CELLULAR AND GENETIC REQUIREMENTS FOR GRAFT - VERSUS - HOST REACTIVITY

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AAN INEKE

CONTENTS

ABBREVIATIONS

ALS	Anti-lymphocyte serum
ATS	Anti-thymocyte serum
ATx	Thymectomy of adult mice
BF	Blastogenic factor
CML	Cell-mediated lympholysis
CTL	Cytotoxic T lymphocyte
DNFB	Dinitrofluorobenzene
DTH	Delayed type hypersensitivity
EBV	Epstein-Barr virus
FGG	Fowl gamma globulin
GvH	Graft-versus-Host
H antigen	Histocompatibility antigen
Hh gene	Hemopoietic histocompatibility gene
HSC	Hemopoietic stem cells
HvG	Host-versus-Graft
Ia antigen	I-associated antigen
Ir gene	Immune response (regulatory) gene
ITL	Initiator T lymphocytes
LAD	Lymphocyte activating determinant
LCMV	Lymphocytic choriomeningitis virus
LD antigen	Lymphocyte defined antigen
LT	Lymphotoxin
MHC	Major histocompatibility complex
MIF	Migration inhibition factor
MLC	Mixed lymphocyte culture
MLR	Mixed lymphocyte reaction
Mls locus	Minor lymphocyte stimulating locus
NK cell	Natural killer cell
PHC	Proliferating helper cell
PLN assay	Popliteal lymph node assay

Sc	Subcutaneous
SD antigen	Serologically defined antigen
T cell	Thymus-derived lymphocyte
T1 cell	Sessile T lymphocyte, short-lived after ATx
T2 cell	Recirculating long-lived T lymphocyte, sensitive to ATS *in vivo*
TBMC	Tetraparental bone marrow chimeras

1. AIM OF THE STUDY

The immune response is an important component of vertebrate's defense to infectious agents. Vertebrates with an ill-developed immune system generally die before reaching the reproductive age. In man this is examplified by children with hereditary severe combined immunodeficiency disease.

Immunity is based upon the capacity of lymphocytes to recognize antigens and to make them harmless. This can be done in two ways: via a humoral immune response and via a cellular or cell-mediated immune response. These two limbs of the immune system are based upon two types of lymphocytes: B and T lymphocytes. The bone marrow-derived B lymphocytes are the progenitors of plasma cells which secrete humoral products, i.e. the antigen-specific antibodies. The thymus-derived or T lymphocytes are responsible for cell-mediated immunity. Humoral immunity can be transferred from immune to non-immune individuals by means of serum, whereas cell-mediated immune responses can only be transferred to non-immune individuals by means of lymphocytes. Recognition of an antigen by a T lymphocyte will trigger that cell to mount a specific immune response without the release of antibodies. Examples of cell-mediated immune responses are the defense to viruses (e.g., measles, mumps) and the resistance to intracellularly growing bacteria (e.g., *Listeria monocytogenes*). Furthermore, delayed type hypersensitivity (e.g., the Mantoux reaction in humans infected with tubercle bacilli) and graft rejection (e.g., rejection of a kidney transplant) are caused by cell-mediated immune responses. Recent studies reveal that for optimal activation of the cell-mediated immune system not only the foreign antigen must be recognized by the T lymphocytes, but also a self-component. These self-components are cell surface markers which can be different for different individuals of a certain species. They are called histocompatibility antigens. Each individual of a certain species can mount an extremely strong cell-mediated immune response against those histocompatibility antigens of most other individuals of that species. This often occurs after organ and tissue transplantation when donor and recipient are genetically unrelated.

When immunocompetent lymphoid cells are transplanted into a recipient which is incapable of rejecting them, the grafted cells can survive. In case the recipient has different histocompatibility antigens, the transplanted cells can recognize these histocompatibility antigens of the host and react immunologically against them. Thus, in stead of a normal transplantation reaction of Host-versus-Graft (HvG), the reverse

occurs, which is called the Graft-versus-Host (GvH) reaction. In man this type of reaction may arise in immunologically anergic subjects receiving bone marrow grafts, e.g., in case of combined immunodeficiency disease, red cell aplasia caused by a radiation accident, and in treatment of certain leukaemias. The GvH reaction is thought to be mainly due to immunocompetent T lymphocytes present in the bone marrow graft.

The experiments described in this thesis were intended to characterize the lymphocytes involved in the specific anti-host immune reactivity of the GvH reaction, and to investigate which histocompatibility antigens are the principal inducing elements of that antigen-specific reactivity. In this study the mouse was chosen as the experimental animal since large numbers of genetically well defined strains of this species are available.

2. *T LYMPHOCYTES*

2.1. *Ontogeny of T cells*

In mammals the thymus-derived lymphocytes or T cells originate from stem cells of the hemopoietic organs. The first hematopoietic organ, and thus the first site of blood cell production, during ontogeny is the yolk sac, an extraembryonic tissue (Moore and Metcalf, 1970). At about 11 days of gestation, the function of the yolk sac as site of blood cell production is taken over by the fetal liver. In the late fetal period and throughout adult life the bone marrow is the major site of hemopoietic stem cell localization and of blood cell production (Metcalf and Moore, 1971). Based upon studies with chromosome markers and upon tissue culture studies it has been argued that the migrant stem cells that enter the epithelial thymic tissue are large basophilic cells (Moore and Owen, 1967; Owen and Ritter, 1969). Observations of Roelants et al. (1975) suggest that thymic stem cells are already precommitted to T cell differentiation prior to migration to the thymus. While proliferating within the superficial thymic cortex the blast cells give rise to a progeny of small lymphocytes (Weissman, 1973). During differentiation in the thymic cortex the differentiating lymphocytes express specific determinants on their cell surface: Thy-1, Tla, Gv-1, Lyt, and a relative low amount of H-2 antigens (Klein, 1975). The thymic medulla constitutes a more mature pool of thymocytes which have lost their Tla antigens, have reduced their content of Thy-1 antigen, and increased their content of H-2 antigens. Weissman (1973) has shown that the more immunocompetent thymocytes residing in the thymic medulla are the progeny of the immuno-incompetent cortical thymocytes. Shortman and Jackson (1974) have presented evidence that in the population of medullary thymocytes also a subpopulation of self-replicating lymphocytes is present. This suggests that there are two separate lines of T cell differentiation (Schlesinger, 1972). A distribution pattern of mouse thymocytes according to size and amount of Thy-1 is shown in Fig. 1.

2.2. *T cell heterogeneity*

After leaving the thymus the T cells migrate to the peripheral lymphoid organs, where a further differentiation may take place. Peripheral T lymphocytes in the mouse represent a heterogeneous population. A part of the T lymphocytes can migrate from blood into peripheral lymphoid tissues, and re-enter the

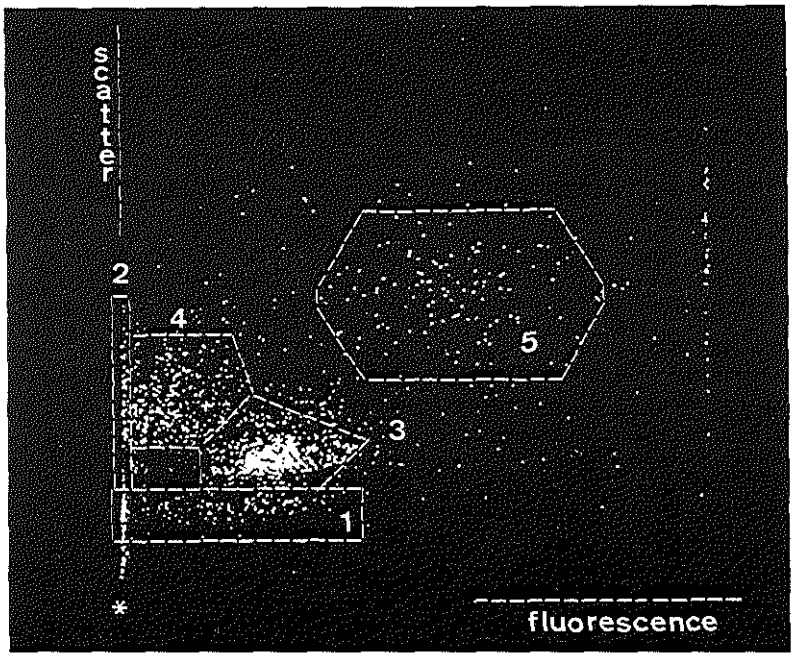

Figure 1
Distribution pattern of mouse thymocytes according to size (ordinate) and amount of Thy-1 surface antigen (absciss).

Thymocytes were stained in an indirect membrane fluorescence procedure with a rabbit anti-mouse thymocyte serum (ATS) and a FITC-conjugated goat-anti rabbit immunoglobulin serum and analyzed for size (scatter) and fluorescence using a cytofluorograf FC-200/4800A.

Five different cell populations can be distinguished:
1. Dead cells (low scatter signal);
2. Cells showing no fluorescence signal;
3. Small cells with medium fluorescence intensity;
4. Medium-sized cells with small fluorescence intensity;
5. Broad population with large scatter and large fluorescence signals.

blood again via the thoracic duct lymph. This migration pattern is called recirculation. These T cells continue to recirculate in this fashion throughout their life span, which is at least several months in the mouse (Sprent and Basten, 1973). Recirculating T lymphocytes are immunocompetent. Their cell surface is characterized by a low amount of Thy-1 antigen and a high amount of H-2 antigens. Herein they resemble the T lymphocytes of the thymic medulla. Besides this long-lived, recirculating T cel subpopulation, there exists a short-lived, non-recirculating T cell subpopulation (Stutman, 1975). These cells are thought to leave the thymus as functionally immature cells, but they can further differentiate in the periphery under thymic hormonal influence. This sessile T cell subpopulation re-

sides mainly in the spleen and has a high amount of Thy-1.2 antigen. Due to the short half life of about 3-4 weeks, these cells can be eliminated by thymectomy. By using the fluorescence activated cell sorter both these T cell subpopulations can be separated from each other and from other nucleated cells, according to their relative amounts of Thy-1 antigen (Cantor et al., 1975). According to these characteristics Raff and Cantor (1971) have proposed to subdivide peripheral T lymphocytes into T1 and T2 cells. T1 cells are short-lived cells, which disappear within 4-6 weeks after adult thymectomy; they are sessile cells which reside predominantly in the spleen. T2 cells are long-lived, recirculating T cells; they are sensitive to treatment with ATS *in vivo*, in contrast to T1 cells.

Several experimental approaches have been used to show that there exists a functional heterogeneity among T cells. Thus, primed helper T cells and cytotoxic T lymphocytes (killer cells) are distinct cell types. Hirst et al. (1975) have demonstrated that different antigen doses can favour the development of either active helper cells or killer cells. Similarly, it has been shown that spleen cells from skin grafted mice appear to have mainly helper activity 13-28 days after grafting, while spleen cells immunized *in vitro* to the same alloantigens show strong cytotoxicity, but no helper activity (Dennert, 1974). These functionally different subsets of T lymphocytes can be classified according to distinct Lyt surface markers. Three T cell subsets can be distinguished based upon the Lyt system, namely Lyt-1^+, Lyt-23^+, and Lyt-123^+ T cells (Cantor and Boyse, 1977).

Lyt-1^+ T cells account for approximately one-third of all peripheral T cells. They are especially programmed to help or enhance the functional activity of other cells. They include the T cells which help B cells in the humoral immune response, enhance the generation of T killer cells, and activate macrophages in delayed type hypersensitivity reactions. Lyt-1^+ T cells also account for the large part of the proliferative activity in mixed lymphocyte cultures.

Lyt-23^+ T cells account for approximately 5-10% of the total peripheral T cell pool. They represent both the killer T cell population and the suppressor T cell population. These last cells can among other things inhibit T helper function. Cytotoxic Lyt-23^+ cells can be distinghuished from the Lyt-23^+ suppressor cells by the presence of Ia antigens on the latter population (Murphy, 1978). Both Lyt-1^+ T cells and Lyt-23^+ T cells belong to the T2 cell population (Feldmann et al., 1977). Studies of Huber et al. (1976) indicate that cells of the Lyt-1^+ sub-

class can not give rise to Lyt-23$^+$ cells and the other way around, which suggests that both these subpopulations represent separate sublines of thymus dependent maturation.

Lyt-123$^+$ T cells account for approximately half of all peripheral T cells. A portion of the Lyt-123$^+$ T cells can be eliminated by adult thymectomy, and thus probably corresponds to the T1 population. Feldmann et al. (1977) reported that these short-lived Lyt-123$^+$ T cell population can amplify helper T cells and suppresor T cells during antibody formation. The remainder of the Lyt-123$^+$ T cells probably belong to the recirculating T2 subpopulation.

According to Stutman and Shen (1979) both Lyt-1$^+$ and Lyt-23$^+$ T cells arise from Lyt-123$^+$ postthymic precursor cells in the periphery. It is not clear whether this applies to all Lyt-1$^+$ T cells, since other studies suggest that not only Lyt-123$^+$ T cells are formed in the thymus, but also Lyt-1$^+$ T cells (Mathieson et al., 1979; Scollay et al., 1978). These latter studies did confirm the generation of Lyt-23$^+$ cells from Lyt-123$^+$ T cells in the periphery (Cantor and Boyse, 1976).

3. *HISTOCOMPATIBILITY ANTIGENS OF THE MOUSE*

3.1. Major histocompatibility antigens

Histocompatibility (H) antigens can be subdivided into major and minor H antigens according to the strength of the graft rejection they induce. In the mouse the antigens belonging to the major histocompatibility complex (MHC) are called H-2 antigens. The H-2 complex of the mouse is situated on chromosome number 17. Incompatibilities for the H-2 system present a stronger H barrier to successful (skin) transplantation than any other H locus of the mouse (Counce et al., 1956). H-2 gene mapping has originally been started by the analysis of recombinant haplotypes which arise when, during meiosis, crossing over occurs within the H-2 complex in heterozygous mice. More recently, intra H-2 recombinant inbred lines have also become available. By testing such recombinant mice the individual H-2 regions and subregions have been studied and mapped as shown in Fig. 2. For reviews see Klein (1975) and Van der Kwast (1979).

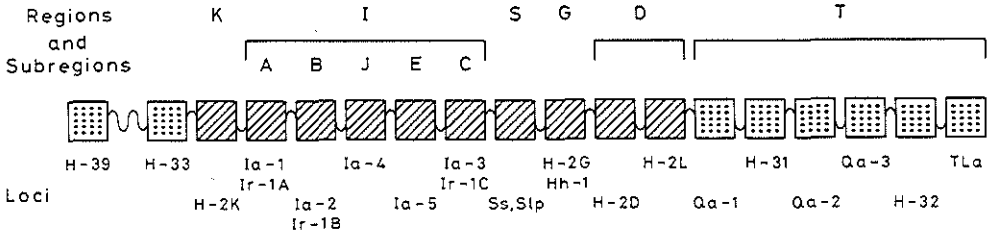

Figure 2
Genetic map of the H-2 complex of the mouse and its vicinity.

Skin graft studies revealed that H-2K and H-2D end incompatibilities are of similar strength (Klein, 1966). Both loci code for complex surface antigens, against which antibodies can be raised. Therefore, these antigens are also referred to as serologically defined (SD) antigens. Each SD antigen has a strong antigenic determinant, the private specificity, which defines a particular allele. On the same molecule other antigenic determinants are found which are common to one or more unrelated mouse strains. These antigenic determinants are called public specificities. Some of these public specificities are restricted to the products of one SD locus, while others are common to both the H-2K and the H-2D region (Murphy and Shreffler, 1975; Démant and Néauport-Sautès, 1978). The surface antigens coded for by the I region of the H-2 complex were initially not detectable serologically.

Rychlikova et al. (1970) found that the H-2K end encodes for antigens, which *in vitro* can induce much stronger proliferation of allogeneic T lymphocytes than the H-2D end. These so-called lymphocyte activating determinants (LAD) appeared to be situated in the I region. Genes encoding for determinants stimulating the strongest mixed lymphocyte reaction (MLR) map in the I-A subregion, while I-B and I-C subregions also encode for LAD's which are, however, less stimulatory. Nowadays it is also possible to detect I region coded antigens serologically (Shreffler et al., 1974); these antigens are called I-associated antigens or Ia antigens. The S region bears the Ss locus which controls the level of a serum substance and the Slp locus which controls the level of a sex-limited protein (Pasmore and Shreffler, 1970). Démant et al. (1973) discovered that the Ss protein is a complement component which later appeared to be homologous to that of C4. The G region bears the H-2G locus, which encodes for an antigen that has so far been detected only on erythrocytes and thus behaves as a typical blood group antigen (Snell and Cherry, 1974). However, recent studies could not confirm the separation of the H-2G locus from the Ss-Slp loci (Huang and Klein, 1979). The H-2L locus encodes for a serologically detectable determinant, that has been detected only recently (Néauport-Sautès and Démant, 1977).

McDevitt and Benacerraf (1969) found striking differences between different inbred mouse strains in antibody production against a particular synthetic polypeptide. Subsequent genetic studies showed that the responsiveness was controlled by a single gene, which the authors named Ir-1 (immune response-1). Later this Ir-1 gene was shown to be located within the H-2 complex between the H-2K and the S region. This is the I region of the H-2 complex. Dorf, Stimpfling and Benacerraf (1975) showed that the immune response to another synthetic polypeptide is controlled by two genes, one in the I-C/S region and another in the K/I-A region. In addition to the Ir genes which are linked to H-2, there are also a number of Ir genes which are not linked to H-2. Some of them are linked to the immunoglobulin heavy chain allotype genes, others are on the X chromosome (Klein, 1975).

3.2. Minor histocompatibility antigens

Distal to the H-2 complex the T region is situated, consisting of the Tla locus and some Qa loci. The Tla locus codes for serologically detectable antigens on thymic leukemia cells and on thymocytes of some strains, while the Qa loci code for serologically detectable antigens on some peripheral lymphocytes

(Flaherty, 1978). In the T region and at the left hand side of the K region also a number of other H loci are situated. These loci encode for minor H antigens. In the mouse about 40 of such loci have been defined so far. Genes encoding for non-H-2 alloantigens are spread over the entire genome of the mouse. Skin grafts transplanted across non-H-2 differences are usually rejected later than in 3 weeks, and in some combinations the grafts are not rejected at all. The most polymorphic locus coding for non-H-2 alloantigens has only five alleles. This stands in clear contrast to the extremely polymorphic and genetically complex H-2 system. So, extensive "cross-reactivity" exists between the non-H-2 alloantigens of different strains of mice (Bevan, 1975). The minor H loci are not involved in MLR, with one exception: the Mls locus situated on chromosome 1. The Mls locus encoded products can induce strong proliferative activity of unprimed H-2 compatible T lymphocytes (Festenstein, 1973). Five alleles have been described for the Mls locus, based on the stimulatory capacity in different strain combinations. Mls locus coded products are expressed mainly on B lymphocytes and on macrophages, but not on T lymphocytes.

