generated in MLR. Based on these findings, an allogeneic MLR might be a good in vitro system to study the diminished DTH function in old mice.

$CD4^+$ T cells play a central role in the induction of DTH effector T cells and in proliferative responses to alloantigens. In these systems, one of the functions of $CD4^+$ T cells is the production of IL-2. In old mice, the ability of spleen cells to produce IL-2 in response to concanavalin A (Con A) is decreased (4). Also, proliferative responses to alloantigens are decreased in old mice (5,6). In these studies, unfractionated spleen cells were used as responder cells. As a consequence, in such an experimental set up possible suppressive effects exerted by $CD8^+$ T cells are not excluded. This is of importance since increased suppressor activity in spleen cells from old mice has been reported (7-9).

Alternatively, impaired T cell function might be a manifestation of a reduced frequency of cells capable to respond in an antigen-specific fashion. Decreased cytotoxic responses mediated by T cells should be due to a decline in precursor frequency (10,11). So far, to our knowledge, no studies were performed, in which age-related changes in the proliferative capacity of $CD4^+$ and $CD8^+$ T cells were investigated separately. In order to exclude possible age-dependent suppressive effects, $CD4^+$ and $CD8^+$ T cells should be fractionated and tested separately for their proliferative capacity.

We choose an alternative approach by determining absolute frequencies of alloreactive purified $CD4^+$ and purified $CD8^+$ T cells in young and old mice by limiting dilution analysis (LDA). The results show that impaired allogeneic

responses of $CD4^+$ and $CD8^+$ T cells from old mice are partly due to a decreased frequency of antigen-specific T cells and a decline in IL-2 production.

MATERIALS AND METHODS

Mice. Male CBA/BrARij (abbreviated in the text as CBA/Rij) and BALB/c mice were bred and raised under SPF conditions in the TNO-REP Institutes, Rijswijk, The Netherlands. Aged mice were obtained from cohorts of mice that were barrier maintained. Aged mice were inspected at the time of death for signs of tumors and examined histologically for age-related pathology (12). If any tumors were found, they were excluded from experiments.

Preparation of cells. Spleen cells from young (20 weeks of age) or old mice (120 weeks of age) were washed twice with culture medium (RPMI 1640 supplemented with 5% fetal calf serum (FCS; Seralab), L-glutamine (2 mM), Hepes (20 mM), penicillin (100 U/ml), streptomycin (100 µg/ml) and 2-mercaptoethanol ($5x10^{-5}$ M). Splenic T lymphocytes were purified by nylon wool passage according to the procedure of Julius et al. (13). An additional depletion of B cells was performed as described by Wysocki (14). Petridishes (Greiner, Nurtingen, FRG) were coated (24 h, 4°C) with 100 µg purified rat-anti-mouse-kappa-light chain monoclonal antibody (187-1) in 10 ml 50 mM $NaHCO_3$ (pH 9.6). These cell populations were further depleted of $L3T4^+$ or $Lyt-2^+$ cells by an indirect "panning" technique. T cells were incubated (30 min, 4°C) with either anti-Lyt-2 (clone 53-6.7 (15)) or with anti-L3T4 (clone GK1.5 (16)), washed and then transferred to petridishes coated with 250 µg affinity-purified goat-anti-rat-Ig. The non-adherent cells were collected. These cells are further referred to as $CD4^+$ (Lyt-2-depleted) or $CD8^+$ (L3T4-depleted) T cells, respectively. Viable cell counts were based on trypan blue dye exclusion. In order to determine the purity of the cell populations after panning, the cells were analyzed with a FACS as previously described (4).

Mixed leukocyte reaction (MLR). Unless stated otherwise 10^5 responder cells were stimulated in round bottom microtiter plates (Greiner) with 2 x 10^5 irradiated (25 Gy) BALB/c spleen cells in a culture volume of 200 µl.

Where indicated, cultures were supplemented with conditioned medium to a concentration of 50 U IL-2/ml including 10 mM α methyl-mannoside at the initiation of the cultures. Con A-free rat spleen cell supernatant was used as IL-2 source. Cells were cultured for 3-8 days. After 6 days, cultures were pulsed with 0.25 μCi ^{3}H-thymidine (^{3}H-TdR) (Amersham) and harvested 6 h later.

IL-2 assay. Supernatants from MLR cultures were collected at day 4 of culture. IL-2 was assayed by determining the ability of supernatants to stimulate proliferation of CTLL-2 cells (17). For this bioassay, 100 μl of the culture supernatant was serially diluted in culture medium. Five thousand CTLL in a volume of 100 μl were added to each well and the plates were incubated at 37 °C for 24 h. The last 4 h of culture 0.25 μCi ^{3}H-TdR was added to each well. Rat IL-2 served as a standard. One unit of IL-2 activity was defined as the amount of IL-2 inducing half-maximal proliferation of the CTLL.

LDA. Individual microcultures were set up in 96-well, round bottom microtiter plates (Costar). The various T cell populations of individual old and a pool of young mice were tested in 24 replicate cultures. Different cell numbers were stimulated with $2x10^{5}$ irradiated (25 Gy) BALB/c spleen cells. Cultures were performed in the presence of 50 U rat IL-2/ml. After 7 days, cultures were pulsed with 0.25 μCi ^{3}H-TdR and harvested 6 hours later.

Statistical analysis. Calculations of frequencies from LDA were based on Poisson distribution. The values for the frequencies and 95% confidence limits were calculated as described by Taswell (18).

RESULTS

Impaired alloreactivity in old mice.

The ability of either total spleen T cells, Lyt-2-depleted T cells and L3T4-depleted T cells from aged CBA/Rij mice to proliferate in an antigen-specific way was studied in an MLR. For total T cells and Lyt-2-depleted (further referred to as $CD4^{+}$) T cells from a pool of old mice the proliferative response to BALB/c spleen cells was compared to the response

by T cells from young CBA/Rij mice (Fig. 1A). Under the culture conditions employed the MLR responses of T total cells and $CD4^+$ T cells from young and old mice were maximal at day 5–6. The L3T4-depleted (further referred to as $CD8^+$) T cells of young and old mice did not proliferate, which is indicative for an efficient depletion of $CD4^+$ T cells. The low proliferative reaction by cells from old mice might be a reflection of a deficiency in IL-2 production capacity. Therefore, in a subsequent experiment we studied the IL-2 production ability. After stimulation with antigen, total T cells or $CD4^+$ T cells from young mice produced about eight times more IL-2 (4.4 U/10^5 responder cells and 6.4 U/10^5 responder cells, respectively) than similar cultures containing responder cells from old mice (0.6 U/10^5 cells and 0.8 U/10^5 cells, respectively). As expected, $CD8^+$ T cells from young as well as old mice did not produce detectable amounts of IL-2. Furthermore, depletion of $CD8^+$ T cells did not result in an improved proliferative response nor in an improved IL-2 production. This indicates that the decreased proliferative response and IL-2 production by $CD4^+$ T cells from old mice is not due to suppressive effects exerted by $CD8^+$ T cells.

Since IL-2 is required for proliferation of T cells, the decreased levels of IL-2 in MLR-supernatant might account for the low proliferative response of T cells from old mice. So, we studied the possible restorative effect of IL-2 on allogeneic responses of total T cells, $CD4^+$ T cells and $CD8^+$ T cells from a pool of young and a pool of old mice. It appeared that in the presence of an excess of IL-2 (50 U/ml) the proliferative response of T cells from old CBA/Rij mice to BALB/c spleen cells was still lower than that of T cells from young CBA/Rij mice (Fig. 1B). Comparable results were obtained for $CD4^+$ T cells and $CD8^+$ T cells, when tested separately. As expected, the proliferative responsiveness of $CD8^+$ T cells from young as well as old mice was completely dependent upon exogenous IL-2 as shown by the increased proliferation after addition of IL-2. Our data further show that in old mice especially $CD4^+$ T cells are impaired in their proliferative capacity. Under the culture conditions employed the MLR responses of total T cells and T cell subsets from young as well as old mice was maximal on day 5. Therefore, in the subsequent experiments the MLR responses of total T cells and the T cell subsets were determined after 5 days of culture.

Interindividual variability in responsiveness of old mice.

The previous experiments were done with pooled cells from young and old mice. For young mice this approach will reflect the response pattern of the individual mice, since they respond quite similar to allogeneic cells. However studying old mice one should be carefull because a greater interindividual variability might be expected as well as possible suppressive effects exerted by cells from individual mice on the pooled cells. Therefore, in subsequent experiments old mice were studied separately. In Figure 2A, the proliferative responses of individual old mice (n=5) and by pooled spleen cells from young mice (n=5) are shown. Indeed extensive interindividual variation was found for old mice. The variability of the 5 individual old mice tested was most clearly for the proliferative responses of total T cells.

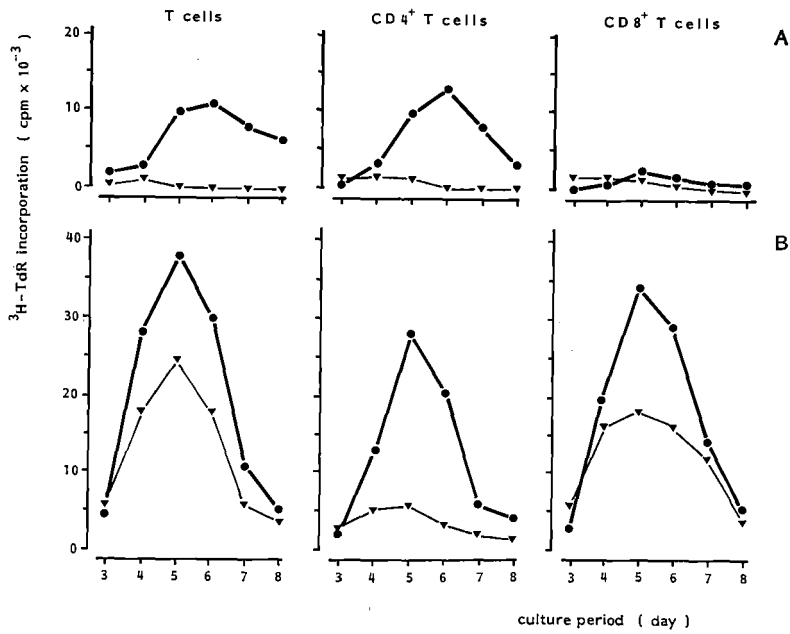

Fig.1 *Time course of MLR of pooled purified T cells, pooled purified CD4+ and pooled purified CD8+ T cells from young (n=5, 20 weeks) and old (n=5, 120 weeks) CBA/Rij mice, stimulated by irradiated (25 Gy) BALB/c spleen cells. In Figure 1A no IL-2 was added, whereas in Figure 1B IL-2 was added to the cultures. The data show mean responses from triplicate cultures. ●, young responders; ▼, old responders.*

With respect to the T cell subsets, $CD8^+$ T cells did not proliferate in the absence of exogenous IL-2, while responses of $CD4^+$ T cells were low. Addition of IL-2 did not very much alter the variability of the T cells, e.g. the response of cells from mouse number 2 remained low (Fig. 2B). In the presence of IL-2, $CD8^+$ T cells did proliferate. In this case an even greater variability was found. Using 10^5 cells from old mice the ^{3}H-TdR incorporation ranged from 2,000 cpm (nr.2) to 25,000 cpm (nr.1), while the response by