Cudkowicz (1968) described the hemopoietic histocompatibility (Hh) genes. Hh-1 and Hh-2 are located at the D end of the H-2 complex, Hh-3 at the K end. Hh genes encode for H antigens which are responsible for the rejection of hemopoietic cells. They behave as recessive antigens, i.e., they are expressed only in the cells of homozygotes. In contrast to most other tissues, bone marrow grafts derived from parental strains can be rejected by F1 hybrid recipients due to the expression of Hh genes. However, the situation is complicated since only some mouse strains are able to reject Hh incompatible bone marrow grafts. Kiessling and Wigzell (1979) found a positive correlation between natural killer cell activity and the "hybrid resistance" phenomenon. However, natural killer cell activity is not limited to targets expressing Hh locus products.

4. *T CELLS IN HOST DEFENSE MECHANISMS AGAINST VIRUSES AND BACTERIA*

4.1. Introduction

The role of T cells in the defense against infectious diseases has been studied most extensively after infection with ectromelia virus and after infection with *Listeria monocytogenes*.

Ectromelia virus is a poxvirus of the vaccinia sub-group (Fenner, 1968), closely related to vaccinia and variola viruses. It is a natural mouse pathogen. Infection with ectromelia results in rapid lysis of the infected cells. The natural port of entry appears to be through skin abrasions. The virus disseminates via lymph and blood to the liver and spleen. Recovery depends on control of virus growth in the liver parenchyma (Blanden, 1970). The recovery process begins between 4 and 6 days after infection and seems to depend mainly upon the presence of activated T cells, which recognize antigen in the liver lesions (Blanden, 1970, 1971a, 1971b). Macrophages may be present in the early infiltrate, they appear to ingest and destroy infectious virus and necrotic tissue. Antibody does not appear in the circulation before the 7th or 8th day after infection and does not seem to play a role in recovery from primary infection.

Listeria monocytogenes is a small Gram-positive, rod-shaped, facultative intracellular bacterium. It is an ubiquitous organism, like ectromelia virus it grows in the liver and spleen (Gray and Killinger, 1966). However, in contrast to ectromelia, *Listeria* lesions are rapidly invaded by inflammatory cells within some hours after infection, at first by polymorphonuclear leukocytes, later on by monocytes (Mackaness, 1962). For 2-3 days the bacteria grow within the phagocytes; recovery from infection depends upon improvement of the bactericidal capacity of these macrophages. This process appears to be T cell dependent (Lane and Unanue, 1972; North, 1973a, 1973b).

4.2. Antigen reactive T cells
4.2.1. Precursor T cells

Blanden and Langman (1972) presented evidence that thymus-derived cells play an essential role in immunity to *Listeria*. Irradiated adult thymectomized (ATx), bone marrow reconstitu-

ted mice were unable to generate antibacterial immunity. However, responsiveness was restored by injection of thymocytes. Mice, only thymectomized 6 weeks before immunization had unimpaired responses to *Listeria*.

The precursors of the effector T cells are probably mainly located in the recirculating pool of antigen-reactive cells. These cells enter the blood via the thoracic duct and return to lymph via the high endothelial venules in the mid cortex of the lymph nodes (Gowans, 1959). In the mouse 60-80 per cent of the thoracic duct cells are T cells (Raff and Wortis, 1970). The majority of these T cells have a lifespan of 12-16 weeks (Sprent and Basten, 1973).

The assumption that these T cells are the precursors of effector cells is based upon the suppressive action of anti-thymocyte serum (ATS) on cell-mediated immunity (Medawar, 1969). Since ATS does not penetrate lymphoid tissue to any significant extent (Denman and Frenkel, 1968), it probably acts upon cells while they are free in circulation, either by promoting the phagocytosis by fixed macrophages in the liver (Martin and Miller, 1969), or by cytotoxic activity.

ATS treatment of mice prior to infection has been shown to impair their response to *Listeria*, as reflected in a reduced ability to control bacterial growth in the liver and spleen (Zinkernagel et al., 1974). In ATS-treated mice infected with ectromelia virus, similar effects are found (Blanden, 1970). In contrast to the poxvirus model where T cell function is obligate to recovery, T cell function in influenza infection appears to be one of several factors, both immune and non-immune (Yap et al., 1979). A variety of other mechanisms including interferon, resistance genes (Haller and Lindenmann, 1974; Lindenmann et al., 1978) and nonspecifically activated macrophages (Shayegani et al., 1974) may be equally important. To this point it has been reported that in neonatally thymectomized mice (Takeya et al., 1968) and in irradiated bone marrow reconstituted ATx mice (Zinkernagel et al., 1974) the non-immunological resistance to *Listeria* infection is more pronounced.

4.2.2. Effector T cells

Cell transfer experiments provide direct evidence for the generation of sensitized T cells in the early stages of *Listeria* infection (Zinkernagel et al., 1974). Spleen cells harvested 3 days after immunization with *Listeria* and cultured *in vitro* for

22 hr, in order to eliminate most of the contaminating viable *Listeria*, were highly capable of conferring an anti-bacterial effect onto infected recipients. Mackaness (1969) reported a lack of transferable anti-bacterial effect in spleen cells harvested earlier than the 5th day after *Listeria* infection, probably caused by large numbers of viable bacteria present in *Listeria* immune spleen cells (North, 1973b; Zinkernagel et al., 1974). Bacterial numbers in the livers and spleens of ATS-treated mice were greater than in NRS-treated controls within 3 days after infection, suggesting that effector T cells are produced within 3 days. Their numbers reach a peak at 6 to 7 days and then decline; low levels are attained by day 10 (Mackaness, 1969; North, 1973b). Mackaness (1962) and Van der Meer (1980) clearly demonstrated that elimination of *Listeria* bacteria from the liver coincided with the acquisition of delayed type hypersensitivity (DTH) to *Listeria* antigens. Both T cell functions depend upon recruitment and activation of macrophages, presumably via the secretion of soluble factors, which are called lymphokines (North, 1970). Using the DTH assay, North (1973b) has shown that in mice the anti-*Listeria*-directed effector T cells harvested 6 days after immunization are dividing cells. However, when such immune T cells are harvested 10 days after immunization they are non-dividing and resistant to X-irradiation (Youdim et al., 1973). Many of the effector T cells generated during the response are functionally short-lived cells (McGregor and Logie, 1973).

For virus the kinetics of the specific T cell response has been studied most extensively by Yap and Ada (1978) and Yap et al. (1978). These authors used influenza-infected mice. The protective capacity of immune T cells upon adoptive transfer peaks 5 or 6 days postimmunization. Adoptively transferred immune T cells taken 21 or more days after exposure of the donor to infectious influenza virus were unable to give any protective effect. Transfer of these immune T cells to influenza-infected nude mice enhanced the survival of these mice and reduced the influenza virus levels in the lungs (Yap et al., 1979). Using ^{51}Cr release assays of virus-immune T cells against virus-infected cells, Gardner et al. (1974a) found significant cytotoxic activity for virus-infected target cells in the spleen of ectromelia-infected mice as early as 2 days after infection. Maximum of cytotoxic activity was attained by day 6. It is likely that immune T cells themselves can kill the target cells by a single hit (Gardner, 1974b).

Effector T cells enter the circulation within 3 days after ectromelia infection, as is shown by the effect of ATS given on

day 3, 4 or 5 after infection. No effect is seen when ATS is given on day 1, 2 or 6 after infection (Blanden, 1974).

These data clearly demonstrate that (1) effector T cells migrate from lymphoid tissues via the blood towards the site of infection, and (2) this migration mainly takes place from day 3 to 5 after infection.

In summary, in defense against intracellularly growing bacteria, antigen-specific T effector cells release lymphokines that activate macrophages to increased bactericidal activity. This bactericidal activity by itself is not antigen-specific. Virus-specific T effector cells kill by direct cell-cell contact virus-infected target cells in an one hit kinetic pattern.

4.3. *Role of major histocompatibility antigens in cell-mediated immunity against viruses and bacteria*

Lilly and Pincus (1973) and Tucker et al. (1977) have observed that in some mouse strains the susceptibility to induction of viral leukemogenesis segregates with the murine histocompatibility complex (H-2). The involvement of self cell surface components in cell-mediated immunity has also been studied by Svet-Moldavsky et al. (1968). They reported that skin grafts from inbred mice that were congenitally infected with oncogenic virus, were rejected by syngeneic non-virus-infected mice, just as allogeneic skin grafts are usually rejected.

Zinkernagel and Doherty (1974a, 1974b) found that virus specific cytotoxic T cells (CTL) lyse syngeneic virus-infected target cells, but that they were 30-300 times less efficient in destroying allogeneic target cells infected with the same virus. Analysis of this restricted virus specific killing of infected target cells has shown that the restriction mapped to the H-2 complex, and more precisely to the K and D, but not to the I region of H-2 (Blanden et al., 1975; Koszinowski et al., 1976). Yap et al. (1978) demonstrated that the protective effect of transferred immune T cells only occurred when the immune cell donor and infected recipient shared genes in the K/D regions of the H-2 complex. K and D region coded antigens must be recognized in combination with virus-specific antigens, in order to elicit optimal T cell mediated immunity. The same K and D region coded antigens were found to restrict CTL that were primed *in vivo*, and restimulated *in vitro* against minor histocompatibility antigens (Bevan, 1975; Gordon et al., 1975) and CTL that were generated *in vitro* against trinitro-

phenylated syngeneic spleen cells (Shearer et al., 1975). However, CTL responses to syngeneic target cells coupled with high doses of hapten were found to be less H-2 restricted (Haas et al., 1979). A lack of restriction is also described for human infectious mononucleosis. There, T cells specifically cytotoxic for Epstein-Barr virus (EBV) not only kill syngeneic, but also allogeneic EBV-infected target cells (Lipinski et al., 1979). This is probably due to the fact that EBV-infected cells express as much as 36 times more HLA molecules at their surface than normal lymphocytes (McCune et al., 1975), so that cross-reactivity between different HLA antigens can be revealed which is otherwise undetectable. Furthermore, Doherty and Bennink (1979) reported that BALB/c ($H\text{-}2K^d/D^d$) T cells, filtered through irradiated (C3H x BALB/c)F1 mice in order to remove anti-$H\text{-}2^k$ alloreactive precursors, and then exposed to vaccinia virus for 6 days in irradiated A/J ($H\text{-}2K^k/D^d$) recipients, were able to develop a strong cytotoxic response against both virus-infected $H\text{-}2D^d$ and allogeneic $H\text{-}2K^k$ target cells. However, when other H-2 haplotypes were used in the same system, no cytotoxic activity against allogeneic H-2 virus infected target cells could be detected (Bennink and Doherty, 1978).

Zinkernagel et al. (1977) found that effector T cells involved in the protection against *Listeria*, are specific for the *Listeria* antigens in association with H-2I region coded self markers. These effector T cells are non-lytic, but produce several lymphokines that attract phagocytic cells. The similarity of these T cells with those involved in DTH reactions has already been mentioned (North, 1970). The transfer of DTH to conventional antigens such as fowl-γ-globulin is also H-2I restricted (Miller et al., 1975). H-2I region encoded determinants are thought to be expressed on very few tissues including lymphocytes, macrophages, sperm, epidermal and endothelial cells (Gose and Bach, 1979). Bacteria associate predominantly, if not exclusively, with phagocytic cells. Such antigen-pulsed macrophages become immunogenic for T cells (Pearson and Raffel, 1971). The T effector cells subsequently formed do not lyse the phagocytic cells, but trigger them in such a way that their phagocytic and digestive capacity is improved. Thereby the bacteria are eliminated. Zinkernagel (1978) described H-2I coded structures as "receptors" for such non-lytic differentiation signals.

H-2K and H-2D coded cell-surface markers have been demonstrated on all nucleated cells, although the quantity varies in different tissues (Klein, 1975). Viruses can actively infect phagocytic as well as nonphagocytic cells. These viruses can rapidly induce the appearance of virus specific cell-surface

antigens (Ada et al., 1976; Koszinowski et al., 1977). These changes make the cell susceptible to immunologic attack. Viruses can be efficiently eliminated by target cell lysis, if it occurs during the eclipse phase of the virus infection, i.e., after penetration and uncoating, and before reassembly of the infectious progeny. H-2K and H-2D coded structures associated with virus-coded antigens can be seen as "receptors" for T cell mediated target cell destruction (Zinkernagel, 1978). However, such an auto-agressive reaction may be only required in case of infections with acute viruses, which themselves cause rapid lysis of the infected cells. Ectromelia infection is a clear example of this group (Doherty et al., 1976). Lymphocytic choriomeningitis virus (LCMV), on the other hand, tends to cause persistant infections, often without lytic effects. Crowle (1975) described that mice infected with LCMV developed a state of DTH to the virus. This DTH reactivity might recruite phagocytic cells which eliminate the non-cytolytic viruses in a less auto-agressive way. Both cytotoxic T cell reactivity against virus-infected cells and adoptive transfer of DTH to virus antigens is restricted by the K and D region of the H-2 complex (Zinkernagel, 1976).

In the first 3 days of acute LCMV infection of mice heterospecific cytotoxic cells are generated. These cells kill many types of infected or uninfected cultured cells, including syngeneic cells (Pfizenmaier et al., 1975; Blanden and Gardner, 1976; Welsh and Zinkernagel, 1977). Probably this is due to activation of natural killer (NK) cells by the virus. These NK cells account for the early, non-H-2 restricted, nonspecific cytotoxicity observed in the early stages of LCMV infections (Miller, 1978). For reviews about NK cells see Herberman et al. (1979) and Kiessling and Wigzell (1979).

4.4. Antigen recognition structures of T cells

The antigen recognition structures of T cells are continuously generated by the cells self, and not passively adhered to the cell surface (Von Boehmer et al., 1979). The phenomenon of H-2 restriction has led to the theory that T cells, upon reacting to non-H-2 antigens, react simultaneously to H-2 coded antigens. Each clone of responding cells is stimulated by both types of antigens or some jointly formed interaction product. Two major forms of recognition are proposed: the *altered-self hypothesis* and the *dual recognition hypothesis*.

The *altered-self model* states that non-H-2 antigens closely associate with H-2 determinants to create new antigenic determinants recognized by one T cell receptor. Strong evidence in favour of a peculiar molecular association was obtained by the demonstration of co-capping of virus antigen and H-2 products (Senik and Neauport-Sauter, 1979; Schrader et al., 1975). However, modification of H-2 products due to a virus infection is less likely, because viruses inactivated by β-propriolactone or ultraviolet light can still provoke H-2 restricted lysis in the absence of viral protein synthesis (Koszinowski et al., 1977; Schrader and Edelman, 1977; Ennis et al., 1977). Fox and Weissman (1979) found that Moloney virus-induced cell surface antigens and H antigens are located on distinct molecules, which could be separated by detergent extraction. No evidence was found for an "altered-self" type molecule.

The *dual recognition model* states that each T cell has two distinct receptors: one directed to non-H-2 antigens and another to a H-2 determinant. One of the main problems of the two receptor theory is that it is difficult to explain the results of cold target inhibition experiments with this model. Unlabeled cells were found to inhibit lysis of ^{51}Cr-labeled target cells, but only if they had both the appropriate H-2 and virus determinants (Zinkernagel and Doherty, 1975; Forman, 1978). It must be assumed therefore that the two receptors can work only as a paired unit. Therefore the two determinants must be in close proximity (Miller, 1979). So, it is very difficult to unequivocally support one of the two models.

The level of the immune response to a large number of thymus-dependent antigens is controlled by Ir genes, which map within the I region (Katz and Benacerraf, 1975) as well as within the K and D regions (Zinkernagel et al., 1978a; Von Boehmer, 1978) of the H-2 complex. It has been proposed that Ir genes do not code for the T cell receptor for antigen (Von Boehmer, 1978), but code for some structure which ensures a stable molecular interaction between the H-2 product and the antigen, so that the two can form a complex which is immunogenic for T cells (Miller, 1979). Alternatively, Ir gene products and the antigens responsible for H-2 restriction are the same. The suggestion that H-2 linked Ir genes exert their influence at the level of antigen-presentation to T cells, does not exclude the possibility that Ir genes are expressed at the level of generation of the T cell repertoire in the thymus. The model for this has been proposed by Miller (1979). It is based on a positive selection for T cells expressing anti-H receptors against H-2 structures displayed by the thymus epithelium. Furthermore, only one

variable (*V*) region gene pool for both the anti-H-2 and the anti-antigen (X) receptor is assumed. The germ line contains one C gene for the constant part of anti-self-H-2, one C gene for the constant part of anti-X, and a *V* region gene pool that can code for structures which are essentially complementary to the various H-2 alleles of the species. In the thymus selection would take place for those cells, which by chance have translocated the C gene for the constant part of the anti-self H-2 receptor to the correct *V* gene, namely the one which codes for a chain complementary to the H-2 structures expressed on the thymus epithelium. Thereafter each of the other different *V* genes in the *V*-region gene pool might translocate to the C gene for the anti-X receptor to allow production of the various anti-X receptors within the population of T cells. The *V* gene coding for the anti-self-H-2, can probably also code for a structure complementary to a certain antigen. But then this *V* gene can no longer be used for the production of the anti-X receptor. This can explain the H-2 (strain) linked Ir gene effect, namely that certain strains of mice are low responders to a particular antigen, while most other strains are high responders. Von Boehmer et al. (1978) demonstrated that when stem cells from low responder mice are allowed to differentiate in a thymus of a high responder strain, phenotypically high-responder T cells are generated. The polymorphism of the H-2 complex is advantageous for the maintenance of the species; it minimizes the incidence of low responsiveness to potentially pathogenic organisms (Zinkernagel, 1977).

5. *T CELLS IN ALLOGRAFT IMMUNITY*

5.1. Introduction

Antigens of the MHC were originally discovered as the primary barrier to allograft transplantation (Snell and Stimpfling, 1966). Graft rejection was subsequently defined as a T cell response (Miller and Osoba, 1967; Wortis, 1971). Two basic forms of cellular immunity probably play a central role in allograft reactions: development of delayed type hypersensitivity (DTH) and the generation of cytotoxic lymphocytes (Huber et al., 1976). Allogeneic skin grafts can induce a state of DTH to H antigens in many species, including mice (Hoy and Nelson, 1969) and guinea pigs (Brent et al., 1962). Al-Askari et al. (1965) showed that inhibition of macrophage migration *in vitro* can be applied to detect a state of allograft immunity. DTH reactive T cells release, upon antigenic stimulation, mediators like migration inhibition factor (MIF) and chemotactic factors which arrest large numbers of macrophages at the reaction site. By arming the macrophages with an antigen-specific cytotoxic factor released from antigen-activated T lymphocytes, macrophages can be induced to lyse the specific target cells (Lohmann-Matthes and Fischer, 1973). After skin allografting in mice (Canty and Wunderlich, 1971) and in rats (Peter and Feldman, 1972) graft specific cytotoxic lymphocytes are generated as well. Cytotoxic activity is usually assessed by measuring the release of ^{51}Cr from labeled target cells *in vitro*. Cytotoxic T cells are autonomous killer cells with an extremely high lytic capacity. In a comparitive study of the cytotoxic and MIF activities of spleen cells during the primary response to alloantigens, Brunner and Cerottini (1971) found that both activities reached peak values at the same time and could be abrogated by treatment of the spleen cells with anti-Thy-1 serum and complement. Evidence that DTH and cytotoxic responses are mediated by distinct T cell subclasses came from work of Huber et al. (1976). The ability to generate graft-specific DTH is confined to T cells with Lyt-1^+ determinants on their membranes. The cytotoxic activity to the allogeneic target cells, on the other hand, is build up and mediated by Lyt-23^+ T cells (Cantor and Boyse, 1975).

5.2. Cytotoxic T cells

5.2.1. Evidence for cytotoxic T cells in allograft immunity

To study the cytotoxic lymphocyte *in vivo* proved to be extremely difficult, primarily because of the complexity of the

allograft reaction. A combination of *in vivo* and *in vitro* techniques for the detection of cytotoxic reactivity was worked out by Brondz (1964). Spleen or lymph node cells from animals mediating an allograft reaction could kill *in vitro* host macrophages. Brunner et al. (1966) introduced the DBA/2 mastocytoma P-815, which proved to be a superior target in cell-mediated lympholysis (CML) assays. The ^{51}Cr-release method described by Brunner et al. (1970), as modified by Canty and Wunderlich (1970), appeared to be the most sensitive assay. The isotope is believed to be firmly but noncovalently associated with cytoplasmic proteins. Once released from the cells, it is not reutilized, and the release reflects irreversible target cell damage. Häyry and Defendi (1970) and Hodes and Svedmyr (1970) demonstrated that in mixed lymphocyte cultures (MLC) cytotoxic cells are generated, capable of lysing appropriate target cells, e.g., mitogen stimulated lymphoid blast cells (Lightbody et al., 1971). Rouse and Wagner (1972) cultured cortison-resistant CBA thymocytes together with mitomycin C treated BALB/c stimulator spleen cells. The CBA cytotoxic T cells which were generated in these cultures specifically rejected BALB/c allografts when they were transferred into thymectomized, lethally irradiated, bone marrow reconstituted CBA mice.

It is rather difficult to isolate allograft-infiltrating cells in a functionally viable state. Roberts and Häyry (1976) developed the so called "sponge-matrix" allograft method, in order to produce large numbers of infiltrating cells for experimentation. In this assay a viscous cellulose sponge is infiltrated by fibroblasts and sc transplanted into the neck of an allogeneic mouse. Treatment of the sponge-matrix-allograft-infiltrating cells by an anti-T cell serum and complement *in vitro*, completely abolished the lytic activity as tested in a CML assay. Tilney et al. (1975) have reported that the cells infiltrating Ag-B incompatible rat heart allografts 5 days after transplantation are predominantly T lymphocytes. These T cells are able to display direct cytotoxic activity *in vitro*. Immunofluorescent studies have shown an abundance of T cells in early cellular infiltrates of rat renal allografts (Balch et al., 1973). Andersson (1973) demonstrated with velocity sedimentation studies that cytotoxic T lymphocytes may be morphologically large cells (lymphoid blasts) or small cells (lymphocytes) depending on the stage of the response. During the early stage of the anti-allograft response, the cytotoxic activity is primarily due to blast cells, whereas later on the cytotoxic activity is mediated by small cells. It has been shown *in vivo* that only lymphoblasts can migrate into inflam-

matory sites (Russel et al., 1975). Sprent and Miller (1976) have shown that isotope-labeled H-2 activated T lymphocytes display a very slight tendency to selectively localize in specific skin allografts, whereas Emeson (1978) and Chang and Sugarbaker (1979) presented evidence that specific anti-allogeneic lymphocytes are selectively recruited to lymphoid cell allografts. There is no rigorous evidence that specifically reactive lymphocytes are actually attracted to the site of sequestered antigen (Ford, 1975). It is more likely that they are selected from the normally heterogeneous pool of circulating lymphocytes as they percolate through the tissue (Cahill et al., 1976).

Strom et al. (1975) have shown that the majority of the killer cells infiltrating human kidney allografts at late stages of rejection are non-T cells. Experiments with sponge-matrix allografts have revealed that the early killer cells are predominantly T lymphocytes, whereas later on several classes of killer cells may invade the allograft (Wiktorowicz et al., 1978). These killer cells include real cytotoxic T cells, non T lymphocytes, monocytes and macrophages. Microscopic studies have shown that monocytes with a high acid phosphatase activity dominate the cellular infiltrate of mouse skin allografts 10 days after transplantation (Poulter et al., 1971).

Results of adoptive restoration of the capacity of heart graft rejection in irradiated rats, establish a central role for the long-lived recirculating T lymphocytes in first-set allograft rejection (Hall et al., 1978). The concommitant production of alloantibody in animals also infused with B lymphocytes had no effect on the tempo of rejection (Hall et al., 1978). However, the hyperacute rejection of allografts is exclusively dependent on alloantibodies (Koene et al., 1973; Pedersen and Morris, 1974; Guttmann, 1977). The failure to abrogate skin allograft rejection by procedures which only deplete the recirculating pool from otherwise intact animals might suggest that first-set allograft rejection is achieved by very few recirculating lymphocytes (McGregor and Gowans, 1964; Roser and Ford, 1972).

5.2.2. Lytic mechanism of cytotoxic T cells

Operationally, the lytic mechanism can be divided in three discrete stages: (1) specific binding of the cytotoxic T cell to the target cell, (2) the lethal hit administered by the cytotoxic T cell and (3) the release of intracellular (^{51}Cr-labeled) components into the medium fluid as the result of membrane damage.

The requirement of divalent cations in the binding of the killer cell to the target cell has been studied by the extent of adhesion of lytically active cells to allogeneic monolayers in media of defined cation content (Plaut et al., 1976). Mg^{2+} ions appeared to be required in the binding of killer to target cells. The subsequent lytic event itself is absolutely dependent on Ca^{2+} ions.

Kinetic studies of the release of various isotopic indicators of target cell damage have suggested that the first, and perhaps the only, lesion is an alteration of the membrane permeability for ions (Henney, 1973; Martz, 1976). Mayer (1977) suggested that cytotoxic T cells possess membrane-associated proteins which, upon interaction with target cells, undergo conformational changes and expose hydrophobic peptides which insert in the lipid bilayer of the target membrane and result in the formation of transmembrane channels. These channels allow free passage of water and other small molecules across the membrane. The lethal hit step can be completed within a few minutes (MacDonald, 1975; Martz, 1975). A single cytotoxic T cell can sequentially destroy several target cells (Zagury, 1979). After the lethal hit, colloid osmotic lysis is responsible for actual desintegration of the target cell (Mayer, 1977).

Cytotoxic T cells become capable of effecting non-specific lysis in the presence of lectins, such as phytohemagglutinin and concanavalin A (Bevan and Cohn, 1975). These lectins are cell agglutinins, and can act as ligands between effector cells and target cells. This clearly indicates that the lethal hit stage itself is nonspecific. Synthesis of DNA or RNA by the effector cells is not required (Brunner et al., 1968), while the data of Thorn and Henney (1976) strongly argue against the requirement for *de novo* protein synthesis.

5.2.3. Genetic requirements

Cytotoxic T cells can have reactivity for H-2 alloantigens (other then self) or for non-H-2 antigens. CBA ($H\text{-}2^k$) mice immunized to DBA/2 ($H\text{-}2^d$) produce cytotoxic T cells which kill any target bearing either $H\text{-}2K^d$ or $H\text{-}2D^d$, no matter what other cell surface antigens these targets may have. Alloreactive T cells are thus clearly not self H-2 restricted (Miller, 1979). However, the response to other antigens generally is self H-2 restricted. For example, CBA mice recovering from infection by LCMV have cytotoxic T cells which attack LCMV infected $H\text{-}2K^k$ or $H\text{-}2D^k$ targets, but not uninfected $H\text{-}2^k$

targets, nor LCMV infected targets of any other H-2 haplotype (Miller, 1979).

Mutations in either H-2K or H-2D loci result in a strong CML in combination with their corresponding standard strains (Klein, 1978a). Presumably, a single aminoacid substitution change in a H-2 antigen can be sufficient to be recognized by precursor cytotoxic T cells. Such CML can be inhibited by antibodies against the mutant H-2K or H-2D antigens (Nabholz et al., 1975).

Studies with mouse strains which have point mutations in K or D region of the H-2 complex, lead to a splitting of a CML target antigen into individual determinants. A single H-2K or H-2D molecule carries several antigenic determinants, which can be recognized by T cells as more or less independent entities (Melief et al., 1977). The relationship between these determinants (defined by cytotoxic T cells) and the serologically detectable antigens is not clear. The importance of serologically detectable private and public determinants on H-2K or H-2D molecules as target antigens in CML has been tested among others by Peck et al. (1976). Cytotoxicity to targets which shared only the public specificities is generally low or undetectable. Strong cytotoxic activity is observed only against those target cells carrying a private determinant in common with the original stimulator strain. However, this is not always the case. In approximately one-fourth of nearly 500 different combinations in which stimulator and target cells shared no private specificity, low but significant CML is detected. Cross-killing can be detected against four (possible five) of the public specificities (Peck et al, 1976).

By serological methods cross-reactions between the products of H-2K, H-2D and H-2L loci have been studied (Hansen et al., 1979). One evolutionary model explaining such homologies would be that the three genes arose from a common ancestral gene, as was previously proposed for H-2K and H-2D (Snell et al., 1971).

The involvement of the H-2I region in allograft reaction was first demonstrated *in vivo* by skin grafting between H-2I region congenic lines (Klein et al., 1974). Thyroid allografts that genetically differ from the recipient at the H-2I region only, are rejected as rapidly as those that differ for the entire H-2 complex (Gose and Bach, 1979). The I region of the H-2 complex contains at least two H loci: a strong (H-2A) locus in the I-A subregion, and a weak (H-2C) locus in the I-C subregion (Klein et al., 1976). Until now no H loci have been detected

in the I-B, I-J or I-E subregion. Optimal cytotoxic activity against I region coded determinants as measured in CML is obtained only after priming *in vivo* (Klein, 1978b) or *in vitro* (Wagner et al., 1975). Data from Klein et al. (1977) revealed that cells with H-2I-A region coded antigens do not require H-2K/D region identity with the cytotoxic T cells in order to function as targets in the CML assay. The Qa-1 and Qa-2 locus in the T region of the H-2 complex also code for determinants against which CML has been demonstrated after *in vivo* priming (Klein, 1978b; Forman and Flaherty, 1978). This reactivity is also unrestricted. In CML reactions against minor H antigens, the non-H-2 alloantigens are recognized by T cells in conjunction with syngeneic K or D region coded antigens (Bevan, 1975; Gordon et al., 1975). However, data from Forman and Streilein (1979) indicate that in neonatally tolerized mice two sets of T cells co-exist, one capable of recognizing minor H antigens in the context of the host haplotype, and the other able to recognize antigens in the context of the tolerated allogeneic H-2 haplotype. T cells do not seem to be able to recognize minor H antigens only. Probably non-H-2 alloantigens belong to the same category of antigens as viral cell surface antigens (Zinkernagel and Doherty, 1975) and haptens (Shearer et al., 1975). Minor H-antigens may be enzymes, structural or transport proteins, or other components which happen to show allelic differences and are expressed on the cell surface (Bevan, 1976).

Recently it was demonstrated that allogeneic skin grafts differing at major and minor H-antigens can prime for second set rejection of skin grafts differing from the skin graft recipient only at minor H-antigens (Murasko, 1978). Allogeneic presensitization of females *in vivo* to H-Y antigen in combination with H-2 antigens and subsequent challenge *in vitro* with syngeneic male cells, generate cytotoxic cells specific for syngeneic male target cells (Gordon et al., 1976). The same holds for minor H antigens (Bevan, 1976). This suggests that *in vivo* H-2 restriction is of little relevance in allograft rejection probably because of host macrophage processing of the allogeneic H-2 incompatible cells, thereby expressing allogeneic minor H-antigens in combination with self K or D region coded antigens.

Studies of the ontogeny of the T cell subsets responsible for generating alloreactive cytolytic activity indicate that early (1-3 wk) after birth alloreactive cytotoxic T cells are generated from Lyt-123$^+$ T cells. At approximately 3-5 weeks of age, alloreactive pre-killer cells shift from the Lyt-123$^+$

compartment into the Lyt-23$^+$ compartment (Burakoff et al., 1978). Priming of Lyt-123$^+$ T cells with autologous virus-coated cells in adult animals stimulates the generation of Lyt-23$^+$ T cell clones that lyse both syngeneic virus-coated targets and, although at a lower frequency, distinct sets of noninfected allogeneic targets (Finberg et al., 1978). Also alloreactive cytotoxic T cells can cross react with chemically modified syngeneic targets (Müllbacher and Blanden, 1979; Lemonnier et al., 1977), and about 1% of the killer cells reactive to minor H antigens can also cross react with the H-2 antigens of an allogeneic target (Bevan, 1977). The frequency of precursor cytotoxic T cells in the Lyt-23$^+$ population capable of developing into cytotoxic effector cells after stimulation with H-2 without preceding immunization is high. For a single MHC haplotype difference this figure has been estimated at approximately 1-3% of all peripheral T cells (Lindahl and Wilson, 1977). Burakoff et al. (1978) suggest the following model to account for the presence of large numbers of alloreactive T cell clones in adult animals. The continuous stimulation of Lyt-123$^+$ cells by autologous MHC antigens associated with foreign materials (e.g., virus) would result in the formation of a Lyt-23$^+$ progeny carrying receptors that have a high degree of cross reactivity for unrelated MHC products. It must be stressed, however, that the phenotype of cytotoxic effectors induced by a whole MHC difference is Lyt-23$^+$, whereas Lyt-123$^+$ T lymphocytes are required at all levels of the immune response to mutant H-2K/D (Wettstein et al., 1979) and normal H-2K/D coded antigens (Bach and Alter, 1978).

5.3. DTH reactive T cells

Brent et al. (1962) found a histologically typical DTH reaction after sc injection of allogeneic cells in guinea pigs, which were transplanted earlier with an allogeneic skin graft from the same donor strain. For elicitation of the DTH reaction intact lymphoid cells as well as crude membrane extracts could be used. Later on, Dekaris and Allegretti (1968) succeeded in inducing a state of DTH to H antigens in mice by allogeneic skin grafts. This state of DTH could be expressed by sc injection of viable spleen cells from the same donor strain in the footpad, but not with disrupted cells. Hoy and Nelson (1969) were able to induce a state of DTH in mice by tumor allograft transplantation. Again some years later, Kon and Klein (1976) studied the induction of DTH to H-2 and non-H-2 antigens by skin graft transplantation in mice across H-2 or non-H-2 barriers. These authors elicited DTH responses by challenge in

the footpad with the same H antigens present on intact spleen cells or solubilized from a methylcholanthrene-induced fibrosarcoma. Sc injection of viable allogeneic spleen cells in cyclophosphamide pretreated mice can induce a state of DTH (Smith and Miller, 1979a). DTH was tested by injecting the antigen into one ear and ^{125}IUdR intraperitoneally, and removing and determing radioactivity in both ears 24 h later (Vadas et al., 1975). However, when mice not pretreated with cyclophosphamide are sc immunized with alloantigens, a state of DTH can occur as well. This can be detected by direct measurement of the swelling in the footpad 24 h after local application of the challenge (Van der Kwast et al., 1979). As compared to the radiometric ear test, the foot swell test has the advantages of (1) a linear relationship between the number of reactive T cells and the height of the response, (2) giving reproducible absolute responses, and (3) enabling longitudinal studies of the same mouse. Vadas et al. (1977) have studied the H-2 requirements for the transfer of DTH in mice. They studied the response to two antigens: fowl gamma globulin (FGG) which is injected sc, and dinitrofluorobenzene (DNFB) which is painted on the skin. Spleen or lymph node cells were harvested 5 days later and injected into naive mice. For both antigens, transfer between mice with multiple non-H-2 disparities was successful, but transfer between mice with disparity across the full H-2 complex was unsuccessful. However, the restriction elements are different for the two antigens tested. In the case of cells sensitized to DNFB, identity at the K, D or I-A regions is sufficient, while for FGG, identity at I-A is both necessary and sufficient. The requirement in the case of FGG for I-A identity recalls the H-2I restriction of T helper function in the humoral immune response to proteins (Katz and Benacerraf, 1975). This is compatible with the fact that both functions are mediated by Lyt-1$^+$ T cells (Vadas et al., 1976). The effectiveness of H-2I, H-2K and H-2D matching in the case of DNFB may mean that DTH to DNFB can be mediated by two subsets of T cells: H-2K or H-2D restricted Lyt-23$^+$ T cells and H-2I restricted Lyt-1$^+$ T cells (Vadas et al., 1977). Alternatively, this agent may have altered a variety of cell surface components, including H-2K and H-2D, probably by binding to the ε-animo groups of lysine (Forman et al., 1977) and by this way they are recognized as unrestricted H-2 alloantigens. Smith and Miller (1979b) have found that both Lyt-1$^+$ and Lyt-2$^+$ T cells were involved in successful transfer of DTH to H-2 incompatible cells. DTH reactivity can be induced against H-2I, H-2K and H-2D region coded antigens (Smith and Miller, 1979c). Probably Lyt-1$^+$ T cells are responsible for DTH reactivity against H-2I region coded determinants, while Lyt-2$^+$ T cells

give rise to DTH reactivity to H-2K and H-2D. Swain and Panfili (1979) showed that H-2K and H-2D activated helper T cells for the primary *in vitro* response to sheep erythrocytes have the Lyt-123 phenotype. This might suggest that Lyt-123$^+$ T cells can mediate DTH reactivity against H-2K or H-2D region coded determinants and that these T cells probably are restricted by these regions. Van der Kwast (1980) brought evidence that DTH T effector cells reactive to minor H antigens are restricted by H-2K or H-2D region coded antigens.

Upon antigenic stimulation DTH reactive T cells release mediators like MIF and chemotactic factors which arrest large numbers of macrophages at the reaction site. Maximum release of chemotactic factors in the supernatant of MLC is caused by H-2I region disparity (Cheung and Sundharadas, 1979).

In mouse skin Ia antigens are predominantly expressed on dendritic macrophages (Langerhans cells) (Tamaki et al., 1979). These cells are involved in the uptake and transport of antigenic material from the epidermis to the lymphatic system. Thus, they are probably also involved in the afferent phase of graft rejection (Rowden et al., 1978). Farr et al. (1979) provided evidence that cytotoxic macrophages are involved in rejection of allografts. They showed that immune T lymphocytes upon interaction with the specific antigen and Ia-positive macrophages from non-immune mice, *in vitro* could produce a factor which induces cytolytic activity in Ia-negative macrophages. Ia-positive macrophages were essential for antigen presentation to the immune T cells. Antigen associated with Ia-negative macrophages was ineffective.