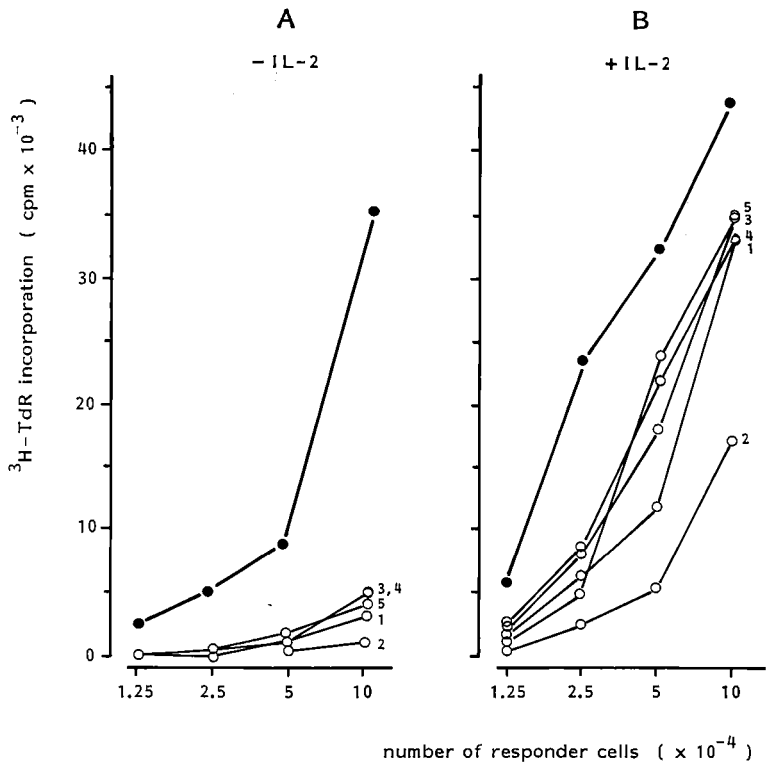

Fig.2 *Comparison of the MLR reactivity of pooled purified T cells from young (n=5, 20 weeks) and old (n=5, 120 weeks) CBA/Rij mice to irradiated (25 Gy) BALB/c spleen cells. In Figure 2A no IL-2 was added, whereas in Figure 2B IL-2 was added. The data show mean responses from triplicate cultures. ●, young responders; ○, old responders.*

cells from young mice was 40,000 cpm (Fig. 3A). The $CD4^+$ T cells of old mice, in contrast to $CD8^+$ T cells, showed only partly improved proliferative responses in the presence of exogenous IL-2 (Fig. 3B). Using 5 x 10^4 cells, a ^{3}H-TdR incorporation of about 5,000 cpm was found with cells from old mice, whereas with cells from young mice a ^{3}H-TdR incorporation of 35,000 cpm was observed. In conclusion, addition of IL-2 does not completely restore the low alloreactive responses of $CD4^+$ T cells and $CD8^+$ T cells from old mice.

The cells used to study alloreactivity were not entirely pure. FACS-

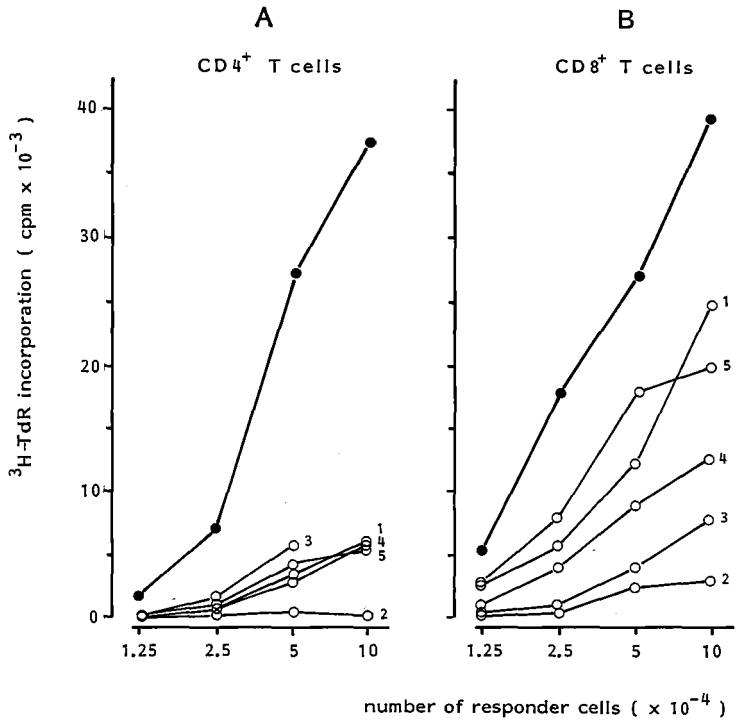

Fig.3 *MLR activity of purified $CD4^+$ T cells (A) and purified $CD8^+$ T cells (B) from young (n=5, 20 weeks) and old (n=5, 120 weeks) responders in the presence of exogenous IL-2. Responder cells from young donors were pooled. Old mice were individually tested. The data show mean responses from triplicate cultures. ●, young responders: ○, old responders.*

analysis with monoclonal antibodies (Mab) towards Thy-1, Lyt-2, L3T4 and mouse-Ig showed that a subpopulation of the cells did not react with these monoclonal antibodies. This is shown in Table 1. According to immunocytological analysis these cells were mostly granulocytes and sometimes monocytes (data not shown). Since these cells might influence the responses studied, the effect of these cells on the proliferative responses of young and old mice was tested (Table 2). Lyt-2$^-$, L3T4$^-$, Ig$^-$ cells did not

Table 1: FACS-analysis of purified spleen cells from young and old mice.

Spleen donor		Cell type									
		$Ig^{-a)}$				$Ig^-L3T4^{-b)}$			$Ig^-Lyt\text{-}2^{-b)}$		
		$L3T4^+$	$Lyt\text{-}2^+$	Ig^+	–	$Lyt\text{-}2^+$	$Gara^{c)}$	–	$L3T4^+$	$Gara^{c)}$	–
young (n=5)		55[d]	23	6	16	48	7	47	64	1	35
old	1	39	31	7	23	44	11	45	54	1	42
	2	29	37	6	38	45	14	41	53	8	39
	3	46	40	7	7	50	11	39	67	6	27
	4	43	33	5	17	45	9	46	63	7	30
	5	46	31	7	16	50	7	43	66	7	27

a). Nylonwool purified T cells from young (n=5, 20 weeks) and 5 individual old (120 weeks) mice were further depleted of Ig+ cells by panning using rat-anti-mouse kappa light chain (187-1) Mab (referred to as T cells).
b). Further depletion of CD4+ and CD8+ cells was performed by panning using anti-L3T4 and anti-Lyt-2 supernatants, respectively (referred to as CD8+ and CD4+ T cells).
c). Using FITC-conjugated goat-anti-rat (Gara) antibodies, cells stained with anti-L3T4 or anti-Lyt-2 supernatant for an indirect panning by GARA, which were not depleted, could be detected.
d). Percentage of cells positive for the determinant against which the Mab are directed, are shown.

proliferate in response to alloantigens (data not shown); in the presence of IL-2 a weak proliferation of these cells (both from young and old mice) was seen. More important, these cells did not suppress the proliferative response of spleen cells from young and old mice.

Decreased precursor frequency of alloreactive $CD4^+$ and $CD8^+$ T cells in old mice.

Because the diminished T cell reactivity in old mice may be due to a decrease of the frequency of responding T cells, we performed LDA to determine the precursor frequency of the alloreactive T cells. This was done in the presence of an excess of exogenous IL-2, because of the age-related

Table 2: Influence of $Ig^-L3T4^-Lyt\text{-}2^-$ spleen cells on the proliferative responses of spleen cells from young and old mice.

donors of spleen cells	Addition of Ig^-, $L3T4^-$,$Lyt\text{-}2^-$ cells	^{3}H-TdR incorporation (cpm)
–	$+^{a)}$	6,071 ± 2,006
–	$+^{b)}$	4,550 ± 956
young	–	39,291 ± 4,647
	$+^{a)}$	53,639 ± 1,246
	$+^{b)}$	56,637 ± 5,509
old	–	23,265 ± 4,368
	$+^{a)}$	29,177 ± 3,454
	$+^{b)}$	29,903 ± 2,586

Spleen cells ($4x10^4$/well) from young (n=5, 20 weeks) and old (n=5, 120 weeks) mice were used to determine the influence of Ig^-,$L3T4^-$,$Lyt\text{-}2^-$ cells ($2x10^4$/well) on the proliferative capacity of the responder cells. MLR activity of these cells was investigated in the presence of exogenous IL-2.
a) Ig^-,$L3T4^-$,$Lyt\text{-}2^-$ cells derived from young mice (n=5, 20 weeks).
b) Ig^-,$L3T4^-$,$Lyt\text{-}2^-$ cells derived from old mice (n=5, 120 weeks).

defect in the production of this lymphokine. T cells and T cell subpopulations from individual old CBA/Rij mice and from a pool of young CBA/Rij mice (n=5) were tested for their reactivity towards BALB/c spleen cells. For T cells of young mice, the frequency of alloreactive T cells appeared to be 1 in 340 splenic T cells (Fig. 4a). In old mice this frequency was two to ten fold lower, ranging from 1 in 730 to 1 in 3600 splenic T cells. The frequency calculated from the pooled data (n=5) was 1 in 1280. With the $CD8^+$ T cell population, results were obtained similar to total T cells. The frequency in a pool of $CD8^+$ T cells from young mice (1 in 200) was eight times higher than the frequency calculated from the pooled data from 5 individually tested old mice (1 in 1600) (Fig. 4b). The individual frequencies of responding $CD8^+$ T cells in old CBA/Rij mice varied from 1 in 750 to 1 in 3800 $CD8^+$ T cells. The frequency of $CD4^+$ T cells that responded to BALB/c spleen cells was lower than the frequency of $CD8^+$ alloreactive T cells. In young mice the frequency of alloreactive $CD4^+$ T cells (1 in 870) was three times higher than the frequency calculated from pooled data of individual old mice (1 in 2900), with an individual variation of 1 in 1000 to less than 1 in 6400 (Fig. 4c).

As discussed above, the $Ig^-CD8^-CD4^-$ cells did not suppress the allogeneic responses of young and old CBA/Rij mice. Therefore, it seems justified to correct the frequencies by using the real percentage (measured by FACS-analysis, Table 2) of total T cells, $CD4^+$ T cells or $CD8^+$ T cells in the cell suspensions used for LDA. The corrected frequencies are depicted in Table 3. The frequency of alloreactive total T cells from young mice was 4 times higher than the frequency calculated from the pooled data of 5 old mice. In young mice the frequency of $CD4^+$ T cells was 3.5 times higher than the frequency calculated from the pooled data of 5 old mice. For the $CD8^+$ T cell population, in young mice the frequency was 9 times higher than the frequency in old mice.

DISCUSSION

In an MLR the proliferative T cell response is comprised of proliferating $CD4^+$ T cells and $CD8^+$ T cells, the latter being dependent on IL-2 production by the $CD4^+$ T cells. In the present study we show that T cells from aged mice exhibit an impaired T cell reactivity towards alloantigens.

This impaired response is partly due to the impaired IL-2 production ability of $CD4^+$ T cells from old mice and partly due to the decreased proliferative response by the alloreactive $CD4^+$ T cells and $CD8^+$ T cells, even in the presence of IL-2. Limiting dilution analysis showed that the latter is at least partly caused by a decreased incidence of $CD4^+$ and $CD8^+$ alloreactive T cells in aged mice.

In old mice the IL-2 production is diminished, e.g. $CD4^+$ T cells from old mice produce low levels of IL-2 compared to $CD4^+$ T cells from young mice. This observation extends reports by Chang et al. (19) and Thoman and Weigle (20), in which IL-2 production ability of unseparated spleen cells was determined. Using purified $CD4^+$ T cells, the production ability of IL-2 can be more reliably determined, since interference of IL-2 consumption by $CD8^+$ T cells is circumvented. In addition, the possibility that IL-2 production by $CD4^+$ T cells is suppressed by $CD8^+$ T cells is excluded. The decreased IL-2 production is most likely not due to an inadequate IL-1 production ability, since the addition of rIL-1α could not restore the impaired IL-2 production ability by cells from old mice. This finding is supported by a study of Rosenberg et al. (21), who reported that old rats have the same IL-1 production ability as young rats. Furthermore, we used purified T cells so that macrophages from the stimulating BALB/c spleen cells had to serve as IL-1 source for the responding cells.

Because of low IL-2 levels the proliferation of activated T cells is smaller in old mice than in young mice. Insufficient IL-2 production by $CD4^+$ T cells from old mice therefore contributes to the low proliferative capacity of both $CD4^+$ T cells and $CD8^+$ T cells. To overcome this defect, IL-2 was added to the cultures. The IL-2 we used was obtained from Con A-free rat spleen supernatant. Comparison of rat IL-2 and rIL-2 in a proliferative assay revealed that the effect of rat IL-2 and rIL-2 on the proliferative capacity of cells from young and old mice was comparable.

In the presence of exogenous IL-2 the magnitude of the proliferative response of $CD8^+$ T cells from young and old mice exceeded that of the $CD4^+$ T cells from young and old mice. The $CD8^+$ T cells from old mice showed a decreased proliferative response compared to $CD8^+$ T cells from young mice. For the $CD4^+$ T cells from young as well as from old mice the proliferative

response was slightly enhanced by addition of exogenous IL-2, but the threefold difference between cells from young and old mice remained. A similar observation was made for the impaired DTH responses which are mediated by $CD4^+$ T cells (Chapter 3). These responses could neither be improved by addition of IL-2 to the cultures, in which the DTH effector T cells were generated. Thus both types of impaired responses mediated by $CD4^+$ T cells cannot be restored by addition of IL-2. A partial restorative effect of IL-2 on age-associated loss of proliferation has been reported by several groups (19,20,22).

These studies together with our own results show that there is a deficit in T cells from old mice, which cannot be overcome by the addition of

Table 3: Corrected frequencies for total T cells, $CD4^+$ and $CD8^+$ T cells from young and old CBA/Rij mice capable to proliferate against irradiated (25 Gy) BALB/c spleen cells in the presence of exogenous IL-2.

Cell type	age	F	95% CL
total T cells	young	1/250	1/210 -1/350
"	old	1/950	1/840 -1/1120
CD4+ cells	young	1/550	1/440 -1/760
"	old	1/1900	1/1520-1/2300
CD8+ cells	young	1/95	1/75 -1/125
"	old	1/800	1/730 -1/860

Frequencies for young mice (20 weeks) were obtained from pooled cells, frequencies for old mice (120 weeks) were calculated from the pooled data of individual mice. F = frequency; 95% CL = 95% confidence value for the frequency.

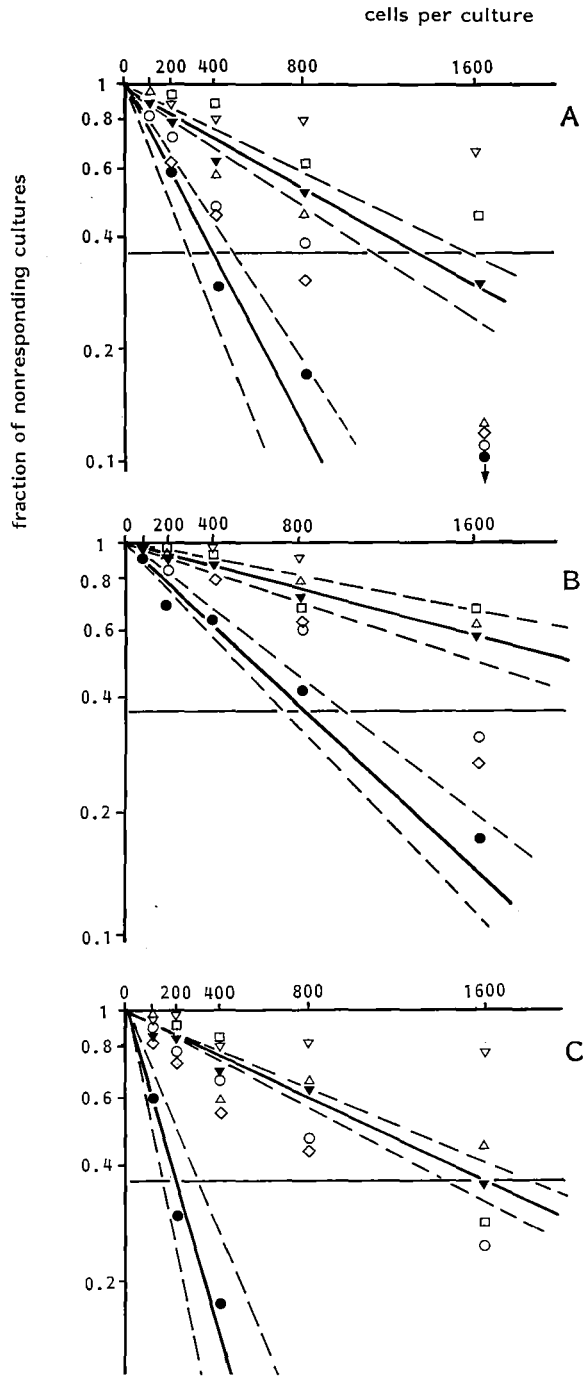
cells per culture
fraction of nonresponding cultures
0
200
400
800
1600
1
0.8
0.6
0.4
0.2
0.1
A
B
C

Fig.4 Comparison of the frequencies of alloreactive T cells from young (n=5, 20 weeks) and old mice (120 weeks) mice. Limiting dilution assay was performed in the presence of exogenous IL-2. Frequencies for young mice were obtained from pooled cells, frequencies for old mice were calculated from pooled data of individually tested mice. Upper panel: T cells, middle panel: CD4+ T cells, lower panel: CD8+ T cells. Closed symbols represent pooled data, open symbols represent individual mice.

exogenous IL-2. Other studies (10,11,23) and our present results indicate that this deficit is in part due to at least a reduction in the frequency of antigen-specific T cells. A decreased precursor frequency in old mice has also been noted by Ashman (10), who determined the precursor Tc (pTc) frequency towards influenza virus. A study of Nordin and Collins (23) demonstrated a lower alloreactive pTc frequency. In addition, Miller (24) showed a decline in precursor frequencies for several different cell-mediated responses: IL-2 production in response to T cel mitogens, the mitogen-stimulated nonspecific CTL response, proliferation to Con A and KLH specific Th cells. In the present paper, we show that the allogeneic precursor frequencies of total T cells, $CD4^+$ T cells and $CD8^+$ T cells from old mice in comparison to young mice are decreased. The precursor frequency we found for alloreactive T cells derived from young mice is consistent with data from the literature (25-27).

The decline in precursor frequency of $CD4^+$ and $CD8^+$ T cells accounts to a great extent, but not completely, for the observed reduction in alloreactive proliferation of $CD4^+$ and $CD8^+$ T cells from old mice in bulk cultures. The decline in apparent precursor frequency may reflect a requirement for several cycles of proliferation before an activated precursor can give rise to a detectable clone of effector cells. Aging might lead to a decrease in the proportion of T cells able to enter into an extended proliferative phase that preceeds differentiation into effector cells and thus indirectly lower the precursor frequencies. Cell cycle kinetics studies with human peripheral blood lymphocytes (PBL) (28,29) have suggested that fewer cells from older donors are able to proceed through repeated mitotic cycles compared with cells from young individuals. Reduced precursor frequencies can also be the result of a decline of the functional activity on a per cell basis. For IL-2 production, it has, however, been reported by Miller (24) that the amount of IL-2 generated per cell is unaltered during aging.

Recent studies have shown that the particular pattern of H-2 alloreactivity displayed by T cells correlates closely with the expression of CD4 and CD8 molecules (31-33). CD4 and CD8 are viewed as accessory molecules which increase the overall avidity of the binding of antigen by T cells. Antigen recognition and T cell activation are mediated through the antigen T cell receptor (TCR) and the CD3 complex (34,35). Thus, an additional explanation for impaired T cell responses might be a reduced avidity of accessory molecules like CD4 and CD8 and consequently a decline in the function of the TCR. Alternatively, impaired T cell responses might be due to an inefficient transmembrane signal transduction and/or generation of second messengers involved in cell proliferation (30).

Whether the impaired responsiveness to alloantigens besides impaired IL-2 production and reduced precursor frequency is due to an inefficient signal transduction or to a defect at T cell-receptor-level is subject of further study.

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CHAPTER 5

ACTIVATION WITH PMA AND IONOMYCIN AS AN APPROACH TO ANALYSE IMPAIRED T CELL RESPONSIVENESS IN OLD MICE

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SUMMARY

Immune responsiveness declines during aging as measured by alloantigen induced and mitogen induced proliferation. Since the impaired immune responses might be due to a reduced signal transduction, we studied T cell activation using phorbol 12-myristate 13-acetate (PMA) and ionomycin. T cell activation was studied by measurement of IL-2 production, IL-2 receptor (IL-2R) expression and proliferation of purified CD4+ and CD8+ T cells from young (20 weeks of age) and old (120 weeks of age) mice. The results demonstrate that following stimulation with PMA and ionomycin the IL-2 production by CD4+ T cells from young and old mice was comparable despite a threefold and twofold decline found after stimulation with BALB/c spleen cells and Con A, respectively. However, after PMA and ionomycin stimulation the proliferative responses of CD4+ T cells from old mice were substantially lower than those from young mice, whereas proliferative responses of CD8+ T cells from old mice were only slightly lower than the responses from young mice. Furthermore, the reduced proliferative capacity of CD4+ T cells was not reflected by a reduced expression of IL-2R. The expression of IL-2R by CD8+ T cells from old mice, however, was lower than that by CD8+ T cells from young mice.

INTRODUCTION

The immune responsiveness of mammals decreases with increasing age (1–6). Our previous results showed that the in vivo and in vitro induced DTH reactivity to alloantigens declines during aging (Chapter 3). It appeared that the decreased DTH reactivity induced in vitro, was due to an impaired function of $CD4^+$ T cells at the level of DTH effector cells and/or a lower frequency of antigen–specific DTH cells (Chapter 3). The latter possibility was supported by our finding that allogeneic responses of $CD4^+$ T cells as well as of $CD8^+$ T cells were decreased and this was not due to suppressive effects exerted by $CD8^+$ T cells but in part due to a reduced precursor frequency and reduced IL–2 production (Chapter 4). Therefore, we assumed that the age–related impaired T cell function might be due to a defect in T cell activation.

T cell activation is initiated by binding of antigen to the T cell receptor (TCR) (7–10), which is noncovalently associated with CD3. As a result of binding of antigen, phosphodiesterase (PDE) catalyzes the hydrolysis of phosphatidylinositol biphosphate (PIP2) into inositol triphosphate (IP3) and diaglycerol (DAG) (11,12). IP3 causes the release of Ca^{2+} from intracellular stores, whereas DAG activates protein kinase C (PKC) activity (11–13) (Fig. 1). The increase in intracellular Ca^{2+} and activation of PKC leads to the initiation of second stage events, in which previously untranscribed genes, like genes encoding IL–2 and IL–2 receptor (IL–2R) become expressed.

Therefore, improper triggering of the TCR and as a possible consequence a reduced capacity to generate second messengers, e.g. PKC and increase of cytoplasmic Ca^{2+}, might be a reason for impaired immune responses in old mice as measured by proliferation and IL–2 production. To investigate this we used an alternative pathway of T cell activation, by which the TCR is bypassed. Such an alternative activation of T cells can be achieved by the synergistic cooperation of phorbol 12–myristate 13–acetate (PMA), which activates PKC directly, and calcium ionophore (e.g. ionomycin), which increases cytoplasmic Ca^{2+} (14, 15).