5.4. T-T interactions

When a small number of peripheral T cells is mixed with thymocytes, and the cell mixture is immunized *in vitro* against cell-bound alloantigens, a cytotoxic activity is generated exceeding 10-20 fold the values that can be explained by a pure additive effect (Wagner, 1973; Häyry and Andersson, 1974; Tigelaar and Feldmann, 1973). Such experiments have lead to the supposition that T2 cells include precursor cells of cytotoxic lymphocytes and that thymocytes are a source of T1 cells which mainly act as amplifier cells. Results of Glaser and Law (1978) and Glaser (1979) indicate that also in rejection of syngeneic SV40-induced sarcoma in mice, there is a cooperative cell interaction between *in vitro* immunized thymocytes and peripheral T cells.

The participation of two collaborating cell types in the induction of a cytotoxic response has also been studied by Bach et al. (1976). They have found that in a MLC in which only the H-2K or H-2D portions of the H-2 complex were mismatched, little cytotoxicity developed. When, in addition, the stimulatoring cells varied from the responder cells at the H-2I region, maximal cytotoxic responses were observed. In their proposed model Lyt-1^+ T helper cells, upon reacting to H-2I region coded antigens, proliferate and produce mediators that amplify the proliferation and differentiation of cytotoxic Lyt-23^+ T cells (Fig. 3). From work of Wagner et al. (1976) it can be concluded that the collaborative effect of I region activated T cells upon the generation of anti-H-2K and H-2D CTL, takes place *in vivo* as effectively as *in vitro*. Okada et al. (1979) showed that production of the amplifying mediator did not require cell division and that the factor was produced within 15 hr of establishing a murine MLC. The production is dependent upon the presence of Lyt-1^+ T cells; Lyt-2^+ T cells are not needed for mediator production, but serve as the target cell population on which the factor exerts its action. However, there is no absolute requirement for I region differences in order to generate cytotoxic responses to H-2K/D region encoded antigens (Bach and Alter, 1978). Moreover, cytotoxic responses to H-2I region coded antigens can also occur (Klein et al., 1977; Billings et al., 1977).

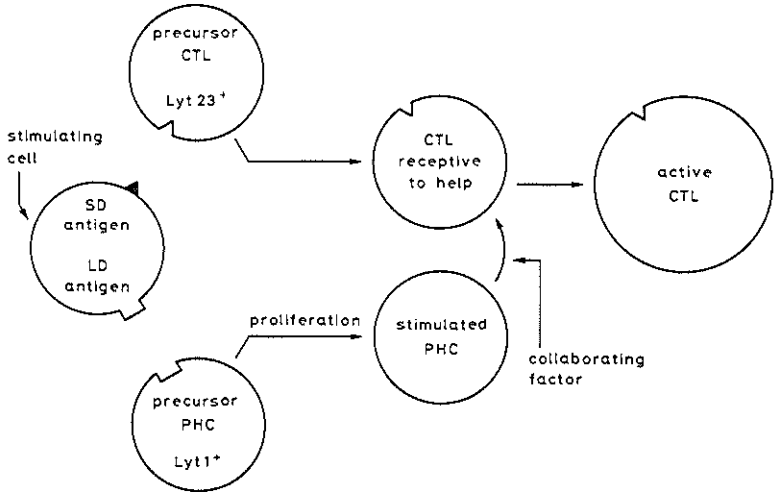

Figure 3
Model for the cooperation of two T cell types in the induction of optimal cytotoxic activity.

CTL: cytotoxic T lymphocyte, LD: lymphocyte defined, SD: serologically defined, PHC: proliferating T helper cell.

As to secondary CTL responses, Wagner and Röllinghoff (1978) and Alter et al. (1976) have reported that CTL specific for H-2K or H-2D alloantigen can be induced by restimulating MLC primed cells with stimulator cells sharing only the I region, but not the K and D region with the stimulating cells used in the primary MLC. They have found that activated Lyt-1^+ T cells release a nonspecific soluble product which triggers antigen primed Lyt-23^+ T cells to cell proliferation and cytolytic activity without further need for the presence of the specific antigen.

For the generation of cytotoxic T cells from thymocyte precursors *in vitro* alloantigen-specific helper T cells are required (Baum and Pilarski, 1978). Thymocytes alone are unable to generate cytotoxicity in response to allogeneic stimulator cells (Shortman et al., 1975). Wagner et al. (1979) found that *in vitro* the rate-limiting factor for the differentiation of Lyt-123^+ cortical thymocytes into CTL is the presence of the helper factor from activated Lyt-1^+ T cells. Cortical thymocytes, which comprise 90-95% of all thymocytes, express the Lyt-123 phenotype (Mathieson et al., 1979) and are immunologically incompetent. Presumably Lyt-123^+ cortical thymocytes belong to the T1 population, and Lyt-1^+ peripheral T cells to the T2 population. Therefore, the finding of Wagner et al. (1979) can provide an explanation for the earlier mentioned T1-T2 synergism in the generation of cytotoxicity in allograft reactions.

6. *T CELLS IN GRAFT-VERSUS-HOST REACTIONS*

6.1. Introduction

According to Billingham (1968) a Graft-versus-Host (GvH) reaction can only be initiated when immunologically competent lymphocytes are introduced into a host that confronts the graft with a large degree of histoincompatibility, and is unable to mount a similar immunologic attack against the intrusive donor lymphoid cells.

Among the most widely acknowledged forms of GvH reaction are *runt disease*, in which an immunologically immature animal is placed at risk by immunocompetent lymphoid cells from a histoincompatible adult; *secondary disease*, in which an animal initially is protected from the lethal effects of ionizing irradiaton (primary disease) by engraftment of hemopoietic stem cells (HSC) from an allogeneic donor, and subsequently is subjected to an attack by mature immunocompetent cells; *parabiosis intoxication*, which can occur in case of chronic cross-circulation of peripheral blood between two immunologically competent, but histoincompatible, individuals; and *F1 hybrid disease*, which occurs following the injection of parental strain lymphoid cells into an F1 hybrid animal derived from two histoincompatible inbred strains (Grebe and Streilein, 1976). From these the parabiosis intoxication does not fulfill the criteria set by Billingham (1968).

Two distinct types of the GvH reaction can be recognized, the acute and delayed form. The acute or early type of GvH reaction causes mortality of the host within about 15 days following transplantation. The delayed type of the GvH reaction has its onset at about three weeks following transplantation and does not necessarily lead to mortality of the host. The early or acute type of the GvH reaction is caused by grafts containing large numbers of lymphocytes (e.g., mouse spleen, and monkey and human bone marrow) (Table 1). In principle, it can be prevented by elimination of most of the lymphocytes from the graft prior to transplantation, even in histoincompatible donor-recipient pairs (Van Bekkum et al., 1979). This can be achieved by selective separation of the lymphocytes from the graft by using velocity sedimentation (Phillips and Miller, 1970; Amato et al., 1972) or density separation (Dicke and Van Bekkum, 1971; Yoshida and Osmond, 1971). However, this separation only eliminates cells capable of eliciting the acute GvH reaction. This procedure does not abrogate the delayed GvH reaction. The delayed type of the GvH reaction ensues after transplants with

TABLE 1

BONE MARROW COMPOSITION AND GVH REACTION IN VAROUS SPECIES

Species	Proportion of immunocompetent lymphocytes	MHC identity	GvH reaction	
			Type	Severity
Mouse	low	yes	delayed	mild if at all
		no	delayed	severe
Dog	intermediate	yes	delayed	mild
		no	subacute	severe
Monkey	high	yes	-	-
		no	acute	severe
Human	high	yes	subacute/delayed	mild/severe
		no	acute	severe

From Van Bekkum et al. (1979).

relatively low numbers of immunocompetent cells. Examples in the mouse are bone marrow and fetal liver grafts, and in man grafts of purified bone marrow cells (Table 1). It is assumed that the delayed GvH reaction can also be provoked by immunocompetent cells which are not present in the graft at the time of cell transfer, but which develop in the host from a subpopulation of the transplanted cells. The nature of the responsible cells is unknown. They might represent pluripotent HSC as well as more or less mature cells of the T lymphocytic series (Löwenberg et al., 1977).

The pathology of the GvH reaction can be studied most successfully in radiation chimeras. A radiation chimera is an animal which carries a foreign hemopoietic system, as a result of whole body irradiation followed by transplantation of hemopoietic cells derived from another animal (Ford et al., 1956). The first signs of the secondary disease are degenerative changes in the intestines, liver and skin. These pathologic changes are used for determing the severity of GvH disease in man (Table 2). Ultimately, infections occur which cause many inflammatory changes (Van Bekkum and De Vries, 1967). The most regular finding in mice which have died from secondary disease is lymphoid hypoplasia (Micklem and Loutit, 1966). Not all cells are at equal risk to the attack of GvH reaction, although H

antigens are present on the surface of virtually all cells in the body. Lymphatic cells, hemopoietic cells, and cells belonging to the mononuclear phagocyte system are almost always involved. Also skin, gastrointestinal tract epithelium and liver are frequently damaged. Muscle, bone, endocrine gland and cells of the nervous system are almost never involved (Grebe and Streilein, 1976). Van Bekkum and Knaan (1977) have developed a model for experimental studies of the intestinal lesion caused by GvH reactions. They established in F1 hybrid hosts, that had conventional or sterile gastrointestinal tracts, grafts of fetal intestine from syngeneic or semi-allogeneic origin. Subsequently, a GvH reaction was induced by injecting these F1 mice with parental lymphocytes. Many necrotic foci were detected in the crypts of both parental and F1 intestinal grafts in mice with a conventional flora, but they were very much reduced in mice that had sterile intestines. This indicates the complexity of the pathogenesis of lesions caused by GvH reactions. Injecting F1 mice with parental lymphoid cells can elicit the formation of host-derived *auto*antibodies resulting in hemolytic anemia or complex glomerulonephritis (Gleichmann, 1979). Also the activation of latent viruses in mice undergoing a GvH reaction has been described (Armstrong et al., 1972; Hirsch et al., 1972).

TABLE 2

CLINICOPATHOLOGIC GRADING OF THE SEVERITY OF HUMAN GvH DISEASE

Parameter	Grade 1	Grade 2	Grade 3	Grade 4
Skin rash	Mild	Moderately severe	Moderately severe	Severe
Gastrointestinal tract abnormalities	Nil	Mild	Moderately severe	Severe
Liver disfunction	Nil	Mild	Moderately severe	Severe
Overall clinical status	Unchanged	Unchanged	Significant impairment	Marked impairment

From Storb et al. (1974).

6.2. Assay systems for GvH reactivity

Splenomegaly assay

Simonsen and Jensen (1959) described a phenomenon which has grown out to one of the best known and widely used assays of the potency of lymphoid cell inocula to induce GvH reactions. After inoculation of allogeneic lymphoid cells into neonatal mice, rats and chickens, a reproducible splenomegaly occurs (Simonsen, 1962). This splenomegaly shows a logarithmic linear relationship to the dosage of lymphocytes injected. Donor cell proliferation is an associative response in the development of a GvH reaction. This has been shown after injection of chromosomally marked (T6T6) CBA spleen cells into neonatal C57BL mice, which are different from CBA at the MHC (Nakić et al., 1967). Nisbet and Simonsen (1967) and Scollay et al. (1974) have found that splenomegaly can also be induced in the absence of donor cell proliferation. Splenic hypertrophy is not only due to a specific immunologic reactivity, since there is evidence that this increase in size can be largely attributed to a response of host lymphoid and/or hemopoietic cells (Piguet et al., 1977). Therefore, comparison of spleen indices of hosts belonging to different populations with respect to genotype and age of inoculation of lymphoid cells, are invalid. Furthermore, the accompanying runt disease may confuse the results (Elkins, 1971).

Popliteal lymph node assay

Levine (1968) observed that when rat parental strain lymphoid cells are injected into the feet of F1 hybrid hosts, the draining popliteal lymph nodes (PLN) enlarged. This assay has been adapted for use in mice by Hardt and Claësson (1971). One of the advantages of the PLN assay above the splenomegaly assay in quantifying the GvH reactivity of lymphoid cells is, that the lymph nodes can enlarge to an extent that is many times larger than can be obtained in the splenomegaly assay. This is even true in mice, where the PLN assay does not give such high ratios as in rats. One of the problems of this assay is that F1 hybrid cells injected into the footpad of parental mice also give PLN hypertrophy, due to a Host-versus-Graft (HvG) reaction (Twist and Barner, 1973). According to Grebe and Streilein (1976), PLN hypertrophy is highly dependent on host radiosensitive cells. Host radioresistant cells appear to be important as an allogeneic stimulus. Two cellular mechanisms play a role in the enlargment of the draining lymph nodes: proliferation *in situ* and trapping of recirculating cells. Piguet et al. (1977) and Rolstad (1976) have reported a close correlation between enlargment and proliferation, although Emeson and Thursh (1973)

have demonstrated that a large portion of GvH-induced lymphadenopathy is due to the trapping of circulating lymphocytes.

Phagocytic index assay
Howard (1961) reported that two weeks after injection of mouse parental lymphoid cells into F1 hybrids, these recipients were capable to clear intravenously injected colloidal carbon from the blood stream at an enhanced rate. This rate of phagocytosis is a measure in the phagocytic index assay. This technique provides useful quantitative data without the need to sacrifice the animal. However, there is no way to distinguish alterations in the mononuclear phagocyte system resulting from the GvH reaction itself, and those resulting from the complications of the GvH reacion, such as infection (Elkins, 1971).

Chorioallantoic membrane assay
Murphy (1916) placed spleen and bone marrow fragments onto the chorioallantoic membrane of an allogeneic chick embryo. The fragments developed into nodules, which later appeared to be individual foci of clonally expanded antigen reactive cells. According to Burnett and Boyer (1961) each "pock" represents the site where a single donor cell, competent to recognize the H antigens of the host, has lodged and reacted. This *in vivo* technique measures donor cell proliferation directly.

Inhibition of hemopoiesis
Blomgren and Andersson (1972, 1974) made use of the inhibiting effect of a developing GvH reaction on the generation of erythrocytes from syngeneic HSC in irradiated mice. They measured the erythroid cell growth by ^{59}Fe incorporation into the spleens of irradiated mice reconstituted with syngeneic bone marrow cells and the allogeneic inoculum.

The *in vitro* MLC reflects the afferent arm or initiation phase of the *in vivo* GvH reaction (Wilson, 1967; Wilson and Nowell, 1970, 1971). Coculture of lymphocytes from two allogeneic individuals (two-way MLC) or culture of one population of lymphocytes together with an allogeneic lymphocyte population which is incapable to proliferate (one-way MLC) will result in blast transformation and proliferation. This proliferative reactivity as measured by tritiated thymidine (^{3}H-TdR) incorporation is a thymus dependent phenomenon (Rodey et al., 1974). During such a MLC cytotoxic T cells are generated which can specifically lyse among other things lymphoblasts, derived from the original stimulator individual (Cerottini et al., 1970). The peak cytotoxic reactivity as measured in the CML assay occurs after the peak proliferative reactivity (Cerottini and Brunner, 1974). This CML

assay may reflect the efferent arm or the effector phase of the GvH reaction, namely, the allograft reaction causing the disease and ultimate death of the recipients. Cytotoxic lymphocytes, generated during an acute GvH reaction *in vivo* do not produce GvH reactivity in a splenomegaly assay (Rouse and Wagner, 1972), indicating that different lymphoid cell populations are responsible for afferent and efferent arms of the GvH reaction.

6.3. Cellular requirements

Small lymphocytes have emerged as the *sine qua non* of GvH reactivity (Gowans, 1962). Peripheral blood lymphocytes, thoracic duct lymphocytes, lymph node cells and spleen cells are potent initiators of GvH reactivity. Treatment of parental spleen or lymph node cells with anti-Thy-1 serum and complement prevents subsequent GvH activity in neonatal F1 hosts (Argyris, 1974; Cantor, 1972), suggesting that T cells are required for GvH reactivity. In rodents the hemopoietic tissues and the thymus are poor sources of cells which initiate GvH reactivity (Kerckhaert et al., 1973). They are, however, rich in the precursors of these GvH reactive T cells as has been demonstrated by Taylor (1963), Tyan (1968) and McGregor (1968). These authors were able to show that bone marrow cells, although incapable of mounting GvH reactions directly, are the source of precursor cells, which ultimately become GvH-inducing T cells after the imposition of thymic influence.

Thymectomy of rodents and birds in neonatal life abolishes the capacity of peripheral lymphocytes to induce GvH reactions and to proliferate *in vitro* to allogeneic cells (Cooper et al., 1966; Miller and Osoba, 1967; Wilson et al., 1967). These responses can be restored by thymic grafts, suspensions of thymus cells, and thymic extracts (Law and Agnew, 1968; Miller and Osoba, 1967; Trainin et al., 1969). Furthermore, Press et al. (1977) and Cohen and Patterson (1975) were able to show that *in vitro* pre T cells, which normally occur in the bone marrow, are readily inducible to maturity by a variety of other stimuli, including phytohemagglutinin.

Goedbloed and Vos (1965) studied the influence of thymectomy of the host before irradiation and allogeneic bone marrow transplantation on the incidence of secondary disease, and Van Putten (1964) studied this effect in a xenogeneic combination. In both these studies it was found that thymectomy of the recipients prior to the bone marrow transplantation could

delay the occurrence of mortality, but not the final cumulative incidence of it. However, it is difficult to distinguish between death caused by secondary disease and death caused by a wasting syndrome due to thymectomy. Chen et al. (1972) also found that in mice host thymectomy only influences the rate of development of secondary disease, but not the final cumulative mortality. The ability of mouse bone marrow to induce secondary disease in thymectomized, irradiated mice, can be attributed to the minor population of mature reactive T cells in the marrow. Sprent et al. (1975) and Norin and Emeson (1978) were able to eliminate the ability of mouse bone marrow cells to induce GvH mortality in lethally irradiated allogeneic hosts by pretreatment of the bone marrow inoculum *in vitro* with anti-Thy-1 serum and complement. Furthermore, treatment of donor mice with anti-lymphocyte serum (ALS) *in vivo* abolishes the GvH-inducing capacity of their bone marrow cells (Ledney and Van Bekkum, 1969). This suggests that the GvH reactive T cells in the bone marrow belong to the pool of potentially recirculating cells (Lance et al., 1973). The secondary disease induced by bone marrow cells from young and old donors differs in tempo as well as in severity of the reaction (Chen et al., 1972). This in contrast to the GvH inducing capacity of spleen cells from senescent mice. This is compatible with the incidences of T cells in spleen and bone marrow of young and old mice (Benner and Haaijman, 1980).

The role of proliferation of donor cells in the development of the GvH reaction can be tested more precisely by organ weight or lymphoid cell number measurements in lethally irradiated recipients. Such data rather good approximate the proliferative activity of donor lymphocytes. Strong et al. (1975) measured the donor proliferative activity in lethally irradiated mice after allogeneic and syngeneic spleen cell transplantation by ^{3}H-TdR incorporation. At 5 days after transplantation they found the same proliferative activity in both combinations, probably because of a prevailing hemopoiesis. Hilgard (1970) reported the absence of host splenomegaly in 500 rad X-irradiated mice transplanted with allogeneic spleen cells, despite the fact that GvH disease developed. However, although not always obligate, donor cell proliferation may be essential for the development of the full-blown GvH syndrome. Cheever et al. (1977) studied the effect of elimination of the proliferating, specifically activated T cells on the ability of donor spleen cells to induce fatal GvH reaction. This was done by ^{3}H-TdR suicide of cells proliferating upon stimulation by allogeneic host cells *in vitro*. They found that elimination of the specifically activated, proliferating T cells greatly

diminished the ability of the residual cells to induce a GvH reaction.

In MLC, the *in vitro* analogue of the GvH reaction, spleen cells from athymic nude mice can respond quite well when cultured with mitomycin C-treated or X-irradiated allogeneic cells (Wagner, 1972). F1 hybrid spleen cells can also respond well to similarly inactivated parental cells (Adler et al., 1970; Harrison and Paul, 1973). Closer examination of these phenomena has shown that T cells are necessary for the recognition of allogeneic stimuli and that these cells, although prevented from DNA synthesis, can exert a mitogenic effect on other cells to proliferate in MLC (Harrison and Paul, 1973; Von Boehmer, 1974). It is likely that these effects are mediated by lymphokines, which are produced by T cells either due to specific or to nonspecific stimulation (e.g., mitogen). Pick and Turk (1972) described that MIF, blastogenic factor (BF) and lymphotoxin (LT) can be produced during a MLC reaction. Furthermore, after interaction of allogeneic cells factors can be released that activate macrophages to nonspecific cytotoxic activity (Fung and Sabbadini, 1976). MIF release already occurs within 6 to 8 hr after antigenic stimulation (Rocklin, 1974). BF appears to selectively activate only a small proportion of lymphocytes to divide (Chan and Gordon, 1971). It has been suggested by Grebe and Streilein (1976) that many of the effects of GvH disease may be attributed to the activity of various lymphokines.

During MLC and acute GvH reactions also cytotoxic T lymphocytes are generated (Cerottini and Brunner, 1974; Cerottini et al., 1970). A clear role of these cytotoxic T lymphocytes in the efferent arm of the GvH reaction remains doubtful. Cytotoxic T lymphocytes generated *in vivo* and *in vitro* can accelerate graft rejection, but do not result in any measurable activity in the splenomegaly assay (Rouse and Wagner, 1972). *In vitro* elimination of cytotoxic lymphocytes does not alter the proliferative activity of the residual cells. Schnagl and Boyle (1979) were able to adsorb cytotoxic precursor cells on preincubated monolayers of allogeneic lymphoid cells. This treatment failed to reduce the proliferative response of the nonadherent population upon allogeneic stimulation *in vitro*. Rubin (1975) could not adsorb specifically GvH-reactive lymphocytes on allogeneic fibroblast monolayers, but could adsorb immune cytotoxic T lymphocytes.

6.4. Genetic requirements

Generally, the most severe GvH reactions occur when the donor and recipient mouse strain differ at the MHC (Hildemann et al., 1967). Since H-2 congenic and intra H-2 recombinant strains became available, it has become possible to pinpoint which regions within the H-2 complex are involved in the GvH reaction. Measurements of splenomegaly, lymph node enlargment and radioactive IUdR incorporation in spleen and lymph nodes following injection of histoincompatible immunocompetent cells all reveal similar results. H-2I region coded antigens appear to be very potent in eliciting GvH reactions, whereas H-2K and H-2D region coded antigens appear to be relatively weak (Livnat et al., 1973; Klein and Park, 1973; Elkins et al., 1973). Bach et al. (1972) and Oppltova and Démant (1973) also found good MLC responses in strain combinations differing at H-2I. H-2K and H-2D region incompatibilities elicit weaker MLC reactions then I region disparities, but they evoke stronger CML responses.

Clark and Hildemann (1977a) demonstrated that the main stimulus for the proliferative phase of the GvH reaction is in most strain combinations provided by an incompatibility in the I-A subregion. Klein (1978c) found in a GvH mortality assay that in some combinations that the mortality in case of an I-C subregion difference was comparable to that of an I-A subregion difference. In the I-C different combination B10.S(7R) - B10.S(9R) the reaction was so strong that almost all the (B10.S(7R) x B10.S(9R)) F1 hybrids injected with parental bone marrow cells died within 3 weeks after the inoculation. The strength of the GvH reactions induced across an I-C region barrier, varies as a function of the interallelic strain combination and is influenced particularly by properties of the recipient I-C determinants. I-C^s determinants on recipient cells lead to strong GvH reactions, while I-C^d determinants induce moderate GvH reactions, even when donors are used which carry different I-C alleles (Clark and Hildemann, 1977b). The predominant stimulator cells in murine MLC are non-T, radiation-resistant splenic adherent cells, which express Ia determinants encoded by genes in I-A and I-E/C subregions (Ahmann et al., 1979). Disparities at the I-B and I-J subregions have not been shown to be able to cause a detectable GvH reaction (Clark and Hildemann, 1977a).

Klein and Chiang (1976) studied the ability of H-2 subregions to induce GvH reactivity as measured in the mortality assay. They have found that K, I and D differences induce about the

same degree of mortality. Furthermore, Klein and Egorov (1973) observed significant GvH reactivity, as measured in the splenomegaly assay, using a combination differing only by a point mutation in the H-2K region.

The specificity and affinity of T blasts, generated in MLC, with respect to unrelated H-2 haplotypes has been studied by Nagy and Elliott (1979) using a plasma membrane vesicle-binding assay. Primary MLC blasts, when tested against limiting concentrations of stimulator and third party membrane vesicles, exhibited a preferential binding of stimulator vesicles. At high concentrations both types of vesicles were specifically bound to almost the same extent. So, receptor specificity within a cell population is manifested only as a higher average affinity for the stimulating haplotype. Receptors of MLC cells cultured for prolonged periods, however do seem to have higher average affinity and more restricted specifity than short term MLC cells.

As stated in Chapter 3.2., not only MHC region coded products give rise to strong proliferative responses in MLC, in the mouse also the products of the Mls locus can stimulate strong MLC responses. This locus is located on chromosome 1. Five codominant alleles termed Mls^a, Mls^b, Mls^c, Mls^d and Mls^e have been described so far, although it has not been formally established that these various stimulatory tissue antigens are all coded for by the Mls locus on chromosome 1. They have been defined by their capacity to stimulate in primary MLC between MHC identical strains of mice (Table 3). These determinants develop late in neonatal life and are predominantly detected on B lymphocytes (Ahmed et al., 1977). T lymphocyte responses to Mls^c locus antigens involve recognition of H-2I region gene products (Peck et al., 1977), while no restriction in responses to Mls^a and Mls^d locus antigens is detectable (Molnar-Kimber and Sprent, 1980). Injection of lymphocytes into Mls locus incompatible neonatal recipients does not give rise to splenomegaly (Huber et al., 1973), probably due to non-expression of Mls locus determinants on neonatal cells (Ahmed et al., 1977). Testing of MHC compatible, Mls locus incompatible lymphocytes in a PLN assay, shows that Mls locus coded antigens are capable of inducing GvH reactivity (Huber et al., 1973; Salaman et al., 1973). Parabiosis of MHC identical mice revealed an equal mortality in Mls locus compatible and in Mls locus incompatible combinations, indicating that differences at Mls loci are not implicated in mortality (Nisbet and Edwards, 1973).

TABLE 3

STRAIN DISTRIBUTION OF Mls DETERMINANTS

Allelic form	stimulatory capacity	Mouse strain
Mls^a	+ + +	DBA/2; AKR; DBA/1; NZB; BRVR/Dk; VmDk; SM/Skc/Dk
Mls^b	-	CBA/H; C57BL/10; C57BL/6; BALB/c
Mls^c	+ +	C3Hf; C3H/He; A
Mls^d	+ +	CBA/J
Mls^e	+ + +	C3H/Tif

It has not been possible to induce cytotoxic effector cells against Mls locus antigens (Peck and Bach, 1974). However, cytostatic effector cells against Mls locus determinants can be generated by immunization of mice with Mls incompatible lymphocytes. These cytostatic effector cells are detected by diminished metabolic activity of macrophage target cells after treatment with immune cells as compared to normal cells (Matossian-Rogers and Festenstein, 1977).

Minor H antigens other than Mls locus products cause minimal reactions in splenomegaly and mortality assays, unless the donors are presentitized (Elkins, 1971; Cantrell and Hildemann, 1972). Korngold and Sprent (1978) studied the effects of bone marrow transplantation across minor H antigens. Their data showed that, at least within certain strain combinations, untreated bone marrow cells did cause some incidence of lethal chronic GvH reaction. This only occurred in case of differences at three or more minor H loci. Mls determinants did not appear to be directly involved. GvH reactivity did not occur when the marrow was depleted of mature T cells with anti-Thy-1 serum and complement.

Studies of Fathman and Nabholz (1977) and Fathman et al. (1978) have demonstrated the expression of murine MLC stimulating determinants on F1 hybrid stimulator cells that are not present on cells from either parental strain. The expression of these hybrid histocompatibility determinants is controlled by at least two nonallelic loci in the H-2 complex. This loci work together in cis or trans position. One of them seems to map in the K or I-A region, and the other in the area between and including the I-B and S region. It is unknown whether these loci can also induce GvH reactivity.

6.5. T-T interactions

T cells from thymus and spleen can be sensitized to allogeneic fibroblasts *in vitro*. When such T cells are injected into the footpad of syngeneic hosts, they are capable to recruit the T cells with GvH potential for the priming alloantigen (Cohen and Livnat, 1976; Livnat and Cohen, 1976a, b). These sensitized cells are called initiator T lymphocytes or ITL. The spleen is several times richer in ITL than the thymus, while no ITL are detectable in the lymph nodes. ITL are relatively resistant to ATS treatment *in vivo* and are rapidly depleted following ATx. These characteristics are reminescent of T1 cells. ITL are found to adhere specifically to the original allogeneic fibroblast monolayer. The nonadhering lymphocytes cannot be sensitized against allogeneic fibroblasts (Cohen, 1973). Sensitization of ITL occurs within 4 to 6 hr, and requires protein but not DNA synthesis. Sensitized ITL injected into the footpad of syngeneic hosts move rapidly into the draining nodes.

The nature of the recruited lymphocytes is different from that of the ITL. They are present in lymph nodes and spleen, are non adherent to nylon wool, and are susceptible to ATS treatment *in vivo*. Belldegrün and Cohen (1979) demonstrated that sensitized syngeneic ITL can recruit precursors of specific GvH reactive lymphocytes from the spleen to the draining lymph nodes. This followed from the marked decrease of the specific GvH potential of the donor spleen cells as tested in the mortality assay.

Data obtained by Cantor and Asofsky (1970, 1972) suggest that two classes of T lymphocytes synergise in the induction of GvH reactions as measured with a splenomegaly assay. One class, T1 cells, is found mainly in the spleen and thymus and is resistant to the *in vivo* effects of ATS. The second class, T2 cells, is extremely susceptible to ATS *in vivo* and is found

mainly in peripheral blood and lymph nodes. Both cell populations must be allogeneic to the host if synergy is to be observed. Synergy between peripheral blood lymphocytes and thymocytes can also be demonstrated in the mortality assay (Tigelaar and Asofsky, 1972). When C57BL thymocytes (as a source of T1 cells) and BALB/c peripheral blood cells (as a source of T2 cells) are inoculated into neonatal F1 recipients, and 9 days later spleen cells from these recipients are inoculated into neonatal BALB/c and C57BL recipients, splenomegaly occurs in the BALB/c recipients, but not in the C57BL recipients. These double transfer experiments were interpreted by Cantor and Asofsky (1972) as to suggest that the cell in excess in the thymus determines the specificity of the immunologic injury leading to enlarged spleens. Thus, the thymus would contain precursors of GvH effector cells. The cells in the peripheral blood, on the other hand, would amplify the reactivity of these first cells. However, these conclusions are questionable, since Blomgren and Andersson (1972) and Sprent and Miller (1972) reported specific loss of GvH reactivity of mouse lymphocytes after passage through irradiated allogeneic hosts.

Synergistic responses between thymocytes and peripheral lymph node cells in murine MLC have been reported by Tittor et al. (1974) and Cohen and Howe (1973). These studies do not bear directly upon the question of which T cell subpopulation delivers the precusors of the proliferating cells and which one amplifies. Wright et al. (1979) demonstrated a differential effect of radiation on the response of mixtures of thymus and lymph node cells in rat MLC. Irradiated thymus cells retained the capacity for synergy in mixtures, whereas irradiated lymph node cells did not. These authors concluded that lymph node cells can proliferate upon stimulation by alloantigen, but are unable to amplify the response of the thymus cells. By contrast, thymus cells which by themselves can respond by proliferation to alloantigen, also provides an amplifier effect independent of their proliferative activity. The amplifier activity of thymus cells requires *de novo* protein synthesis. Thus, these experiments lead to the opposite conclusion of those of Cantor and Asofsky (1972), namely that T1 cells are amplifier cells and that T2 cells are the precursors of the effector cells. This view is supported by the experiments of Andersson et al. (1974), who found no significant effect of ATx upon the proliferative response of spleen cells to alloantigen as measured in an one way MLC, at least not during a period of 90 days after ATx.

7. *FUNCTIONAL T CELL DIFFERENTIATION IN AN ALLOGENEIC ENVIRONMENT*

7.1. Introduction

Between 20 and 30 days after allogeneic bone marrow transplantation into irradiated mice, the faeces usually become abnormal and the animals start to lose weight; also characteristic skin lesions may appear as early as the beginning of the second month. The peak of mortality from secondary disease falls in the second and third month. The animals which survive for more than 100 days, show a very low death rate after this period. At the end of the third month, the diarrhoea and the wasting gradually disappear and it seems that at least partial recovery takes place (Van Bekkum and De Vries, 1967).

The finite duration of the GvH reaction may be ascribed to the generation of suppressor T cells during the GvH reaction (Grebe and Streilein, 1976). Suppressor T cells have been shown to be sensitive to ATx (Okumura and Tada, 1971; Kerbel and Eidinger, 1972), low-dose of irradiation (Taniguchi and Tada, 1971), and drugs such as actinomycin-D and cyclophosphamide (Tada et al., 1971; Rollinghoff et al., 1977). They can be eliminated by ^{3}H-TdR suicide *in vitro* (Moorhead, 1978). These findings indicate that suppressor T cells are short-lived and largely proliferating cells. Shand (1977) reported that suppressor T cells, generated in (CBA x C57BL)F1 mice undergoing a GvH reaction, are Lyt-123^{+}, Ia^{+} T cells. Serum from F1 hybrid mice undergoing a GvH reaction, can suppress the proliferative response of normal syngeneic, allogeneic and parental spleen cells to an allogeneic stimulus (McMaster and Levy, 1975). Elie and Lapp (1977) reported that the suppressive activity, arising during a GvH reaction is due to a cell with monocyte-macrophage properties.

After recovery from the lesions induced by a GvH reaction, a stable radiation chimera is formed. Such chimeras are appropriate models to study the influence of T cell differentiation in an (semi-)allogeneic thymus upon the immune response.

7.2. Anti-host immune reactivity in stable radiation chimeras

Sprent et al. (1975) have shown that lymphocytes from stable semi-allogeneic radiation chimeras, prepared by injecting irradiated F1 hybrid mice with bone marrow cells from one parental strain, have a low, but significant, responsiveness to host type

H antigens in *in vitro* MLC, but do not react in CML. Against third party H antigens, the chimeric cells respond well, both in MLC and CML. From these results Sprent and coworkers concluded that semi-allogeneic radiation chimeras lack host-type MLC stimulating determinants, which seem to be expressed predominantly by cells of the lymphoreticular system. The chimeras are entirely repopulated by lymphoreticular cells of donor origin. Donor-derived lymphocytes will have the potential to respond to host type MLC determinants. Host-type CML determinants, by contrast, not being restricted to the lymphoreticular system, will exist in the chimeras and therefore be available to confront CML reactive cells. This continuous confrontation results in a clonal deletion of lymphocytes reactive to host CML determinants. In chimeras containing a mixture of donor-derived and host-derived (or host-type) lymphoreticular cells, the donor-derived lymphocytes lack the potential to mount an anti-host MLC response. This clonal deletion has been found only in *sub*lethally irradiated recipients (Sprent et al., 1975) and in tetraparental bone marrow chimeras (TBMC), produced by injecting bone marrow cells from both parents into lethally irradiated F1 hybrid mice (Von Boehmer et al., 1975). With regard to CML reactive T cells, it has been demonstrated that when chimeric lymphoid cells are maintained for 2 weeks in an environment essentially free of host CML determinants, the tolerance is not abrogated (Von Boehmer and Sprent, 1976). When chimeric bone marrow cells were transferred into lethally irradiated recipients lacking hosts CML determinants, and after 5 weeks the spleen cells of these secondary recipients were tested in a CML assay, a low but significant lysis of targets of original host origin could be detected (Von Boehmer and Sprent, 1976). These experiments, and the failure to detect suppressor cells or specific inhibitory components in serum from chimeras (Von Boehmer et al., 1975) argue for clonal deletion of anti-host CML reactive T cells in chimeras.

Phillips and Wegmann (1973) have reported that spleen cells from allophenic tetraparental mice are capable of preventing immunocompetent parental spleen cells from responding either to the opposite parent or to the tetraparental cells themselves in MLC. However, Meo et al. (1973) could not confirm these results. Studies of Hellström and Hellström (1973) on fully allogeneic radiation chimeras revealed cytotoxic activity of chimeric lymph node cells to fibroblasts carrying H-2 antigens of the host type. This cytotoxic activity could be blocked by serum of the chimeric mice. On the other hand, Grant et al. (1972) failed to detect anti-host cytotoxic cells

and humoral blocking factors in mouse radiation chimeras. Weiden et al. (1976) produced canine radiation chimeras by transplantation of bone marrow cells from a littermate donor matched at the MHC. Infusion of large numbers of donor blood lymphocytes could not disturb the stable chimeric state. However, infusion of lymphocytes from donors preimmunized against the minor H antigens of the host, caused pertubation of the stable chimeric state and development of a GvH reaction. The authors interpreted these results as being not consistant with the classical theories of tolerance, i.e., elimination or inactivation of potentially reactive cell clones. The presence of an active mechanism suppressing responsiveness against host antigens by the infused donor lymphocytes is suggested; this mechanism might be overcome by infusion of sensitized cells.