In the present study, we investigated PMA and ionomycin induced activation of T cells from young and old mice. Because of our main interest in $CD4^+$ T cells as a cell with a central regulatory function, we studied $CD4^+$

T cells and $CD8^+$ T cells separately.

The results suggest that reduced T cell responses in old mice are in part due to an impaired signal transduction.

MATERIALS AND METHODS

Mice. Male CBA/BrARij mice (abbreviated in the text as CBA/Rij) and BALB/c mice were bred and raised under SPF conditions in the TNO–REP Institutes, Rijswijk, The Netherlands. Aged mice were obtained from cohorts of mice that were barrier maintained. Aged mice were examined histologically for age–related pathology (16) and excluded when a malignancy was found.

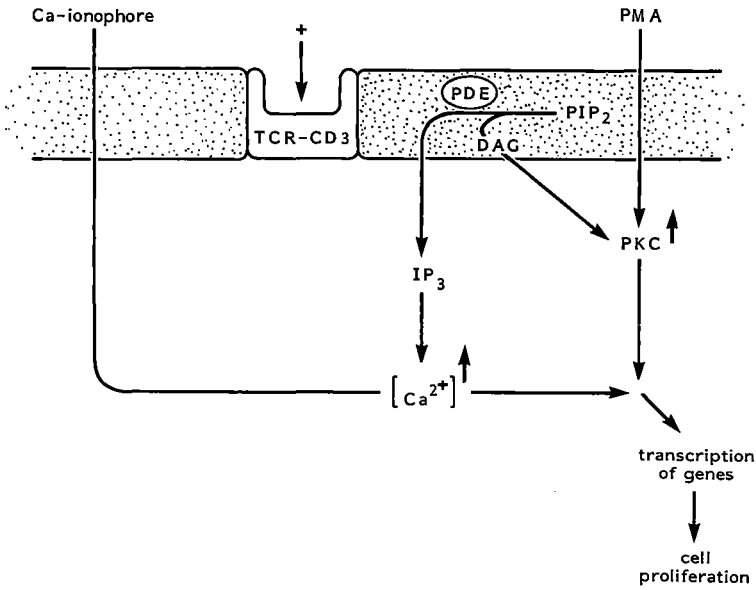

Fig. 1 Schematic representation of T cell activation. Upon appropriate presentation of an antigen pertubation of TCR–CD3 complex takes place. As a result phosphodiesterase (PDE) catalyzes the hydrolysis of phosphatidylinositol biphosphate (PIP2) to inositol triphosphate (IP3) and diaglycerol (DAG). IP3 mobilizes Ca2+ and DAG activates protein kinase C (PKC). Antigen–dependent T cell activation can be mimicked by stimulation with phorbol 12–myristate 13–acetate and ionomycin.

Preparation of cells. Spleens from young (20 weeks old) and old mice (120 weeks old) were prepared for single cell suspensions and washed twice with culture medium. This medium consisted of RPMI 1640 supplemented with 5% fetal calf serum (FCS; Seralab, Oxford, UK), L-glutamine (2 mM), Hepes (20 mM), penicillin (100 U/ml), streptomycin (100 µg/ml) and 2-mercaptoethanol ($5x10^{-5}$ M). Splenic T cells were purified and separated into subsets as described in Chapter 4. The Lyt-2-depleted and L3T4-depleted T cells are further referred to as $CD4^+$ and $CD8^+$ T cells, respectively. Viable cell counts are based on trypan blue exclusion.

Proliferation assay. Responder cells ($3x10^4$/well) were stimulated in the presence of 10^5 100 Gy irradiated syngeneic spleen cells in culture medium in 96 well flat bottom microtiter plates (Costar, Cambridge, MA). Cells were stimulated with 2 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma, Chemical Co., St. Louis, MO) and 300 nM ionomycin (Calbiochem, La Jolla, CA) both diluted from stock solutions made in dimethyl sulfoxide. The plates were incubated at 5% CO_2 in a humidified environment at 37°C for 2 to 5 days. Cultures were pulsed with 0.25 µCi ^{3}H-methyl-thymidine (^{3}H-TdR) (Radiochemical Centre, Amersham, UK) and harvested 6 h later.

IL-2 assay. Culture supernatants were collected after 2 and 3 days of culture with PMA and ionomycin or after 2 days of culture with concanavalin A (Con A) or after 4 days of culture with irradiated (25 Gy) BALB/c spleen cells. IL-2 was assayed by determining the ability of the supernatants to stimulate proliferation of CTLL-2 cells (17). For this bioassay, 100 µl of the culture supernatant was serially diluted in culture medium. Five thousand CTLL in a volume of 100 µl were added to each well and the plates were incubated at 37°C for 24 h. The last 4 h of culture 0.25 µCi ^{3}H-TdR was added to each well. Rat IL-2 served as a standard. One unit of IL-2 activity was defined as the amount of IL-2 inducing half-maximal proliferation of the CTLL.

Flow cytometric analysis of cultured cells. Cells were stimulated with irradiated BALB/c spleen cells as described in Chapter 4 or with PMA and ionomycin as described above. Cells were washed and phenotyped by labelling with FITC-conjugated anti-Lyt-2 or with biotin-conjugated anti-L3T4, and followed by incubation with FITC-conjugated avidin. For the expression of

membrane IL-2 receptor (IL-2R) the cells were incubated (45 min, 4°C) with saturating amounts of culture supernatants containing the monoclonal antibody 7D4 (cells were kindly provided by Dr. E.M. Shevach) (18) for 45 min on ice. The cells were washed and incubated (30 min, 4°C) with FITC-conjugated-rabbit-anti-rat-Ig. The fluorescence was analyzed with a FACS-II (Becton Dickinson, Sunnyvale, CA). Fluorescence histograms were generated from live cells as determined by forward light scatter and propidium iodide-fluorescence.

RESULTS

IL-2 production by $CD4^+$ T cells from young and old mice.

In a previous study we demonstrated that the $CD4^+$ T cells from old CBA/Rij mice were impaired in their ability to respond to alloantigen by proliferation and by IL-2 production (Chapter 4). As can be seen in Table 1, cells from individual young mice produce 51 U/ml IL-2 after stimulation with BALB/c spleen cells, whereas cells from individual old mice produce 16 U/ml IL-2. The impaired IL-2 production was not entirely due to a decline in alloantigen-specific $CD4^+$ IL-2 producers. This appeared from the IL-2 production by similar cells after polyclonal stimulation with Con A. Then $CD4^+$ T cells from young mice produced 262 U/ml IL-2, whereas cells from old mice produced 121 U/ml IL-2. Stimulation of the cells with PMA and ionomycin, however, resulted in 161 U/ml IL-2 for $CD4^+$ T cells from young mice and 142 U/ml for $CD4^+$ T cells from old mice. In separate experiments we used pooled $CD4^+$ T cells. It appeared that $CD4^+$ T cells from young mice produced 85-120 U/ml IL-2 and pooled $CD4^+$ T cells from old mice produced 65-130 U/ml IL-2. Thus, in contrast to allogeneic or Con A-induced stimulation, activation with PMA and ionomycin resulted in comparable IL-2 production by $CD4^+$ T cells from young and old mice. Hence, the reduced IL-2 production ability of old mice upon allogeneic stimulation may be due to an inadequate signal transduction.

Table 1: IL-2 production by $CD4^+$ T cells from young and old mice.

Experiment (no)	Donor mice	Activation	IL-2 production (U/ml)
1	young	BALB/c	51(43-60)
"	old	"	16 (4-37)
"	young	Con A	262(157-480)
"	old	"	121 (83-163)
"	young	PMA/ionomycin	161 (83-290)
"	old	"	143 (84-230)
2	young	"	85
"	old	"	65
3	young	"	150
"	old	"	130

10^5 purified $CD4^+$ T cells from individual young (n=5; 20 weeks) and individual old (n=5; 120 weeks) mice were stimulated either for 4 days with $2x10^5$ irradiated (25 Gy) BALB/c spleen cells, for 2 days with 1 µg/ml Con A or for 2 days with 2 ng/ml PMA and 300 nM ionomycin (exp. 1). Additional experiments (2&3) were performed with pooled cells: $3x10^4$ pooled purified $CD4^+$ T cells from young (n=5) or old (n=5) mice were cultured with PMA and ionomycin. The IL-2 activity in the supernatants was quantified, of which the average and for experiment 1 also the range is given.

Age-related decline in PMA and ionomycin induced proliferation of T cells.

To investigate whether the PMA and ionomycin induced IL-2 production is reflected by proliferation, we studied the proliferation of cells from young and old mice with this type of activation. This was performed with total T cells as well as purified $CD4^+$ T cells and purified $CD8^+$ T cells in the absence and in the presence of exogenous IL-2 (50 U/ml).

Because of the type of activation and because the PMA and ionomycin induced IL-2 production by $CD4^+$ T cells from old mice was

comparable to that by cells from young mice, we expected that the magnitude of proliferation by $CD4^+$ T cells and by $CD8^+$ T cells from young and old mice would be comparable. However, as can be seen in Figure 2, this was only partly true. Maximum responses were observed on day 3. In the absence of IL-2, responses of $CD8^+$ T cells from old mice were only slightly lower than those from young mice: about 40,000 cpm ^{3}H-TdR incorporation by 30,000 cells from old mice was found as compared to 48,000 cpm by a similar number of cells from young mice. Whether these responses were IL-2 independent is unclear, although culture supernatants did not contain detectable amounts of IL-2 (not shown). In contrast to the results obtained with $CD8^+$ T cells, the day 3 responses by $CD4^+$ T cells from old mice were threefold lower than the day 3 responses by $CD4^+$ T cells from young mice. However, in this case maximum responses occurred one day later than observed with $CD8^+$ T cells. In the absence of exogenous IL-2, responses of total T cells were intermediate to those of $CD8^+$ and $CD4^+$ T cells.

Since $CD4^+$ T cells produce IL-2 by themselves, it was not realistic to compare responses of these cells with those of $CD8^+$ T cells. Therefore, proliferative responses were also studied in the presence of an excess of rat IL-2 (50 U/ml). Although the magnitude of the response was higher than found in the absence of IL-2, responses of $CD8^+$ T cells from old mice were only slightly lower than those by $CD8^+$ T cells from young mice. The increase in the responses showed that, in addition to a possible IL-2 independent pathway, a great part of the response was IL-2 dependent. Addition of IL-2 to cultures of $CD4^+$ T cells hardly affected their response as might be expected from the fact that these cells are able to produce IL-2 themselves. In the case of $CD4^+$ T cells from young mice, but not from old mice, the proliferative responses were comparable at day 3 and 4. Total T cells exhibited a type of response intermediate to that by $CD4^+$ T cells and $CD8^+$ T cells.

Thus, the ability of $CD8^+$ T cells from old mice to proliferate in response to PMA and ionomycin appeared only slightly below normal. In contrast, $CD4^+$ T cells from old mice –despite an efficient capacity to produce IL-2 – displayed an impaired proliferative capacity as compared to

$CD4^+$ T cells from young mice. Apparently, the alternative pathway to generate second messengers is sufficient to induce normal IL-2 levels, but not a normal cell proliferation.