7.3. T cell function after differentiation in an allogeneic thymus

The immune response of long-term, allogeneic mouse radiation chimeras to particulate antigens (SRBC, *Salmonella typhosa* bacteria) is characterized by subnormal antibody titers and a slow and often incomplete conversion from 19 S to 7 S antibody synthesis (Gengozian et al., 1965, 1971; Gengozian and Congdon, 1973). Urso and Gengozian (1973) found that adherent cells in radiation chimeras behave as normal cells and that functional B cells are present in normal numbers in the spleen. In contrast, the T cell population in allogeneic chimeras, although quantitatively normal, is defective in their response to SRBC. This might be due to a defective cooperation of chimeric T and B cells (Gengozian and Urso, 1976). Onoë et al. (1980) reported that in fully allogeneic bone marrow chimeras neither direct nor indirect plaque-forming cell responses were observed upon primary stimulation to SRBC. Nonetheless, vigorous responses to this T dependent antigen were observed for both IgM and IgG during the secondary response.

Zinkernagel et al. (1978b) reported that virus-infected A parental mice which had been irradiated with a dose of 900-950 rads and reconstituted with (A x B)F1 hybrid bone marrow cells generate virus-specific cytotoxic T cells which are only reactive to infected A targets. Therefore, the H-2 type of the host determines the H-2 restricted activity of cytotoxic T cells.

The restricted specificity for host type H-2 determinants has also been shown using $H\text{-}2^{bm1}$ mutant mice, which carry a point mutation in the H-2K region. $(H\text{-}2^{bm1} \times H\text{-}2^{b})F1 \rightarrow H\text{-}2^{b}$ chimeras lyse infected K^{b} target cells. However, $(H\text{-}2^{bm1} \times H\text{-}2^{b})F1 \rightarrow H\text{-}2^{bm1}$ chimeras generate no virus specific cytotoxic activity for infected K^{b} targets (Zinkernagel et al., 1978c). From the fact that the mutation has caused only minor changes in the tertiary structure of K^{b}, it can be concluded that the receptor for self is exquisitely specific. Adult thymectomized, irradiated and syngeneic bone marrow reconstituted (A x B)F1 mice, which received a sc transplanted irradiated thymus of A or B origin, generate virus-specific cytotoxic T cells reactive to virus-infected A and B targets, respectively (Zinkernagel et al., 1978b). This result demonstrates the crucial role of thymic epithelial cells in the expression of anti-self H-2 specificities by T cells. The radioresistant portion of the thymus determines these restriction specificities of differentiating T cells during the period of maturation in the thymus.

Lymphocytes from A→(A x B)F1 chimeric mice do produce virus specific cytotoxic T cell activity for infected A but not for infected B cells. However, when those chimeric lymphocytes are sensitized in an acutely irradiated and infected (A x B)F1 recipient, they respond to both infected A and infected B targets (Zinkernagel et al., 1978d). In long-term radiation chimeras the lymphoreticular system has been replaced by cells derived from the reconstituting stem cells. In the acutely irradiated and infected recipient mice, the original lymphoreticular system is still intact at the moment of sensitization. Thus, the thymus determines the spectrum of receptor specificities of differentiating T cells for self H-2, whereas the phenotypic expression of T cell specificity for self + virus is determined predominantly by the H-2 type of the antigen presenting cells of the lymphoreticular system (Zinkernagel et al., 1978d). Therefore, completely H-2 incompatible A→B chimeras fail to generate any measurable virus-specific cytotoxicity (Zinkernagel et al., 1978b).

Similar results were found for genetic restriction of T cells involved in antibody formation. Katz et al. (1978) have reported that T cells from lethally irradiated F1 hybrid mice, reconstituted with bone marrow cells from one parental strain are unable to help antibody formation by B cells from the opposite parental strain. Sprent and Von Boehmer (1979) *sub*lethally irradiated F1 hybrid mice and reconstituted these mice with bone marrow from one parental strain. These chimeras contain two discrete subgroups of H-2 restricted T cells, each capable

to collaborate with B cells from either parental strain. This might be due to the heterozygous cells of the lymphoreticular system which are still present in the *sub*lethally irradiated chimeras. Recently, Singer et al. (1979) proved that for antigen priming, T cells strictly require recognition of MHC determinants on macrophages, but that a similar recognition of MHC determinants on B cells was not a requirement for effective T-B cooperation. Thus, splenic T cells from A→(A x B)F1 chimeric mice collaborate equally well with macrophages from either parental strain. However, (A x B)F1→A splenic T cells collaborate with macrophages from parent A, but not from parent B. In the presence of macrophages from parent A, (A x B)F1→A chimeric helper T cells collaborate *in vitro* equally well with B cells from either parental strain.

Since MHC alloreactivity may not depend upon self recognition but only upon recognition of the alloantigen, T cells from fully H-2 incompatible chimeras can express alloreactivity (MLC and CML) or other similar reactivities, such as PHA stimulation, that do not rely upon self-recognition (Dauphinee and Nordin, 1974). In contrast, these spleen cells are unable to generate a measurable antibody response against the same alloantigens, because in A→B chimeras, the precursor T cells learn to recognize B as self H-2, but since the T cells and the rest of the lymphoreticular system are built up of cells expressing exclusively A, no associative antigen recognition can occur (Zinkernagel et al., 1978d). The same is reported for nude mice, transplanted with an allogeneic neonatal thymus. These mice showed little improvement of the antibody production against T cell dependent antigens such as SRBC. However, these animals' alloreactivity against unrelated skin grafts is surprisingly well developed (Kindred, 1976; Kindred and Loor, 1974). Aisenberg (1970) studied the restoration of immune function in irradiated ATx mice after transplantation of neonatal allogeneic thymuses. Although the thymuses were rejected, a return of the capacity to reject MHC incompatible skin grafts was observed. In contrast, no restoration was found of the capacity to produce hemolytic plaque-forming cells. Thus T cells developing in a fully allogeneic thymic environment may be deficient in MHC restricted immune responses.

In contrast to the data reviewed above, Zinkernagel et al. (1980) showed that restriction specificity for the non-host H-2 of the thymus could not be demonstrated in nude mice after transplantation of an allogeneic fetal thymus. T cells developing in such nude mice were restricted exclusively for the nude H-2. Further experiments that attack the proposed unequivocal role

of the host thymus in determining MHC restriction specificity were reported by Blanden and Andrew (1979). They reported that primary anti-viral cytotoxic responses in (AxB)F1→A chimeras are not absolutely restricted to the strain A H-2 haplotype. Also antigen together with H-2 antigens of strain B are recognized, although those cytotoxic T cells are at least 10-fold less numerous than cytotoxic T cells restricted for the H-2 haplotype of strain A. The difference between these results and those of Zinkernagel (1978b) using similar (AxB)F1→A chimeras might be due to the different assay system used: Blanden and Andrew (1979) used macrophage targets in a 16 hr cytotoxicity assay, whereas Zinkernagel (1978b) used fibroblast targets in a 6 hr cytotoxicity assay. Macrophages are considerably more sensitive targets than fibroblasts (Blanden et al., 1977). Another argument for the possible occurrence of not absolutely H-2 restricted anti-viral cytotoxic responses comes from the work of Doherty and Bennink (1979). These authors filtered lymph node cells through irradiated allogeneic mice in order to deplete the specific alloreactive potential. These negatively selected T cells were then stimulated with vaccinia virus in mice expressing the foreign H-2 determinants encountered previously in the "filter-mouse" environment. Strong virus-immune cytotoxic T cell responses were seen in the context of the H-2 of the "filter mouse". Furthermore, Forman and Streilein (1979) have shown that in neonatally tolerized mice at least two sets of T cells co-exist, one capable of recognizing non-H-2 alloantigens in the context of the host H-2 haplotype, and the other able to recognize antigens in the context of the H-2 haplotype of the neonatally transferred allogeneic cells. They postulated that alloreactive T cells bear receptors with a spectrum of avidities for H-2 antigens. In the tolerance protocol, high-avidity T cells are deleted so that only T cells with low avidity receptors remain. These putative low avidity receptor clones might very well bind a complex of that particular alloantigen and another non-H-2 alloantigen with high avidity. The same might hold for complexes of H-2 and virus.

Taken together, these results suggest that MHC restriction specificity, as determined by host (thymic) epithelium, is not an absolute phenomenon. Furthermore, host thymic tissue may not absolutely be necessary in determining MHC restriction specificity though it remains possible that it is the most efficient and common pathway.

8. *INTRODUCTION AND DISCUSSION OF THE EXPERIMENTAL WORK*

As outlined in chapter 6, GvH disease can occur when a graft containing immunocompetent cells is introduced into immunologically incompetent allogeneic recipients. Usually the severity of a GvH reaction is measured at the syndrome level: body weight change, incidence of disease, mortality rate (Elkins, 1971), splenomegaly (Simonsen, 1962), and lymph node hypertrophy (Ford et al., 1970). However, these symptoms are not specific for this type of immune reaction. Furthermore, the increase in size of the lymphoid organs during a GvH reaction is only indirectly related to the activation of immunocompetent cells by host alloantigens, since there is evidence that this increase can be mediated by host lymphoid and/or hemopoietic cells (Piguet et al., 1977). Some of the symptoms are also greatly influenced by infections for which animals suffering from GvH disease are very susceptible. Therefore, measurement of the *specific immune reaction* underlying GvH disease should give a better indication of the onset of the disease. However, data about the development of specific anti-host immune reactivity are hardly available. Only in a few studies the responding cells were assayed functionally, e.g., in a cytotoxicity assay (Cerottini et al., 1971; Sprent and Miller, 1972; Clark and Nedrud, 1974). In view of this lack of data on the specific anti-host immune response, we developed a DTH assay which reveals another type of anti-host immune reactivity. By using this assay it is possible to study *in vivo* the proliferative activity of *specific* anti-host directed donor T cells. Furthermore, this assay enables studies on the *H antigens* and *T cell subpopulations* which are required for this anti-host immune response.

In Appendix paper I it is shown that during an acute GvH reaction induced by injection of parental spleen cells into lethally irradiated F1 hybrid mice, a state of DTH against host H antigens occurs in spleen and lymph nodes. This was demonstrated by means of transfer of host lymphoid cells into normal secondary recipients, syngeneic to the original spleen cell donor. These secondary recipients received a challenge in the right hind foot with spleen cells, syngeneic to the irradiated primary recipients. The challenge can evoke a DTH response which is maximal 24 hr after the challenge injection. This response is measured as a relative specific increase in foot thickness. A scheme of the experimental system used to demonstrate GvH-related DTH reactivity is shown in Figure 4.

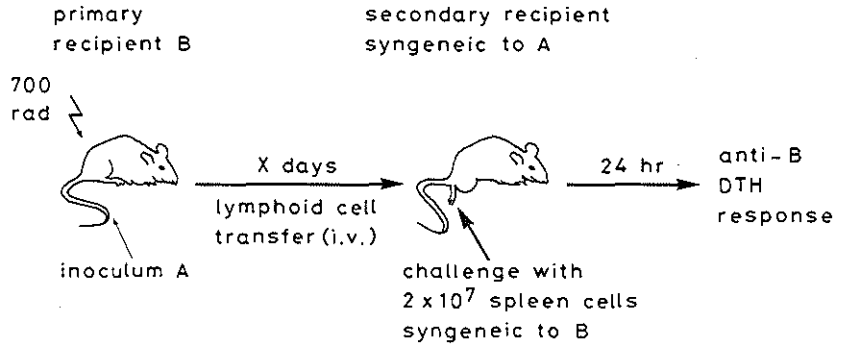

Figure 4
Scheme of the experimental set up used to demonstrate GvH-related DTH reactivity.

The GvH-related DTH reactivity was shown to be antigen specific, and both initiation and transfer of the DTH reactivity were highly dependent on Thy-1$^+$ cells. The DTH reactivity could be demonstrated during the first week after irradiation and semi-allogeneic spleen cell transplantation, with a peak reactivity on the 4th and 5th day for spleen and lymph nodes, respectively. This is somewhat earlier than the appearance of the characteristic GvH symptoms and long before the occurrence of death. This latter is apparent from the observation that in the strain combination used 50% mortality occurs on day 15-16 after irradiation and reconstitution. These results suggest that the development of DTH reactivity during the early phase of a GvH reaction is a temporary phenomenon associated with the initiation of the immune response leading to the characteristic symptoms of GvH disease, and ultimately to death.

GvH-related DTH reactivity appears in the spleen of irradiated and allogeneically reconstituted hosts between 8 - 24 hr after reconstitution. This early onset may indicate that proliferation of the transplanted allogeneic T cells is not a prerequisite for the appearance of this anti-host DTH reactivity. This is consistent with the study of Phillips et al. (1972) on the generation of MIF during a MLC. In their studies MIF production could be demonstrated within 24 hr, and sc injection of the MLC supernatant, containing MIF, could evoke a typical DTH response *in vivo*. Nisbet and Simonsen (1967) and Scollay et al. (1974) found that GvH-induced splenomegaly can occur in the absence of donor cell proliferation. Apparently, upon stimulation by alloantigens, nondividing donor cells may release factors that induce host cells to proliferate.

In Appendix paper II it was investigated to what extent proliferation of specifically reacting donor-derived T lymphocytes occurs during, and is required for, GvH reactivity. Blocking of DNA synthesis of the parental spleen cells by incubation with 25 µg mitomycin C per ml before inoculation into irradiated F1 hybrid recipients did not prevent the anti-host DTH responsiveness in the spleen during the first 2 days, and in the lymph nodes at 3 days after reconstitution. Thereafter, the DTH responsiveness was greatly decreased by the mitomycin C treatment. Blocking of both DNA and RNA and a great part of protein synthesis by pretreatment with 100 µg mitomycin C per ml could completely prevent the development of anti-host DTH responsiveness. This result suggests that RNA and protein synthesis are required for the appearance of the early, proliferation independent, anti-host DTH reactivity in the spleen and lymph nodes. However, full development of specific anti-host DTH responsiveness during an acute GvH reaction does depend on proliferation of the reactive T cells. In both spleen and lymph nodes, elimination of the DNA synthesizing cells at the moment of peak DTH reactivity by ^{3}H-TdR suicide *in vitro* reduced the anti-host DTH response to about 50% of the normal value. This decrease was larger before reaching peak DTH reactivity, whereas thereafter ^{3}H-TdR could no longer affect the height of the anti-host DTH response.

Using the GvH-related DTH assay characterised in the appendix papers I and II, we have analysed the cellular and genetic requirements for this type of GvH reactivity. As described in Chapter 3 there is a great diversity of H antigens in the mouse. Several of these antigens induce different types of immune responses. Antigens coded for by the H-2K, H-2D and H-2I subregion of the MHC of the mouse initiate different *in vitro* immune responses. H-2I region coded antigens activate mainly Lyt-1^{+} T cells to a proliferative response in MLC, which can be seen as an *in vitro* analogue of the GvH reaction. H-2K and H-2D region coded antigens, on the other hand, predominantly stimulate Lyt-23^{+} T cells to become cytolytic effector cells (Bach et al., 1976). In MLC across H-2I+K differences Lyt-1^{+} T cells bind stimulator I region determinants, whereas Lyt-23^{+} T cells bind stimulator K region determinants (Nagy et al., 1976). Prud'homme et al. (1979) have found in an *in vivo* GvH system that Lyt-1^{+} GvH reactive donor T cells can adsorb host cell products encoded by the I-A subregion. Furthermore, daily injection of the irradiated recipients with an antiserum against host Ia antigens causes a marked suppression of the GvH reaction. H-2I region coded antigens also appear to be very potent in eliciting GvH reactions as measured with the spleno-

megaly assay, whereas K and D region coded antigens appear to be relatively weak (Livnat et al., 1973). However, H-2I as well as H-2K and H-2D region coded antigens can account for mortality in GvH reactions (Klein and Chiang, 1976). In skin graft rejection studies also similar responses were found to H-2K, H-2I and H-2D antigens (Klein, 1975), suggesting, but by no means proving, an equal immunogenicity of these antigens.

Proliferative responses in MLC and generation of cytolytic T cells *in vitro* can also be induced by non-H-2 alloantigens. Mls locus products can induce positive MLC responses (Festenstein, 1973), whereas other non-H-2 antigens can give rise to cytotoxic T cell responses *in vitro*, following *in vivo* priming (Simpson and Gordon, 1977). Differences in only minor H antigens are sufficient to cause lethal GvH reactions after allogeneic bone marrow transplantation (Korngold and Sprent, 1978). When MHC compatible, Mls locus incompatible lymphocytes were tested in a PLN assay, GvH reactivity was found (Huber et al., 1973; Salaman et al., 1973). Nisbet and Edwards (1973) parabiosed parental mice to both Mls locus incompatible and Mls locus compatible F1 backcross mice. These authors found an equal mortality in both groups, indicating that a difference at the Mls locus is not implicated in mortality.

In Appendix paper III it was investigated whether this discrepancy between the various H-2 subregion coded antigens and between Mls locus coded antigens and other non-H-2 alloantigens also exists at the level of GvH-related DTH reactivity. In lethally irradiated mice, reconstituted with congenic spleen cells, which were different at the whole MHC or only a part of it, it could be demonstrated that the transferable anti-host DTH reactivitiy is directed exclusively to the I region of the H-2 complex. The H-2K and H-2D antigens did not induce anti-host DTH T effector cells. H-2 compatible, non-H-2 incompatible spleen cell transplantation into lethally irradiated hosts revealed that antigens encoded by the Mls locus can also elicit the development of a transferable anti-host directed DTH reactivity. Non-H-2 alloantigens other than Mls locus products did not contribute to the expression of the anti-host DTH reactivity. Apparently, Mls locus products and H-2I region coded antigens have a similar capacity to induce anti-host directed DTH T effector cells. H-2K and H-2D antigens and non-H-2 alloantigens other than Mls locus coded products are much less able to do so. This correlates well with the inability of K and D products and non-H-2 alloantigens other than Mls locus coded products to stimulate efficiently T cell proliferation *in vitro* (Schendel and Bach, 1975). In contrast, in HvG reactions induced by sc

injection of allogeneic spleen cells, H-2K, H-2I and H-2D antigens as well as non-H-2 alloantigens can give rise to anti-graft DTH T effector cells.

In the appendix papers IV and V this differential responsiveness to different H-2 and non-H-2 coded antigens in GvH and HvG reactions was studied more thoroughly. The finding that H-2K and H-2D antigens did not give rise to any measurable anti-host DTH responsiveness, might be ascribed to a relatively small pool of anti-H-2K/D reactive T cells. However, in Appendix paper IV we have shown that after enlargement of the pool of potentially reactive T cells by preimmunization, also no measurable anti-host DTH response occurs. In contrast, under HvG conditions preimmunization of responder mice against H-2K/D antigens does result in a clear secondary type DTH response. This suggests that under GvH conditions not a quantitative but a qualitative incapability exists to induce anti-host DTH reactivity against H-2K/D antigens. Furthermore, under GvH conditions may be only Lyt-1^+ DTH T effector cells are activated (anti-H-2I response), whereas under HvG conditons two subsets of T cells mediating DTH might be activated, namely, anti-H-2i Lyt-1^+ T cells and anti-H-2K/D Lyt-2^+ T cells. Evidence for this supposition concerning the participation of different T cell subsets in HvG-DTH reactivity comes from work of Smith and Miller (1979b).

In H-2K and H-2D mutant strains (Wettstein et al., 1979), and in recombinant strains differing at H-2K or H-2D (Bach and Alter, 1978), it has been shown that the only cells which can proliferate in a murine MLC across a disparity at the H-2K or H-2D region are Lyt-123^+ T cells. These Lyt-123^+ T cells probably belong to the T2 cell subpopulation (Swain et al., 1979). Possibly, under GvH conditions the excess of H-2K/D antigens drives Lyt-123^+ cells to become cytotoxic active cells, whereas under the limited antigenic pressure of the HvG condition these Lyt-123^+ cells become DTH reactive cells. This view would be in harmony with the observation of Swain and Panfili (1979) that mitomycin C treated Lyt-123^+ T cells can release humoral factors with helper activity in humoral immune response, whereas unblocked Lyt-123^+ T cells generate cytotoxic T cells upon *in vitro* stimulation with H-2K/D antigens.

In Appendix paper V it is shown that Mls^a and Mls^c coded antigens initiated both a postive MLC response and a distinct GvH-related DTH reactivity. Mls^b locus antigens were not able to initiate *in vitro* proliferation, which was associated with a marginal and short-lasting GvH-related DTH reactivity. The role

of Mls locus products in the development of HvG-directed T effector cells is completely different from that in the GvH situation. In the HvG the role of Mls locus differences is of minor importance. Other non-H-2 differences are probably responsible for most of the DTH reactivity after sc immunization with H-2 identical spleen cells. These non-H-2 alloantigens other than Mls locus products then evoke a primary proliferative response as measured in the regional lymph nodes.

The appendix papers III, IV and V document the dominant role of the I region of the H-2 complex and Mls locus coded antigens in GvH-related DTH reactivity. Thereafter we investigated whether different T cell subpopulations can contribute to the development of anti-host DTH reactivity. A synergistic effect of short-lived, sessile, ATx sensitive T1 cells and long-lived, recirculating, ATS sensitive T2 cells has been demonstrated for the splenomegaly GvH assay (Cantor and Asofsky, 1970, 1972) and the *in vitro* MLC (Tittor et al., 1974; Wright et al., 1979).

In Appendix paper VI the role of T1 and T2 cells in the development of specific anti-host DTH reactivity was studied in lethally irradiated hosts. It appeared that elimination of T2 cells by *in vivo* treatment of spleen cell donor mice with ATS completely prevents the development of anti-host immune reactivity. T1 cells, although by themselves incapable of displaying anti-host DTH reactivity, were shown to amplify the response of T2 cells. The T1 cell-mediated amplification is most apparent when only small numbers of T2 cells are present in the transplant. It was found that more differentiated T cells than those residing in the thymus are needed for optimal amplifier activity. These amplifier cells probably belong to the population of post-thymic Lyt-123$^+$ T cells in the spleen, which are ATx sensitive (T1 cells) (Feldman et al., 1977; Stutman, 1977).

In appendix paper III it was shown that generation of GvH-related DTH effector cells can only be initiated by H-2I region coded antigens. In appendix paper VI it is shown that H-2I region differences do not induce amplifier activity in T1 cells. On the other hand, a H-2K region difference, next to a H-2I region difference, does induce amplifier activity in T1 cells, indicating that T1 amplifier cells are activated by H-2K antigens. The fact that the collaborating T1 and T2 cells are activated by different H-2 subregion coded antigens implies, that they must belong to different T cell differentiation pathways. Observations of Scollay et al. (1978) and Mathieson et al. (1979) suggest that there are two separate lines of intra-

thymic differentiation, one leading to Lyt-1$^+$ T cells and the other to Lyt-123$^+$ T cells. The latter cells might further differentiate into Lyt-23$^+$ T cells after their migration as Lyt-123$^+$ cells to the periphery (Cantor and Boyse, 1976). A part of this Lyt-123$^+$ population is ATx sensitive, and thus fulfills the criteria of T1 cells (Cantor and Boyse, 1977). We suggest that the same subpopulation of postthymic Lyt-123$^+$ T cells amplify the generation of anti-host DTH T effector cells from (probably Lyt-1$^+$) T2 cells. These T2-derived DTH effector cells can release humoral factors which lead to the expression of GvH-related DTH upon transfer, to the differentiation of Lyt-123$^+$ T cells into MHC restricted and non-restricted CTL (Wagner et al., 1979), and to stimulation of differentiation of Lyt-23$^+$ allo-MHC reactive precursor CTL into active CTL (Bach et al., 1976). A model for the generation and activities of GvH-related DTH reactive cells is shown in Figure 5.

In the previous appendix papers the cellular and genetic requirements for the development of anti-host immune reactivity have been investigated in an acute GvH model. This model is based upon transplantation of allogeneic spleen cells into lethally irradiated hosts. However, the final purpose is to get more insight in the processes which lead to the development of delayed GvH disease and to the ultimate recovery from this disease. Therefore, we tested whether the GvH-related DTH assay could also be used for following the anti-host immune response after allogeneic bone marrow transplantation into lethally irradiated recipients. In Appendix paper VII it is shown that in that case also a DTH reactivity against host H antigens occurs. This anti-host DTH reactivity is maximal around 12 days after bone marrow transplantation and could no longer be demonstrated by day 20. Elimination of recirculating T2 cells from the allogeneic bone marrow donor mice by *in vivo* treatment with ATS, almost completely prevents the development of anti-host immune reactivity. These results show that recirculating T2 cells in the bone marrow inoculum play a major role in the development of anti-host DTH.

Comparison of the anti-host immune response by allogeneic bone marrow transplantation into lethally irradiated sham-thymectomized and ATx mice, revealed that T cells formed under influence of the host thymus affect the height and the onset of the anti-host immune response. This is in harmony with the T1-T2 synergism described in appendix paper VI. These newly formed T cells, which might be equivalent to T1 cells, enhance the anti-host immune response, which was elicited by mature recirculating T cells residing in the bone marrow inoculum. These newly-formed

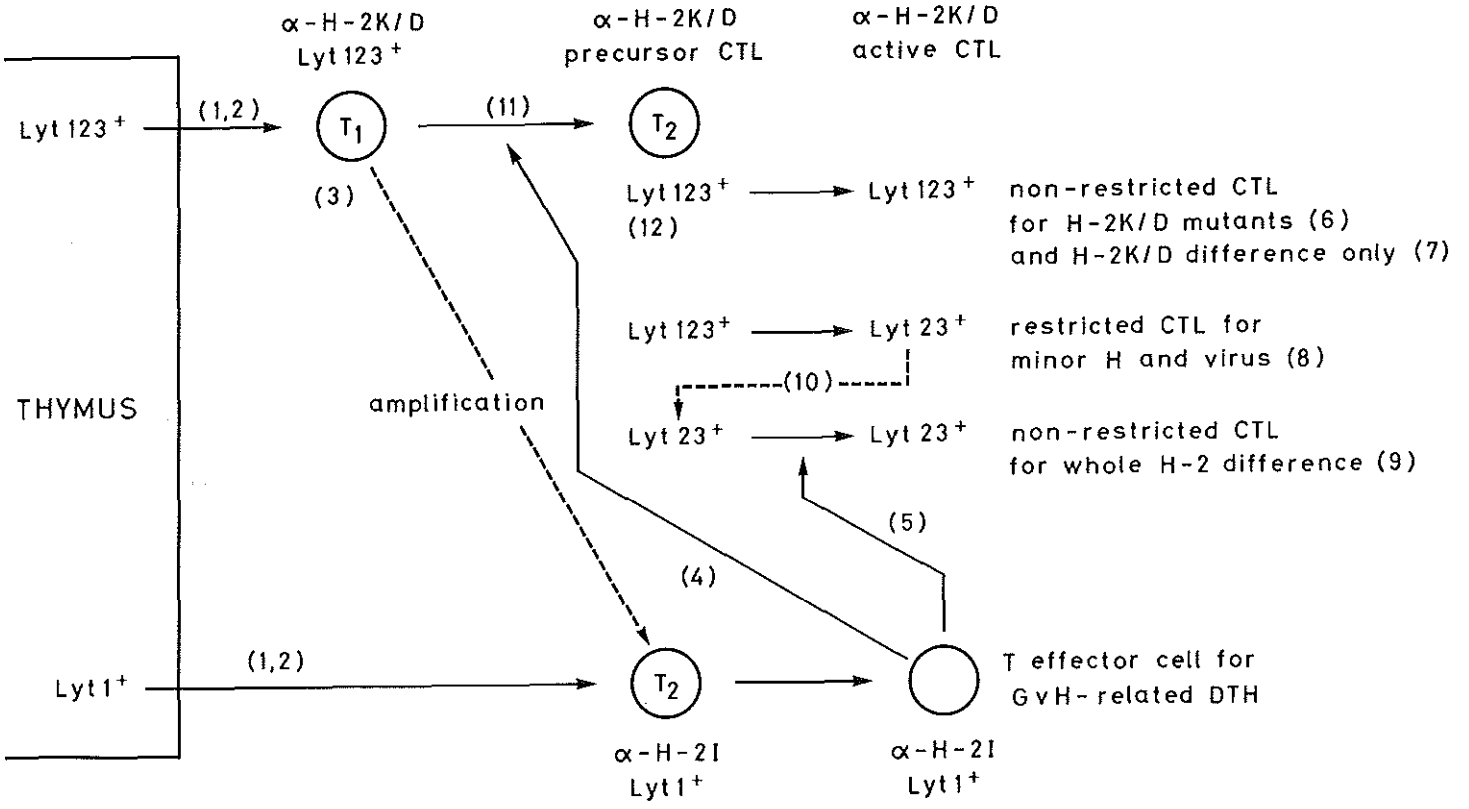

Figure 5
Model for the differentiation pathway and activities of GvH-related DTH reactive cells.

(1) Scollay et al., 1978; (2) Mathieson et al., 1979; (3) Cantor and Boyse, 1977; (4) Wagner et al., 1979; (5) Bach et al., 1976; (6) Wettstein et al., 1979; (7) Bach and Alter, 1978; (8) Cantor and Boyse, 1976; (9) Cantor and Boyse, 1975; (10) Burakoff et al., 1978; (11) T2 precursor CTL arise from either the T1 "amplifier" cell population or from a separate T1 cell population; (12) Probably H-2K/D reactive DTH cell as described in Appendix paper IV.

T cells by themselves are incapable of eliciting anti-host DTH reactivity. Thus, when during a delayed GvH reaction the mature T2 cells become suppressed or clonally depleted, the anti-host DTH reactivity will gradually wane.

Further experiments have to be done in order to elucidate which host H antigens trigger these newly-formed T cells to exert their regulatory function. It has also to be investigated whether these T cells are formed within the host thymus or under hormonal influence of the host thymus, and why they are not immediately tolerized against host (thymic) H antigens.

9. *SUMMARY*

T cells differ fundamentally from B cells in that they have to recognize not only the foreign antigen in order to become activated, but also one of their own histocompatibility (H) antigens which belong to the major histocompatibility complex (MHC); in mice this MHC is called: H-2. Examples of this self MHC (H-2) restriction are found in cell-mediated immune responses against viruses, bacteria and minor H antigens. The MHC codes for H antigens, which can induce by themselves an extremely strong, unrestricted, cell-mediated immune response. A strong host-versus-graft (HvG) reaction developes after organ and tissue transplantation between MHC unrelated individuals of the same species. After transplantation of immunocompetent lymphoid cells into a MHC different recipient which is incapable of rejecting them, the transplanted cells can recognize these H antigens of the host and react immunologically against them. A severe graft-versus-host (GvH) reaction will then occur.

During an acute GvH reaction in lethally irradiated mice a state of delayed type hypersensitivity (DTH) against host H antigens can occur. This can be demonstrated by means of transfer of host spleen and lymph node cells into normal secondary recipients, syngeneic with the original spleen cell donor. These secondary recipients are challenged in one of the hind feet with spleen cells syngeneic to the irradiated host. The increase in foot thickness, measured 24 hr later, is a measure for the height of the anti-host immune response. Initiation and transfer of GvH-related DTH is highly dependent on Thy-1^+ cells. The development of the anti-host immune reactivity starts between 8 and 24 hr after allogeneic spleen cell transplantation and increases during the days thereafter. In the spleen maximal anti-host immune responses are found by day 4 after irradiation and reconstitution, whereas in the lymph nodes maximal responses are found by day 5. Thereafter, the reactivity decreases steadily (Appendix paper I).

Blocking of the proliferative activity of the allogeneic spleen cells before inoculation into the irradiated hosts, does not prevent the development of anti-host DTH responsiveness in the spleen during the first 2 days and in the lymph nodes at 3 days after reconstitution. Thereafter, the anti-host DTH response is greatly decreased. Elimination *in vitro* of DNA synthesizing cells from the spleens of irradiated and allogeneically reconstituted hosts before secondary transfer, reveals that full development of the specific anti-host DTH response during an

acute GvH reaction is dependent on proliferation of the reactive T cells (Appendix paper II).

In lethally irradiated mice reconstituted with allogeneic spleen cells which are different at the whole MHC or a part of the MHC, it is demonstrated that the transferable anti-host DTH reactivity is directed exclusively to the I region of the H-2 complex. H-2K and H-2D subregion coded antigens do not induce anti-host DTH T effector cells. H-2 compatible, non-H-2 incompatible spleen cell transplantation into lethally irradiated hosts reveals that antigens encoded by the Mls locus can also elicit a transferable anti-host directed DTH reactivity. Non-H-2 alloantigens other than Mls locus products do not clearly contribute to the expression of the anti-host DTH response (Appendix paper III).

Comparison of GvH and HvG reactions induced by either a H-2K, H-2I or H-2D difference revealed that in HvG reactions antigens encoded for by each of these regions can give rise to anti-graft DTH T effector cells. Preimmunization of spleen cell donor mice against H-2K or H-2D antigens does not influence the DTH responsiveness to these antigens under GvH conditions. However, the capacity to respond to H-2K and H-2D antigens in a HvG-DTH reaction is markedly enhanced by preimmunization of the responder mice. These results suggest that under GvH conditions only one subset of precursor T cells is activated (presumably anti-H-2I Lyt-1^+ T cells), whereas under HvG conditions two T cell subsets can give rise to DTH T effector cells (presumably anti-H-2I Lyt-1^+ T cells and anti-H-2K/D Lyt-2^+ T cells) (Appendix paper IV).

The capacity of Mls locus antigens to elicit distinct anti-host DTH reactivity correlates with the capacity to induce proliferation in mixed lymphocyte cultures (MLC). Mls^a and Mls^c locus coded antigens elicit both a positive MLC response and distinct GvH-related DTH reactivity. On the other hand, the Mls^b locus antigen is not able to initiate *in vitro* proliferation, which coincides with a marginal and short-lasting GvH-related DTH reactivity. These Mls locus coded antigens seem to play a minor role in HvG reactions. Other non-H-2 alloantigens are responsible for most of the DTH reactivity after sc immunization with H-2 identical, non-H-2 different spleen cells (Appendix paper V).

In donor-recipient combinations differing at the MHC or part of the MHC it can be demonstrated that the anti-host directed T effector cells are the progeny of long-lived, recirculating

T2 cells, activated by antigens encoded by the I region of the H-2 complex. This response can be amplified by short-lived, sessile, post-thymic T1 cells. The amplifier activity can only be detected in case of a H-2K difference next to the H-2I difference, indicating that T1 amplifier cells are activated by H-2K antigens. T1 cells themselves are incapable of displaying anti-host DTH reactivity (Appendix paper VI).

During initiation of a delayed GvH reaction by injection of allogeneic bone marrow cells into lethally irradiated recipients, also a state of DTH against host H antigens occurs. This DTH is maximal around 12 days after transplantation and can no longer be demonstrated by day 20. Elimination of the recirculating T2 cells by ATS treatment of the bone marrow donor mice, almost completely prevents the development of the anti-host DTH reactivity. When the irradiated recipients had previously been thymectomized, the anti-host DTH reactivity was delayed and weaker as compared with non-thymectomized recipients. This result suggests that T cells newly formed under influence of the host thymus affect the height and onset of the anti-host DTH response (Appendix paper VII).

Our studies suggest that when low numbers of mature T2 cells are present in the allogeneic transplant, attention must be paid to the possibility of amplification of the anti-host immune responsiveness by newly-formed T cells.

10. *SAMENVATTING*

T cellen verschillen fundamenteel van B cellen door het feit dat ze niet alleen het lichaamsvreemde antigeen moeten herkennen om geaktiveerd te worden, maar ook nog een van hun eigen transplantatie antigenen die behoren tot het zgn. major histocompatibiliteits complex (MHC); bij de muis heet het MHC: H-2 complex. Voorbeelden van deze MHC (H-2) restrictie zijn te vinden in de cellulaire immuunresponsen tegen virussen, bacteriën en zwakke transplantatieantigenen. Het MHC codeert voor transplantatieantigenen, die zelf een zeer heftige niet "restricted", cellulaire immuunresponse kunnen uitlokken. Een sterke transplantaatafstotingsreaktie (host-versus-graft, HvG) komt tot stand na orgaan- en weefseltransplantatie tussen individuen van dezelfde soort, die wat betreft het MHC van elkaar verschillen. Na transplantatie van immuuncompetente lymfoïde cellen in ontvangers met een vreemd MHC, die niet in staat zijn om het transplantaat af te stoten, kunnen de getransplanteerde cellen de vreemde transplantatie antigenen van de gastheer herkennen en er tegen gaan reageren. Er zal dan een sterke transplantaat-anti-gastheer (graft-versus-host, GvH) reaktie ontstaan.

Tijdens een acute GvH reaktie in letaal bestraalde muizen ontwikkelt zich een staat van vertraagd type overgevoeligheid (DTH) tegen gastheer transplantatieantigenen. Dit kan aangetoond worden door cellen van milt en lymfeklieren van de gastheer in normale secundaire ontvangers te brengen. Deze secundaire ontvangers welke syngeen zijn met de oorspronkelijke donoren van het lymfoïde transplantaat, worden in een van de achterpoten ingespoten met een testdosis ("gechallenged" met) miltcellen welke weer syngeen zijn met de bestraalde gastheer. De toename in pootdikte, welke 24 uur later gemeten wordt, is een maat voor de sterkte van de anti-gastheer immuunresponse. De initiatie en het overdragen van de GvH-gerelateerde DTH is sterk afhankelijk van T lymfocyten. De ontwikkeling van de anti-gastheer immuunreaktiviteit begint tussen 8 en 24 uur na allogene miltceltransplantatie en neemt in kracht toe gedurende de eropvolgende dagen. In de milt wordt 4 dagen na bestraling en reconstitutie een maximale anti-gastheer immuunreaktiviteit gevonden, terwijl in de lymfeklieren maximale reactiviteit wordt gevonden op dag 5. Daarna neemt de reactiviteit gestaag af (Appendix paper 1).