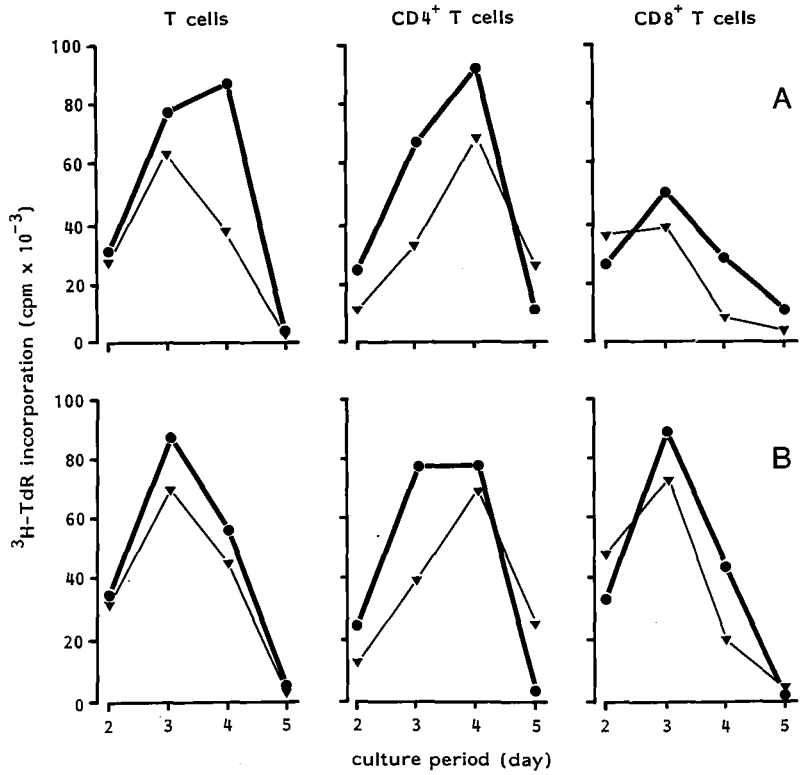

Fig. 2 Nylonwool purified total T cells, purified CD4+ T cells and purified CD8+ T cells from young (n=5; 20 weeks) and old (n=5; 120 weeks) CBA/Rij mice were stimulated with PMA (2 ng/ml) and ionomycin (300 nM) and cultured for 2-5 days in the absence (Fig. 2A) or in the presence (Fig. 2B) of an excess of exogenous rat IL-2 (50 U/ml). Proliferative responses were measured by labelling cells with 0.25 µCi ^{3}H-TdR and harvesting these cultures 6 hours later. Means of triplicate cultures are given. ●, young responders; ▼, old responders.

IL-2 receptor expression.

Cellular proliferation studied so far may in part be IL-2 independent, in addition to the IL-2 dependent pathway. The latter may be reflected by the expression of IL-2R. Therefore, information on the expression of IL-2R may be helpful to elucidate why responses of $CD4^+$ T cells from old mice are lower than the responses of these cells from young mice, even in the presence of IL-2. Thus, we studied the expression of IL-2R as measured by the monoclonal antibody 7D4 on $CD4^+$ and $CD8^+$ T cells from young and old mice after stimulation with either BALB/c spleen cells or with the combination of PMA and ionomycin.

A. IL-2R expression on $CD4^+$ T cells

Allogeneic stimulation of $CD4^+$ T cells in the presence of IL-2 (50 U/ml) resulted in more than 99% $L3T4^+$ cells (Fig.3). On the basis of forward light scatter and fluorescence intensity two distinct $L3T4^+$ populations could be discriminated: a dull cell population reflecting the small resting lymphocytes and a bright cell population reflecting the blastlike cells. The latter population comprised 28% of the cells analysed. Twenty-four percent of the cells, the majority within the blast-like population, expressed IL-2R as measured by Mab 7D4.

After activation of the cells with PMA and ionomycin, more than 99% of the cells were $L3T4^+$ and more than 95% of the cells were $IL\text{-}2R^+$ (Fig. 4). This was true for cells from young as well as from old mice. Allogeneic stimulation of $CD4^+$ T cells as well as stimulation with PMA and ionomycin resulted in comparable binding of 7D4 by cells from young and from old mice, suggesting a comparable IL-2R expression. Addition of IL-2 during the culture period did not influence the percentage of 7D4-positive cells nor the level of IL-2R expression. Thus impaired proliferation by $CD4^+$ T cells from old mice is not reflected by a reduced expression of IL-2R.

B. IL-2R expression on $CD8^+$ T cells

Because $CD8^+$ T cells did not proliferate in the absence of IL-2, allogeneic stimulation was necessarily performed in the presence of

exogenous IL-2. After a culture period of 5 days more than 95% of the cells were Lyt-2^+. As for $CD4^+$ T cells, activated blast-like $CD8^+$ T cells could be discriminated from small resting $CD8^+$ T cells on the basis of forward light scatter and fluorescence intensity (Fig. 5). Stimulation of cells from young mice resulted in the generation of twice as many blast cells than stimulation of cells from old mice (42% versus 20%). Furthermore, the $CD8^+$ T cells from young and old mice exhibited also

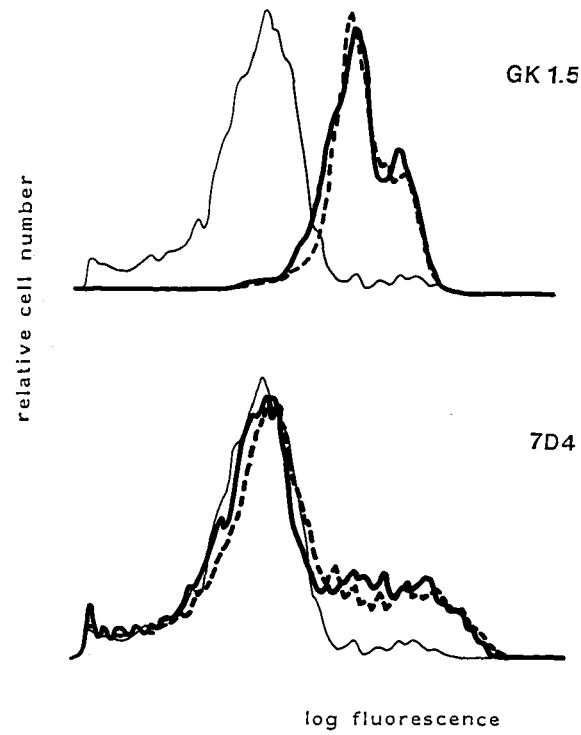

Fig. 3 Purified CD4+ T cells from young (n=5; 20 weeks) and old (n=5; 120 weeks) CBA/Rij mice were cultured with irradiated (25 Gy) BALB/c spleen cells in the presence of IL-2 (50 U/ml). Cells were cultured for 5 days and stained with GK1.5 to assess the percentage of CD4+ T cells and with 7D4 to assess the IL-2R expression. ——— young responders; ----- old responders; ——— negative control.

differences in IL-2R expression. Among cells from young mice 38% $7D4^+$ cells were found, whereas among cells from old mice only 12% were $7D4^+$. Therefore, improper IL-2R expression may explain low proliferative responses of $CD8^+$ T cells after allogeneic stimulation.

When $CD8^+$ T cells were stimulated with PMA and ionomycin in the absence of IL-2, more than 95% of the cells were $Lyt\text{-}2^+$. In the absence of exogenous IL-2 this type of activation resulted in less than 5% $IL\text{-}2R^+$ cells as measured by 7D4 staining (Fig.6). This finding, together with our inability to detect IL-2 in the culture supernatant, suggests that the proliferation we observed with $CD8^+$ T cells in the absence of IL-2 occurred through an IL-2 independent pathway. In the presence of an

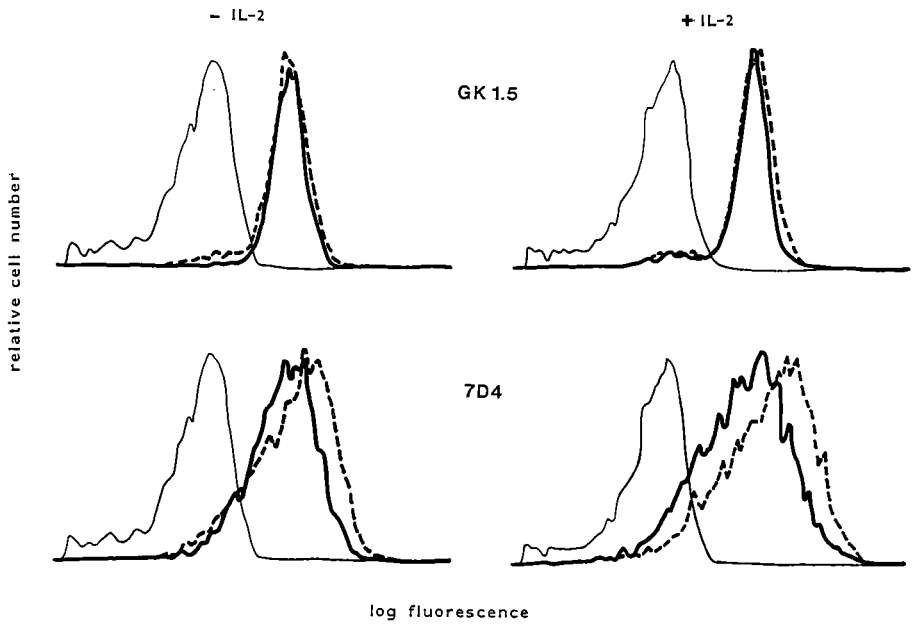

Fig. 4 Purified CD4+ T cells from young (n=5; 20 weeks) and old (n=5; 120 weeks) CBA/Rij mice were stimulated with PMA (2 ng/ml) and ionomycin (300 nM) in the absence or presence of IL-2 (50 U/ml). Cells were cultured for 3 days and stained with GK1.5 to assess the percentage of CD4+ T cells and with 7D4 to assess the IL-2R expression. ——— young responders; ------- old responders; —— negative control.

excess of exogenous IL-2 92% of the cells from young mice and 73% of the cells from old mice expressed IL-2R. This confirms the observation of others that IL-2R expression is an IL-2 dependent event. Cells from young and old mice differred by the density of IL-2R expression: cells from young mice exhibited a higher density of IL-2R than cells from old mice. Apparently, IL-2R expression was only of slight importance for this type of activation since proliferative responses were almost the same.

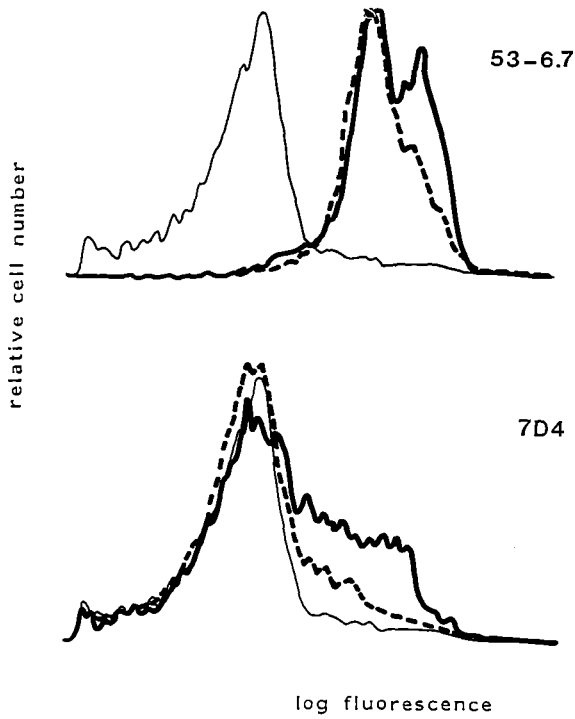

Fig. 5 Purified CD8+ T cells from young (n=5; 20 weeks) and old (n=5; 120 weeks) CBA/Rij mice were stimulated with irradiated (25 Gy) BALB/c spleen cells in the presence of IL-2 (50 U/ml). Cells were cultured for 5 days and stained with 53-6.7 to assess the percentage of CD8+ T cells and with 7D4 to assess the IL-2R expression. ——— young responders; ----- old responders; ——— negative control.

DISCUSSION

Binding of antigen to the T cell receptor (TCR) on the cell surface initiates the production of a series of messenger molecules which in turn activate enzymes that control cellular functions such as IL-2 production, IL-2R expression and proliferation (19,20). This process can be mimicked by substances that directly activate PKC, like PMA, and induce an increase of cytoplasmic Ca^{2+}, like ionomycin.

Immune responses decrease during aging (1-6). Previously, we demonstrated this for in vivo and in vitro induced DTH reactivity to

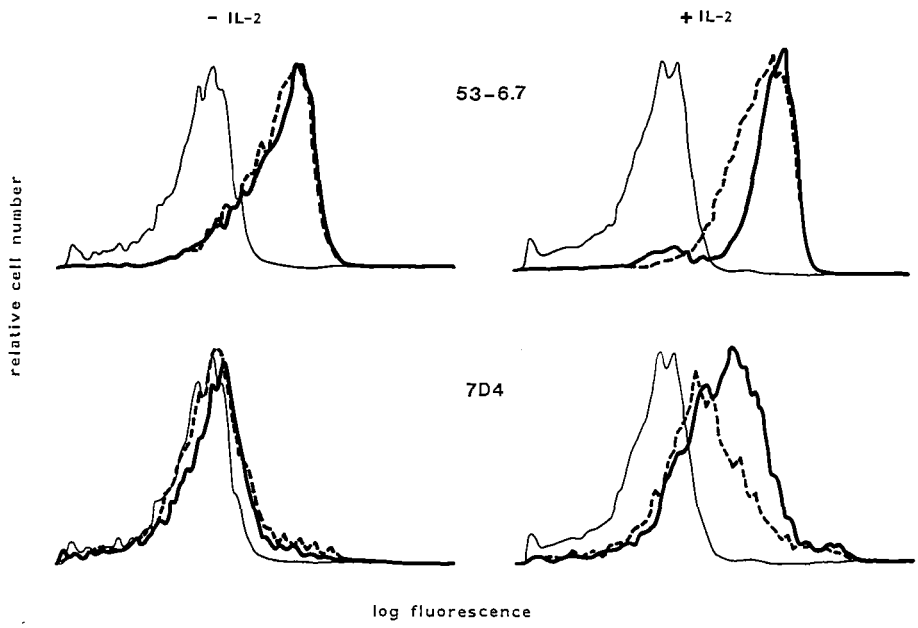

Fig. 6 Purified CD8+ T cells from young (n=5; 20 weeks) and old (n=5; 120 weeks) CBA/Rij mice were stimulated with PMA (2 ng/ml) and ionomycin (300 nM) in the absence or presence of IL-2 (50 U/ml). Cells were cultured for 3 days and stained with 53-6.7 to assess the percentage of CD8+ T cells and with 7D4 to assess the IL-2R expression. —— young responders; ----- old responders; ——— negative control.

alloantigens (Chapter 3). A decreased allogeneic proliferation was also found for an MLR and this appeared to result at least to some extent from a reduced precursor frequency and a reduced ability to produce IL-2 (Chapter 4). This age-related impairment of T cell function could, however, not be explained by these phenomena completely. Therefore, it was hypothesized that impaired immune responses are partly due to an impairment of the cells to transmit extracellular signal across the cell membrane. In order to investigate this possibility, we studied T cell activation by PMA and ionomycin, a procedure that bypasses the TCR.

With respect to IL-2 production, which is almost entirely restricted to $CD4^+$ T cells, it has been reported that the process of aging results in a decrease when studied in models employing allogeneic stimulation (5, 6, Chapter 4). Our present results show that upon PMA and ionomycin activation comparable amounts of IL-2 are produced by $CD4^+$ T cells from young and old mice. This suggests that the capacity to produce IL-2 is intact, provided that the appropriate signals are generated. Possibly, low responses observed after stimulation via the TCR, as is the case for the allogeneic MLR, are due to a failure in the process that leads to the activation of PKC and increase of intracellular calcium rather than a process thereafter. Concerning the generation of second messengers upon allogeneic stimulation of T cells from old mice, no data are available.

In spite of a comparable IL-2 production capacity by $CD4^+$ T cells from young and old mice, PMA and ionomycin activation of $CD4^+$ T cells from young and old mice did not result in comparable proliferative responses. This finding is difficult to explain in view of the observation that PMA induced PKC activity in spleen cells from young and old mice is comparable (21). The fact that responses may be due to two types of activation pathways, i.e. IL-2 dependent and IL-2 independent, makes the situation even more complex. Concerning the IL-2 dependent pathway, progress of T cells from G_0/G_1 into the S phase of the mitotic cycle depends on an interaction between IL-2 and its specific receptor, which is transiently expressed by T cells that have received a stimulatory signal. The work of Cantrell and Smith (22) has shown that the proliferative response of activated T cells depends on a critical threshold of

interaction between IL-2 and IL-2R, and that changes in concentration of either element can influence cell cycling (22). Furthermore, when mitogen-stimulated human T cells were separated by flow cytometry into subpopulations expressing different levels of IL-2R as measured by Tac-expression (p55 subunit), most of the IL-2 supported proliferative response was found to reside in the subset with the highest level of IL-2R expression (22). So, the expression of IL-2R may be considered as a reflection of the extent of activation. However, $CD4^+$ T cells from old mice either after allogeneic stimulation or after PMA and ionomycin stimulation expressed levels of 7D4, which were comparable to those of cells from young mice. One explanation might be that 7D4 is not representative for a functional IL-2R. We determined the expression of IL-2R with monoclonal antibody 7D4, which is directed against the p55 subunit of the IL-2R (18). Using such an antibody it is impossible, however, to discriminate between the high and the low affinity IL-2R. Therefore, it might be that 7D4 expression on $CD4^+$ T cells from young and old mice is comparable, but that the expression of the p75 subunit on cells from old mice is reduced. From the work of Negoro et al. (23) it is clear that PHA activated human peripheral blood lymphocytes (PBL) from old donors express less receptors with high affinity than those from young donors. Another explanation for the difference between $CD4^+$ T cells from young and old mice might be a difference in the IL-2 independent pathway. This can be studied by using antibodies directed against IL-2 and against IL-2R. A third explanation for the discrepancy between the apparently normal IL-2 production and IL-2R expression and the impaired proliferative response might be that the latter are caused by a subset of $CD4^+$ T cells that do not produce IL-2. It is known that $CD4^+$ T cells are comprised of IL-2 producers (Th_1) and IL-4 producers (Th_2)(24). Therefore, information on for example the IL-4 producing $CD4^+$ T cells will be valuable.

We observed a decreased IL-2R expression by $CD8^+$ T cells from old mice after allogeneic stimulation. This finding extends that of other groups (23,25,26). In addition, we found that the IL-2R expression on $CD8^+$ T cells after PMA and ionomycin activation was IL-2 dependent. IL-

2R expression on $CD8^+$ T cells from old mice was decreased in comparison to cells from young mice. Because it has been demonstrated that the induction of IL-2R requires translocation of PKC activity (20) and because PKC activity after PMA activation was equal in spleen cells from young and old mice (21), it might be that in $CD8^+$ T cells from old mice defects in processes different from PKC activation are responsible for the reduced IL-2R expression.

Using PMA and ionomycin as stimuli we observed proliferation of $CD8^+$ T cells in the absence of IL-2. Moreover, this occurred in the absence of a detectable IL-2R expression, suggesting an IL-2 independent pathway. Still, the addition of a high affinity antibody directed against IL-2R, i.e. 5A2 (27) partly inhibited the proliferative responses (data not shown). The fact that no IL-2 was detected in culture supernatants of $CD8^+$ T cells might be due to the complete utilization of IL-2. Therefore, one should be carefull with interpreting this response as completely IL-2 independent. Still, IL-2 independent proliferation of $CD8^+$ T cells has been reported by some groups (28-30). According to the results of Kim et al. (29) and Geller et al. (30) such proliferation can be induced by PMA alone or ionomycin alone. Apparently, both PMA and ionomycin are able to drive $CD8^+$ T cells along an activation pathway involving IL-2 independent proliferation.

The present study shows that especially with regard to the $CD4^+$ T cells, which have a central regulatory role, further studies are needed to increase insight into the age-related deterioration of immune responses. Such studies should focus on intracellular processes.

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CHAPTER 6

GENERAL DISCUSSION

In this thesis the age-related changes of the cellular immune system have been investigated. The experiments have focussed on a variety of functional aspects. Using T lymphocyte activation through allogeneic stimulation in comparison with other types of stimulatory signals as a model, changes with age have been studied both in vivo and in vitro at various levels. As summarized in Table 1 and depicted in Figure 1, changes in T cells throughout aging are multiple and can occur at every level investigated. At the level of functional capacity of T cells we observed an age-related decline in in vivo and in vitro induced delayed type hypersensitivity (DTH) to a variety of antigens (Chapters 2 and 3), including the DTH to alloantigens. It appeared that the impaired DTH to alloantigens is not due to suppressive effects exerted by $CD8^+$ T cells, but is probably due to a defect of the $CD4^+$ T cell population. To get more insight into the possible defects, we studied the proliferative capacity of these cells (Chapter 4). In line with others we observed an age-related decline in the MLR of both $CD4^+$ and $CD8^+$ T cells. The decreased proliferation of $CD4^+$ and $CD8^+$ T cells from old mice could partly, but not completely, be explained by a reduced antigen-specific precursor frequency and by a reduced IL-2 production ability. Based on these findings and other reports (1,2) we hypothesized that also the $CD4^+$ T cells themselves are defective. Activation signals delivered by the TCR-CD3 complex (3) or CD4/8 determinants (4) are transferred into the interior of the cell and lead to the formation of second messengers, i.e. protein kinase C (PKC) and increase of cytoplasmic Ca^{2+} (3,5,6). These second messengers can also directly be generated by the use of phorbol 12-myristate 13-acetate (PMA), which activates PKC, and calcium ionophore (i.e. ionomycin), which increases cytoplasmic Ca^{2+}. The synergistic cooperation of PMA and ionomycin bypasses the TCR. Using this type of activation (Chapter 5) we observed, in contrast to allogeneic stimulation, comparable IL-2 production by $CD4^+$ T cells from young and old mice. However, after PMA and ionomycin stimulation the

Table 1: Summary of age-related changes observed in allogeneic and PMA/ionomycin stimulated $CD4^+$ T cells and $CD8^+$ T cells studied in this thesis.

	$CD4^+$ T cells		$CD8^+$ T cells		Chapter
	allo	PMA/ion	allo	PMA/ion	
T cell functions:					
-DTH	y>>o	-	-	-	2,3
-proliferation	y>>o	y>o	y>>o	y>o	4
Functional aspects of T cells:					
-precursor frequency (1)	y>o	-	y>o	-	4
-IL-2 production (5)	y>o	y=o	-	-	5
-IL-2R expression (6)	y=o	y=o	y>o	y>o	5

y: young mice (20 weeks); o: old mice (120 weeks); - not done; allo: allogeneic; PMA/ion: PMA/ionomycin; (1): Fig. 1.1; (5): Fig. 1.5;(6): Fig. 1.6.

proliferative responses of $CD4^+$ T cells from old mice were lower than those from young mice, whereas proliferative responses of $CD8^+$ T cells from old mice were only slightly lower than the responses from young mice. Furthermore, the reduced capacity of $CD4^+$ T cells to proliferate was not reflected by a reduced expression of IL-2R. While $CD4^+$ T cells from old mice apparently normally expressed IL-2R, the expression of IL-2R by $CD8^+$ T cells from old mice was lower than that by $CD8^+$ T cells from young mice. Thus, quantitative as well as qualitative differences between young and old T cells seem to underly the diminished T lymphocyte functions at old age.

The first obvious conclusion which can be drawn from these results is that dysfunction of the immune system during aging is not caused by one single defect affecting a central key function as has been suggested for instance for IL-2 production (7,8). The results described in this thesis and supported by many other investigations (9,10) strongly indicate changes at various levels of cell function. As will be discussed later, still a common

mechanism causing such a generalised age-associated cellular dysfunction is quite possible.

A second important observation which cannot directly be made from Table 1, but which is obvious from the actual data in the preceeding Chapters, is the high interindividual variation found between the old mice for almost all functions and criteria assessed.

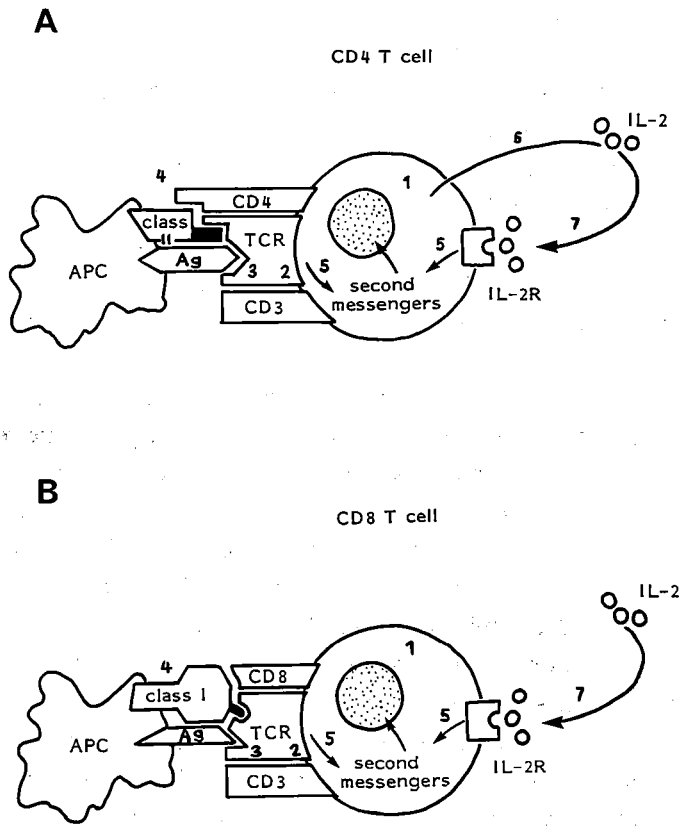

Fig.1 Schematic representation of age-related changes of CD4+ T cells and CD8+ T cells. The cells are affected at the level of precursor frequency (Fig. 1.1), they might be affected at the level of density of TCR-CD3 complex (Fig. 1.2), of antigen recognition (Fig. 1.3), of CD4 and CD8 function in adhesion to class II and class I molecules, respectively, or of the formation of second messengers (Fig. 1.5). Age-related decline is observed for IL-2 production by CD4+ T cells (Fig. 1.6) and IL-2R expression by CD8+ T cells, whereas IL-2R expression by CD4+ T cells did not change during aging (Fig. 1.7).

These differences might range from severely diminished responses to response levels which are comparable to those found in young mice. This variation is highly remarkable if one keeps in mind that the mice used for these studies were highly inbred and kept under identical conditions with regard to housing, feeding and so on throughout their life. Possible immune-associated pathology seems not to be involved in this phenomenon since all mice were screened routinely for tumors or other abnormalities not only by gross examination but also through general histological procedures. Animals displaying irregularities in these screenings were excluded from the results. The observed interindividual variation among old mice as found in these studies for the various immunological parameters resembles the variability in tumor occurrence, which is also found in old animals in a wide variety of highly inbred strains (11). Both the multiple nature of the immune defects as well as the high interindividual variability as found in old age in these studies argue against a common, built-in mechanism controlling aging at the cellular level.

The dysfunctioning of the immune system during aging might also be due to a decrease of positive or an increase of negative signals exerted by other organ systems upon the immune system. In this respect extensive interactions between the endocrine system in general and the neuroendocrine system in special on one hand and the immune system on the other hand have been found (12-14). Since major changes in hormonal levels and other endocrine functions have been described in old individuals (15,16), it is likely that such changes have their impact on the function of the immune system during aging. However, the observed age-associated immunological dysfunction cannot be ascribed entirely to influences of other organ systems. As a matter of fact some of the age-related effects seen in the endocrine system may be due in part to the dysfunctioning of the immune system, since besides influences from the endocrine system upon the immune system also strong reverse effects have been described (17,18). It seems thus likely that diminished T cell function during aging is to a large extent caused by factors within the immune system itself.

In terms of quantity it appears that during aging both the absolute number of cells with a T cell phenotype in peripheral lymphoid organs as well

as the frequency of cells that can respond to certain antigens decreases (20). However, the decline in numbers is not as sharp as is the reduction in frequencies of responsive cells. Especially the latter observation is striking. Since the findings of both ourselves (Chapter 4) and others (21,22) are uniform in this matter one has to assume that the decrease in precursor frequencies as found in our studies for alloreactive T cells is a general phenomenon for all antigens capable to evoke a cellular immune response. Several explanations can be thought of to explain this data.

One obvious possibility would be that at old age a distinct number of T cells would be non-functional. To be more precisely: a significant number of cells with the T cell phenotype may not react functionally in the type of assays used. In this respect one has to keep in mind that most of the functional tests used by us and others are MHC-restricted T cell assays. Perhaps non-MHC restricted T cell reactivity is gaining importance during aging. The recently described TCR γ/δ T cell would fit into this hypothesis, since this cell-type both displays the T cell phenotype as well as responds in a non-MHC restricted manner (24). As a consequence a shift from T-$\alpha\beta$ cells towards T-$\gamma\delta$ cells would be expected during aging according to this theory.

A second explanation for the age-related shift in frequencies might be an expansion of the antigen-receptoire recognized by T cells. In other words less cells can react to more antigens. It has also to be considered that the continuous replacement of T cells making up the available repertoire for the individual, at any given moment goes down to almost zero due to the involution of the thymus (see later) with age. This theory assumes ongoing changes in the available T cell population. Perhaps changes in the process leading to the ultimate specificity of a T cell may cause a shift from the already defined antigenic specificity in such cells towards reactivity patterns with an altered affinity or perhaps even a different specificity. In terms of mechanisms one may think of somatic mutations in (hyper) variable regions of the T cell receptor genome throughout the aging of the cell leading to differences in basic epitope-recognition by such a cell. Although not yet established for the T cell population, the observed increase of anti-self reactivity in the B cell compartment during aging (25) could well be in line with this speculative point of view.

A third option to explain the reduced frequencies of responsive T cells during aging would be again in parallel with the observed changes in the B cell system, namely the existence of one or a few clonally expanded T cell clones. Just as with the homogeneous components of the immunoglobulins (26), the progeny of such cell would make up a significant part of the overall T cell population, thereby lowering the relative contribution of the other T cell clones towards the total T cell response. Since T cells do not produce a cell-specific product which can be detected as easily as the product of B cells (immunoglobulins), the proof for this latter hypothesis has to come from investigations on panels of antigen-specific T cell clones derived at various ages from various organs and analysed at the molecular level for the organisation of their TCR-genes. These types of studies will also clarify the first two types of speculations mentioned above on the nature of the reduced T cell frequencies as observed throughout aging. Probably a combination of two or more of the suggested options will turn out to give the true correct answer.

Besides these quantitative changes also qualitative changes are involved in the diminished T cell function during aging. T cell proliferation after antigenic activation is a complicated and only partly understood process. Components involved include interactions of specific membrane receptors with their ligands (i.e. Ag/TCR, class I MHC/CD8, class II MHC/CD4, IL-1/IL-1R, IL-2/IL-2R, CD2/LFA), signal transduction from these interactions towards the nucleus and activation of perhaps previously untranscribed gene systems, all leading to a specific proliferative response. As summarized in Table I, most of the steps involved in this pathway which have been analyzed in these studies show a more or less pronounced defect during aging. In addition we found, in line with another group (23), a reduced CD3 expression on unstimulated splenic T cells from old mice (data not shown). Since the TCR is always expressed in conjunction with CD3, this finding would implicate that the density of the TCR-CD3 complex may decrease during aging. As mentioned earlier, the simplest explanation for the findings depicted in Table 1 would be a common cause influencing all of the properties of T lymphocytes in an age-related manner. The most plausible theory to fit this description is that of the age-associated accumulation of genomic damage. One can think of for

instance somatic mutation in the coding sequences of the genes involved in the functioning of the immune system. In this way not only the V regions of TCR-genes but also in other directly (IL-2) and indirectly (activation genes such as proto-oncogenes) related gene systems differences should be observed in the type of molecular studies as suggested before. It has been reported that stimulation with IL-2 results in the accumulation of c-myc mRNA in lymphocytes (24). The involvement of other transcripts of proto-oncogenes like c-fos and c-myb has also been shown (25). All of these effects would add up and finally result in a diminished ability to respond with an appropriate proliferative response upon antigenic stimulation. Indeed cell cycle kinetic studies (26,27) have shown that cells from older donors are less capable to proceed through repeated mitotic cycles than cells from young individuals.

Finally, the question remains why all of these defects are found in the aging cellular immune system. In this respect it is obvious that one has to consider the influence of the involution of the thymus on the cellular immune system at old age. Although the precise nature and the causes for the involution of the thymus are far from understood, it is well established that at the age that the animals in the present studies were studied (i.e. 100 weeks of age and older) the size of the lymphoid compartment of the thymus has reduced to less than 5% of its normal (young) value (28). It is unclear whether any functional capacity must be ascribed to these thymic remnants (29). If any, one has to assume that such functions must be quite defective (see Table 1). Along this line the age-associated diminished T cell function would then be due to the impaired rest-activity of the involuted thymus. It seems more likely, however, to expect an almost complete absence of T cell replacement in peripheral lymphoid organs as a result of the severe involution of the thymus. In that case changes in T cell function at old age would not be the result of a malfunctioning of the thymus but due to negative changes in a population of cells that once has been formed and now instead of being replaced regularly should remain functional over a long period of time. During this period, while aging, these cells will then accumulate all types of damage, just as any other somatic cell type which is not part of a cell-renewal system.

The conclusion of this thesis may well be relevant to the pathophysiology of aging in general; e.g. the increased variability in responses of aged individuals, the decrease in frequency of responding cells, and the decreased signal-transduction may apply to other organs and tissues as well.

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SUMMARY

In this thesis an attempt is made to further characterize the age-related changes in T cell-mediated immunity. In Chapter 1 a general introduction is given dealing with several aspects of the immune system during senescence.

In the first part of this thesis the delayed type hypersensitivity (DTH) reaction is used as a read-out system of T cell mediated immunity. In Chapter 2 it is shown that the ability to elicit a DTH reaction to SRBC declines during aging. During the induction of DTH a lymphokine, i.e. IL-2, is required for the proliferation of the DTH reactive T cells. It appeared that the Con A induced IL-2 production by T cells from old mice was decreased. In addition, the proportion of Thy-1^+,Lyt-2^- spleen cells which are able to mediate DTH, also declined during aging although less strongly. From this it was concluded that there is a quantitative as well as a qualitative decrease of T cell function during senescence.

In Chapter 3 the analysis of the age-related decline in DTH was extended to the DTH response to H-2 alloantigens and non-H-2 alloantigens. Since the decline may be a consequence of the impaired IL-2 production observed in old mice, the age-related decline in DTH to alloantigens was studied employing in vitro induction of DTH. Cells generated in an allogeneic mixed lymphocyte reaction (MLR) were capable to induce footpad thickness in young naive syngeneic recipients, if injected together with the antigen. Using this in vitro system it was shown that cells from old CBA mice induced considerably lower DTH responses than cells from young CBA mice. By depletion of the effector cells, T cells for either $CD4^+$ T cells or $CD8^+$ T cells, it was demonstrated that the effector phase was mediated by $CD4^+$ T cells. Lower responses by cells from old mice were not due to increased suppressor cell activity, since removal of the $CD8^+$ T cells at the onset of the cultures did not influence the results. $CD4^+$ T cells from old mice were less effective in the generation of DTH effector T cells than $CD4^+$ cells from young mice. This difference could not be attributed to a defective IL-2 production, since the addition of IL-2 to the in vitro cultures did not improve the effector function, neither in a qualitative nor in a quantitative way. Our data suggest that in aged mice

$CD4^+$ DTH reactive T cells like the $CD4^+$ IL-2 producing T cells, cannot be activated effectively, resulting in a low level of secretion of mediators accounting for the characteristic DTH inflammation.

In order to get more insight into the age-related decline in T cell mediated immunity, we investigated the proliferative capacity of $CD4^+$ T cells as well as $CD8^+$ T cells (Chapter 4). In aged CBA/Rij mice the MLR response to BALB/c alloantigens was impaired. This was demonstrated for the response of total T cells as well as for the responses of $CD4^+$ T cells and $CD8^+$ T cells, respectively. Addition of IL-2 to the cultures could not correct the defect. The lower responses by cells from old mice were not due to increased suppressor activities of $CD8^+$ T cells, since removal of these cells at the onset of the cultures did not influence the results. These lower responses appeared to be due at least in part to a reduced precursor frequency of alloreactive $CD4^+$ T cells. Since neither the reduced IL-2 production nor the reduced precursor frequency could completely explain the decreased MLR, it was suggested that the impaired immune response might be due to a defect in the signal transduction via the TCR.

Therefore, in Chapter 5 activation of $CD4^+$ and $CD8^+$ T cells from young and old mice was studied in a system circumventing the TCR. Using phorbol myristate acetate (PMA), which directly activates protein kinase C (PKC), and a Ca^{2+} ionophore, ionomycin, which increases the cytoplasmic free Ca^{2+} concentration, the induction of IL-2, IL-2R expression and cell proliferation were studied in total T cells, purified $CD4^+$ T cells and purified $CD8^+$ T cells. The results demonstrated that following stimulation with PMA and ionomycin the IL-2 production by $CD4^+$ T cells from young (20 weeks of age) and old (120 weeks of age) mice were similar despite a threefold and twofold decline when compared to stimulation with BALB/c spleen cells and Con A, respectively. However, after PMA and ionomycin stimulation the proliferative responses of $CD4^+$ T cells from old mice were clearly lower than those from young mice, whereas proliferative responses of $CD8^+$ T cells from old mice were only slightly lower than the responses from young mice. Furthermore, the reduced capacity of $CD4^+$ T cells to proliferate was not reflected by a reduced expression of the IL-2R. The expression of the IL-2R by $CD8^+$ T cells from old mice was lower than by $CD8^+$ cells from young mice.

The results presented in this thesis show that the age-related decline in T cell-mediated immunity measured by DTH and proliferation has a complex background. Although a number of defects, like reduced precursor frequency, decline in IL-2 production and responsiveness to IL-2, contribute to the impaired immune responsiveness of old mice, one should be aware of possible defects at the early activation steps. These aspects are discussed in Chapter 6 in relation to other possible characteristics of aging.

SAMENVATTING

Dit proefschrift beschrijft onderzoek naar de leeftijdsafhankelijke veranderingen die tijdens veroudering optreden in het T cel kompartiment. Een algemene inleiding met betrekking tot veranderingen in immunologische reakties tijdens veroudering wordt gegeven in hoofdstuk 1.

Aanvankelijk is als model voor een T cel respons gebruik gemaakt van de vertraagd type overgevoeligheids (VTO; in het Engels: DTH) reaktie. In hoofdstuk 2 wordt aangetoond dat het vermogen tot een DTH reaktie tegen schape rode bloedcellen (SRBC) afneemt bij het ouder worden. Bij de induktie van DTH is het lymfokine IL-2 betrokken. Gebleken is dat het vermogen van Con A gestimuleerde cellen van oude muizen om IL-2 te produceren verminderd is. Ook bleek dat, hoewel minder sterk, de frequentie van Thy-1^+,Lyt-2^- miltcellen die in staat zijn om een DTH te induceren, afnam. Op grond van deze resultaten werd gekonkludeerd dat niet alleen in kwantitatief maar ook in kwalitatief opzicht de T cel funktie tijdens veroudering achteruit gaat.

In hoofdstuk 3 is de leeftijdsafhankelijke afname in DTH verder onderzocht, maar nu gebruik makend van H-2 alloantigenen en non-H-2 alloantigenen. Verondersteld werd dat de leeftijdsafhankelijke afname in DTH mogelijk een gevolg is van de afname in het vermogen van de cellen om IL-2 te produceren. Daarom is een systeem ontwikkeld waarbij de induktie van de DTH in vitro plaats vond, zodat extra IL-2 kon worden toegediend. Op een wijze gelijkend op de "mixed leukocyte reaction" (MLR) werden cellen geaktiveerd, die in staat bleken tezamen met het relevante antigeen een DTH op te wekken in onbehandelde jonge naieve syngene ontvangers. Gebruik makend van dit in vitro systeem bleek dat cellen van oude CBA/Rij muizen een geringere DTH induceerden dan cellen van jonge muizen. Door de effektor cellen te ontdoen van óf $CD4^+$ T cellen óf $CD8^+$ T cellen, bleek dat $CD4^+$ T cellen verantwoordelijk zijn voor de effektorfase van de DTH respons. Verder bleek dat de lage DTH responsen door cellen van oude muizen niet een gevolg waren van verhoogde suppressor aktiviteit van de $CD8^+$ T cellen, omdat verwijdering van de $CD8^+$ T cellen bij het inzetten van de kweken geen effekt had op de DTH respons. De $CD4^+$ T cellen van oude muizen bleken

minder goed in staat te zijn om tot DTH effektor cellen te differentiëren. Dit verschijnsel kon niet worden toegeschreven aan een verminderde IL-2 produktie, omdat toevoeging van IL-2 aan de kweken noch in kwalitatief noch in kwantitatief opzicht de responsen verbeterde. Onze resultaten wijzen er daarom op dat in oude muizen de $CD4^+$ T cellen minder goed kunnen uitgroeien tot klonen DTH effector cellen.

Om beter inzicht te krijgen in deze verouderingsaspekten van T cel responsen, hebben we de proliferatieve capaciteit van $CD4^+$ T cellen, die betrokken zijn bij de DTH respons, en $CD8^+$ T cellen, die suppressieve effekten kunnen veroorzaken, bestudeerd (hoofdstuk 4). Het bleek dat totale T cellen, $CD4^+$ T cellen en $CD8^+$ T cellen van oude CBA/Rij muizen een geringere MLR respons vertoonden ten opzichte van cellen van jonge CBA/Rij muizen. Toevoeging van exogeen IL-2 leidde tot een geringe toename van de MLR maar niet tot korrektie van het proliferative defekt. De geringere reaktie van T cellen van oude muizen bleek ook niet een gevolg te zijn van $CD8^+$ T cellen, die eventueel tot een suppressief effekt in staat zouden zijn. Wel bleek dat de verminderde MLR responsen voor een deel verklaard konden worden uit een verlaagde precursor frequentie. Omdat noch de afname in IL-2 produktie noch de afname in precursor frequentie de verlaagde MLR volledig kon verklaren, werd verondersteld dat de mogelijke oorzaak op het niveau van signaaltransduktie zou kunnen liggen.

In hoofdstuk 5 zijn daarom de $CD4^+$ en $CD8^+$ T cellen van jonge en oude muizen geaktiveerd op een wijze waarbij geen gebruik is gemaakt van de T cel receptor (TCR). Hiertoe zijn de cellen geaktiveerd met phorbol 12-myristaat 13-acetaat, dat direkt proteine kinase C (PKC) activeert, en ionomycine, dat de concentratie van calcium ionen in het cytoplasma verhoogt. Op deze wijze zijn een drietal aspekten bestudeerd, IL-2 produktie, IL-2 receptor (IL-2R) expressie en proliferatie. De resultaten toonden aan dat de IL-2 produktie door $CD4^+$ T cellen van oude muizen vergelijkbaar was met die van $CD4^+$ T cellen van jonge muizen. Dit in tegenstelling tot allogene stimulatie of Con A stimulatie, waarbij een drievoudig en tweevoudig verschil gevonden werd tussen jonge en oude muizen. Echter, de proliferatieve responsen van $CD4^+$ T cellen van oude muizen waren lager dan die van cellen van jonge muizen. Dit in tegenstelling tot de proliferatieve responsen van

$CD8^+$ T cellen, waarbij die van oude muizen iets lager waren dan die van jonge muizen. Verder bleek dat de verlaagde proliferatieve capaciteit van de $CD4^+$ T cellen niet tot uiting kwam in een verminderde IL-2R expressie. Voor de $CD8^+$ T cellen daarentegen, was dit wel het geval.

De resultaten die worden beschreven in dit proefschrift laten zien dat de leeftijdsafhankelijke afname in T cel responsen zoals DTH en proliferatie een complex proces zijn. Hoewel een aantal defekten, zoals afname in precursor frequentie en afname in IL-2 produktie en verminderde IL-2R expressie tot op zekere hoogte bijdragen aan de verminderde immuunrespons van oude muizen, moet de invloed van mogelijke defekten tijdens de vroege fase van T cel aktivatie niet worden onderschat. Deze aspekten worden in hoofdstuk 6 verder besproken in relatie tot andere mogelijke oorzaken die tijdens het proces van veroudering een rol kunnen spelen.

ABBREVIATIONS

APC	antigen presenting cell
AvF	avidin-FITC
BMG	benign monoclonal gammapathies
BSA	bovine serum albumin
CBA/Rij	CBA/BrARij
C57BL/Ka	C57BL/KwRij
C57BL/Rij	C57BL/LiARij
CD	cluster of differentiation
C3H	C3Hf/LwRij
CL	confidence limit
Con A	concanavalin A
CTLL-2	cytotoxic T cell line
γ-IFN	γ-interferon
DAG	diaglycerol
DNP	dinitrofenyl
DTH	delayed type hypersensitivity
F	frequency
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
HBSS	Hank's balanced salt solution
^{3}H-TdR	^{3}H-thymidine
IL-1	interleukin-1
IL-2	interleukin-2
IL-1R	interleukin-1 receptor
IL-2R	interleukin-2 receptor
IP_3	inositol triphosphate
i.v.	intravenously
LDA	limiting dilution analysis
Mab	monoclonal antibody
MG	monoclonal gammapathies
MHC	major histocompatibility complex
MLR	mixed leukocyte reaction
MMG	malignant monoclonal gammapathies
PBL	peripheral blood lymphocytes
PDE	phosphodiesterase
PIP_2	phosphatidylinositol biphosphate
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
pTc	precursor cytotoxic T cell
s.c.	subcutaneously
SRBC	sheep red blood cells
Tc	cytotoxic T cell
Th	helper T cell
Ts	suppressor T cell
TCR	T cell receptor
Tdth	delayed type hypersensitivity T cell

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CURRICULUM VITAE

Christine Sybilla Vissinga werd geboren op 4 november 1958 te Rijswijk. Na het behalen van het gymnasium-β diploma werd aangevangen met de studie Biologie aan de Rijksuniversiteit te Leiden. In mei 1984 werd het doctoraal examen afgelegd, met als hoofdvak Immunologie en als bijvak Moleculaire Plantkunde.

Van mei 1984 tot december 1987 was zij als onderzoeksassistent aangesteld op de afdeling Immunologie van het Instituut voor Experimentele Gerontologie (IVEG), TNO Rijswijk (directeur: Prof. dr. D.L. Knook) op een door MEDIGON (NWO) en later door het IVEG gefinancieerd project. Tijdens deze periode vond het in dit proefschrift beschreven onderzoek plaats.

Vanaf 1 april 1988 is zij werkzaam als "postdoctoral fellow" in het Terry Fox Laboratory in Vancouver, Canada onder leiding van Dr. F. Takei.