De ontwikkeling van de anti-gastheer DTH reaktiviteit kan in de milt gedurende de eerste 2 dagen en in de lymfeklieren op dag 3 na reconstitutie niet voorkomen worden door het delingsvermogen

van de allogene miltcellen te blokkeren, voordat deze in de bestraalde gastheermuizen getransplanteerd worden. In plaats van verder te stijgen neemt de anti-gastheer response daarna echter af, als gevolg van de remming van het delingsvermogen. Door *in vitro* de DNA synthetiserende cellen uit de milten van bestraalde en allogeen gereconstitueerde gastheermuizen te elimineren, voordat deze in een secundaire ontvanger gebracht worden, is duidelijk geworden dat voor een volledige ontwikkeling van de specifieke anti-gastheer immuunresponse gedurende een acute GvH reaktie, proliferatie van de reaktieve T cellen onontbeerlijk is (Appendix paper II).

In letaal bestraalde muizen die gereconstitueerd worden met allogene miltcellen die wat betreft het MHC geheel of gedeeltelijk verschillen, is aangetoond dat de overdraagbare DTH reactiviteit uitsluitend tegen de I regio van het H-2 complex gericht is. Antigenen, gecodeerd door de H-2K and H-2D subregio, kunnen geen anti-gastheer DTH T effector cellen induceren. Na miltceltransplantatie in letaal bestraalde H-2 compatibele, "non-H-2" incompatibele gastheren, kunnen ook antigenen gecodeerd door het Mls locus een overdraagbare anti-gastheer DTH reactiviteit oproepen. Non-H-2 alloantigenen, anders dan Mls locus producten, leveren geen duidelijke bijdrage aan de expressie van de anti-gastheer DTH reactiviteit (Appendix paper III).

Worden GvH en HvG reakties, gericht tegen H-2K, H-2I of H-2D antigenen, met elkaar vergeleken, dan blijkt dat in HvG reacties elk van deze H-2 subregionen kan leiden tot het ontstaan van tegen het transplantaat gerichte DTH T effektor cellen. Preïmmunisatie van miltcel donor muizen tegen H-2K of H-2D regio gecodeerde antigenen, heeft geen invloed op de DTH reaktiviteit tegen deze antigenen in GvH situaties. Het vermogen om een immuunresponse tegen H-2K of H-2D gecodeerde antigenen in een HvG-DTH reaktie te geven, wordt echter sterk verbeterd door preïmmunisatie van de responder muizen. Deze resultaten suggereren dat in GvH situaties slechts 1 subpopulatie voorloper DTH T effector cellen wordt geaktiveerd (vermoedelijk anti-H-2I Lyt-1^+ T cellen), terwijl in HvG situaties 2 subpopulaties T cellen tot DTH T effector cellen kunnen worden geaktiveerd (vermoedelijk anti-H-2I Lyt-1^+ T cellen en anti-H-2K/D Lyt-2^+ T cellen) (Appendix paper IV).

Het vermogen van Mls locus antigenen om anti-gastheer DTH reaktiviteit op te roepen correleert met het vermogen om proliferatie te induceren in een gemengde lymfocytenkweek (MLC). Mls^a en Mls^c locus gecodeerde antigenen kunnen zowel een positieve MLC response als GvH gerelateerde DTH reaktiviteit uit-

lokken. Echter het Mls^b locus antigeen kan geen *in vitro* proliferatie initiëren. Dit gaat samen met een marginale en kortdurende GvH gerelateerde DTH reaktiviteit. Deze Mls locus gecodeerde antigenen induceren nauwelijks enige specifieke HvG-DTH reaktiviteit. Andere "non-H-2" alloantigenen zijn verantwoordelijk voor het grootste gedeelte van de DTH reaktiviteit na subcutane immunisatie met H-2 identieke, "non-H-2" verschillende miltcellen (Appendix paper V).

In donor-ontvanger combinaties die wat betreft H-2 antigenen helemaal of slechts gedeeltelijk van elkaar verschillen, is aangetoond dat de T effector cellen, welke gericht zijn tegen de gastheer transplantatieantigenen, voortkomen uit langlevende recirculerende T2 cellen. Deze T2 cellen worden geaktiveerd door H-2 I regio gecodeerde antigenen. Deze response kan op zijn beurt weer versterkt worden door kortlevende T1 cellen. Deze T1 cellen, welke vooral in de milt voorkomen, zouden kort tevoren de thymus verlaten hebben. Versterking van de reactie wordt echter alleen gezien indien naast het H-2I regio verschil een H-2K regio verschil bestaat, hetgeen betekent dat de amplificerende T1 cellen geaktiveerd worden door H-2K regio gecodeerde antigenen. T1 cellen zelf kunnen geen anti-gastheer DTH reaktiviteit ontplooien (Appendix paper VI).

Tijdens de initiatie van een vertraagde GvH reactie door transplantatie van allogeen beenmerg in letaal bestraalde muizen, kan ook een staat van DTH tegen de gastheer transplantatieantigenen ontstaan. Bij transplantatie van C57BL beenmerg cellen in bestraalde (C57BL x CBA)F1 muizen is deze DTH reaktiviteit maximaal omstreeks 12 dagen na transplantatie, en kan niet langer aangetoond worden op dag 20. Eliminatie van recirculerende T2 cellen door behandeling van de beenmergdonoren met anti-thymocyten serum, kan de ontwikkeling van de anti-gastheer DTH reactiviteit vrijwel volledig voorkomen. Indien de bestraalde ontvangers tevoren gethymectomeerd zijn, ontwikkelt zich een zwakkere en later optredende anti-gastheer DTH response dan in niet-gethymectomeerde dieren. Deze resultaten suggereren dat T cellen, welke recent gevormd zijn onder invloed van de thymus van de gastheer, de sterkte en het begin van de anti-gastheer DTH response kunnen beïnvloeden (Appendix paper VII).

Onze studies suggereren dat indien kleine aantallen rijpe T2 cellen aanwezig zijn in het allogene transplantaat, sterk rekening gehouden moet worden met de mogelijkheid dat de anti-gastheer immuunresponse versterkt kan worden door recent gevormde T cellen.

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CURRICULUM VITAE

Na het behalen van het diploma gymnasium β aan het Sint Thomascollege te Venlo in 1968 begon ik de studie pharmacie aan de Rijksuniversiteit te Utrecht. In 1975 legde ik het doctoraalexamen af met bijvak Immunologie bij Dr. J.M.N. Willers (Immunologische aspecten van chalonen) en bij Dr. J.A.M. Kerckhaert (Onderzoek naar de invloed van cyclofosfamide op de huidtransplantaatafstoting bij de muis). In maart 1976 behaalde ik het apothekersdiploma en in april 1976 trad ik in tijdelijke dienst als wetenschappelijk ambtenaar bij de afdeling Celbiologie en Genetica van de Erasmus Universiteit te Rotterdam. Binnen de afdeling Celbiologie II, onder leiding van Prof.Dr. R. Benner en Prof.Dr. O. Vos, werd het in het proefschrift beschreven onderzoek verricht.

APPENDIX PAPER I

IMMUNOBIOLOGY OF THE GRAFT-VERSUS-HOST REACTION
I. SYMPTOMS OF GRAFT-VERSUS-HOST DISEASE IN MICE ARE PRECEDED BY DELAYED TYPE HYPERSENSITIVITY TO HOST HISTOCOMPATIBILITY ANTIGENS

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SUMMARY

During initiation of an acute Graft-versus-Host reaction by injection of C57BL/Rij spleen cells into lethally irradiated (C57BL/Rij x CBA/Rij) F1 hybrid mice, a state of delayed type hypersensitivity (DTH) against host histocompatibility (H) antigens occurs. This was demonstrated by means of transfer of host spleen and lymph node cells into C57BL/Rij recipients, which received a challenge with CBA/Rij spleen cells. Initiation and transfer of the Graft-versus-Host-related DTH reactivity was highly dependent on Thy-1.2^{+} cells.
The development of DTH reactivity started between 8 and 24 hr after semiallogeneic spleen cell transplantation and increased during the days thereafter. In the spleen maximal DTH reactivity was found on day 4, whereas in the lymph nodes maximal reactivity occurred on day 5 after irradiation and reconstitution. Thereafter, the reactivity decreased until there was no further DTH reactivity demonstrable on day 13.
The specificity of the DTH reactivity for host H antigens was proved by no reactivity to a challenge of DBA/2 and Swiss spleen cells, which are H-2 incompatible with CBA cells.

INTRODUCTION

When a graft containing immunocompetent cells is introduced into immunologically incompetent allogeneic recipients, a Graft-versus-Host (GvH) reaction can occur. The severity of the GvH disease is usually measured at the syndrome level: body weight change, incidence of disease, mortality rate (1), splenomegaly (2), and lymph node hypertrophy (3). An important drawback for studies on GvH disease is that these symptoms are not specific for this type of immune reaction. The increase in size of the lymphoid organs during GvH reaction is only indirectly related to the activation of immunocompetent cells by host alloantigens, since there is evidence that this increase can be attributable to a response of host lymphoid

and/or hematopoietic cells (4). Furthermore, some of the symptoms are highly influenced by infections for which animals suffering from GvH disease are very susceptible. Therefore, measurement of the immune reaction underlying the GvH symptoms might be a better indication of the onset of the disease.

Brent et al. (5) showed that, in guinea pigs, cell-mediated immunity against skin graft antigens coincided with a tuberculin-type inflammatory response of delayed onset to the graft. More recently, Kon and Klein (6) could measure immunity against H-2 and non-H-2 antigens in the mouse with the footpad swelling test. The *in vivo* recognition and proliferation phase of cellular immunity to histocompatibility antigens (graft rejection, GvH) has its *in vitro* counterpart in the mixed lymphocyte culture reaction (MLC) (7). During a MLC a substance is released into the supernatant fluid that can induce histologically a typical delayed type hypersensitivity (DTH) response upon injection *in vivo* (8). These data combined led us to investigate whether GvH reactivity can be measured as a DTH response by means of the footpad swelling test. The results reported here show that during the initiation phase of GvH in mice, an antigen-specific DTH reactivity against host histocompatibility (H) antigens occurs. The kinetic aspects of the development of this reactivity were investigated.

MATERIALS AND METHODS

Animals. Male C57BL/Rij ($H\text{-}2^b$), 10 to 20 weeks old, female CBA/Rij ($H\text{-}2^q$), 30-40 weeks old, male and female (C57BL/Rij x CBA/Rij)F1 ($H\text{-}2^b/H\text{-}2^q$), 10 to 20 weeks old, male DBA/2 ($H\text{-}2^d$), and male Swiss ($H\text{-}2^s$) mice, 10 weeks old, were used. The C57BL/Rij and CBA/Rij mice were purchased from the Medical Biological Laboratory TNO, Rijswijk, and from the Reactor Centre, Petten, The Netherlands. The (C57BL/Rij x CBA/Rij)F1 mice were bred at the Laboratory Animals Centre of the Erasmus University, Rotterdam, The Netherlands. The DBA/2 and Swiss mice were purchased from the Central Institute for the Breeding of Laboratory Animals TNO, Zeist, The Netherlands.

Preparation of cell suspensions. Mice were killed by carbon dioxide. Immediately after killing the organs to be used (spleens, inguinal, axillary, and mesenteric lymph nodes) were removed and placed into a balanced salt solution (BSS). This solution was prepared according to Mishell and Dutton (9). Spleens and lymph nodes were minced with scissors and squeezed through a nylon gauze filter to give a single-cell suspension. Nucleated

cells were counted with a Coulter Counter Model B. Viable cells were counted in a haemocytometer using 0.2% trypan blue in BSS as a diluent.

Irradiation. The recipient (C57BL/Rij x CBA/Rij)F1 mice received 975 rad of whole body irradiation, generated in a Philips Müller MG 300 X-ray machine. Animals were irradiated in well-aerated circular Perspex cages. Physical constants of the irradiation were: 250 kv (constant potential), 10 ma, added filtration of 1.0 mm Cu, irradiation was corrected for field inhomogeneity, and focus object distance was 53 cm. Animals were irradiated at a dose rate of 30 to 35 rad/min. Maximal backscatter was achieved by placing the cage on a layer of 15 cm hardboard. During irradiation the dose was measured with a Baldwin Ionex dosimeter. Radiation control mice died within 10 to 16 days.

Acute GvH reaction. Acute GvH reactions were elicited by iv injection of 5×10^7 nucleated male C57BL/Rij spleen cells in lethally irradiated female (C57BL/Rij x CBA/Rij)F1 recipient mice within 4 hr after irradiation. The cells to be injected were suspended in a volume of 0.5 ml of BSS. In the experimental system 50% of the mice had died from acute GvH disease on days 15 and 16 (Fig. 1). Female (C57BL/Rij x CBA/Rij)F1 mice that were lethally irradiated and reconstitututed with 5×10^7 syngeneic male spleen cells survived for at least 150 days.

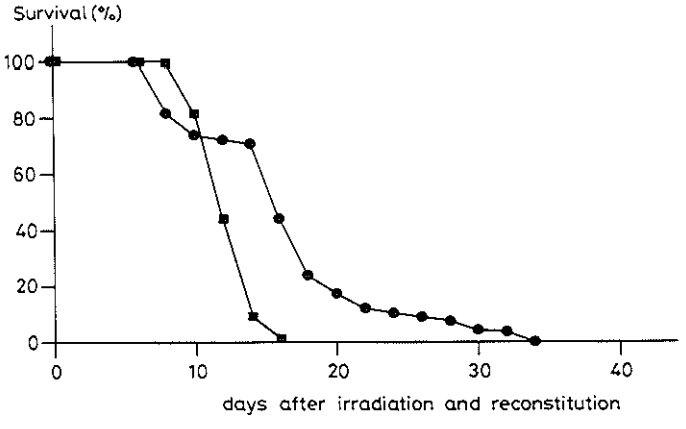

Figure 1.
Survival of lethally irradiated (C57BL/Rij x CBA/Rij)F1 mice (n = 42) undergoing an acute GvH reaction evoked by 5×10^7 C57BL/Rij spleen cells (●). Similarly irradiated nonreconstituted syngeneic mice (n = 38) were used as controls (■). Data represent the arithmetic mean of three different experiments.

Selective elimination of T cells. T cells were eliminated from spleen cell suspensions by treatment with anti-Thy-1.2 antibodies and guinea pig complement *in vitro*. The production of anti-Thy-1.2 sera and their use for selective elimination of T cells have been described previously (10). The amount of anti-Thy-1.2 serum used in the experiments was two or three times higher than that needed to kill more than 95% of corticosteroid-resistant thymocytes (30 mg of dexamethasone sodium phosphate per kg body weight (Merck and Co., Rahway, New Jersey) ip 2 days before harvest).

Mitomycin C treatment. Treatment of spleen cells with mitomycin C (Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan) was carried out as described by Blomgren and Svedmyr (11). Two to 8 x 10^6 spleen cells were incubated for 30 min at 37 C in 20 ml of BSS containing 100 µg of mitomycin C per ml. After treatment with mitomycin C, the cells were washed twice by centrifugation at 1,500 rpm for 10 min and were resuspended in BSS.

Assay for DTH. At various intervals after transfer of male C57BL/Rij spleen cells into lethally irradiated female (C57BL/Rij x CBA/Rij)F1 recipient mice, the recipient spleens were removed and prepared as a single-cell suspension. Unless stated otherwise, one spleen equivalent of the pooled spleen cell suspension was transferred iv into male C57BL/Rij recipient mice. The cells to be injected were suspended in a volume of 1.0 ml of BSS. DTH reactivity of these C57BL/Rij recipient mice was determined by measuring the difference in thickness of the hind feet 24 and 48 hr after subcutaneous (sc) injection of 2 x 10^7 mitomycin C-treated CBA/Rij spleen cells into the instep of the right hind foot. This dose of spleen cells was administered in a volume of 0.05 ml with a tuberculin syringe and a 25-gauge needle. The thickness of the left and right hind feet was measured with a footpad meter as described by Bonta and Vos (12), with some minor modifications. During measurement the mice were anaesthetized by ether. As a control for background DTH reactivity, irradiated female (C57BL/Rij x CBA/Rij)F1 mice were reconstituted with 5 x 10^7 syngeneic male spleen cells. At various intervals thereafter, the recipient spleen cells were transferred into male C57BL/Rij mice. These C57BL/Rij mice showed the same background reactivity after challenge with 2 x 10^7 mitomycin C-treated CBA/Rij spleen cells as did C57BL/Rij mice that only received the challenge dose. Therefore, in all experiments a group of C57BL/Rij mice challenged with 2 x 10^7 mitomycin C treated CBA/Rij spleen cells was used as a control. The relative increase in foot thickness was calculated as:

$$\frac{\textit{Thickness right foot} - \textit{thickness left foot}}{\textit{thickness left foot}} \times 100\%$$

The specific increase in foot thickness was calculated as the relative increase in foot thickness of the immune mice minus the relative increase in foot thickness of the control mice. The swelling in C57BL/Rij control mice challenged with 2×10^7 mitomycin C (100 µg/ml)-treated CBA/Rij spleen cells varied between 14 and 20%. If not treated with mitomycin C, the same dose of CBA/Rij spleen cells gave a foot swelling of 25 to 30%.

RESULTS

Development of GvH-related DTH reactivity in spleen and lymph nodes.

GvH reactions were evoked in (C57BL/Rij x CBA/Rij)F1 mice that had been lethally irradiated and reconstituted with 5×10^7 C57BL/Rij spleen cells. At different intervals after irradiation and reconstitution, the recipient spleens were individually transferred into normal C57BL/Rij mice. Immediately thereafter these C57BL/Rij mice were challenged in the instep of the right hind foot with 2×10^7 mitomycin C-treated CBA/Rij spleen cells. At 24 and 48 hr after challenge, the DTH reaction was measured and expressed as the percentage increase of foot thickness. A scheme of the experimental system is shown in Fig. 2.

One day after initiation of the GvH reaction, DTH reactivity could be demonstrated, when measured 24 hr after challenge (Fig. 3). Such a 24-hr DTH response was not detectable when assayed 8 hr after initiation of the GvH reaction. During the

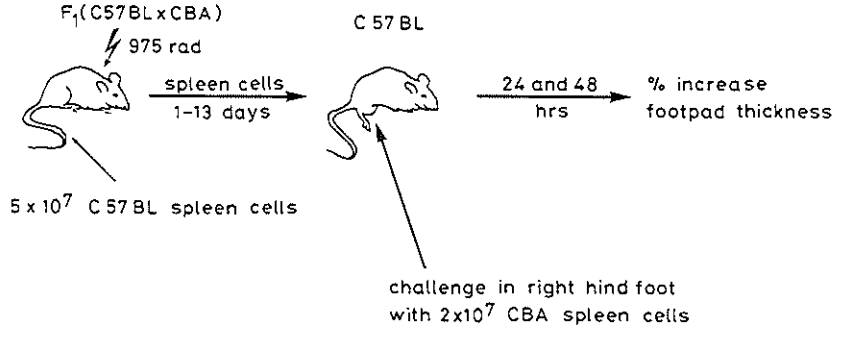

Figure 2.
Scheme of the experimental system used to demonstrate GvH-related DTH reactivity against host histocompatibility antigens.

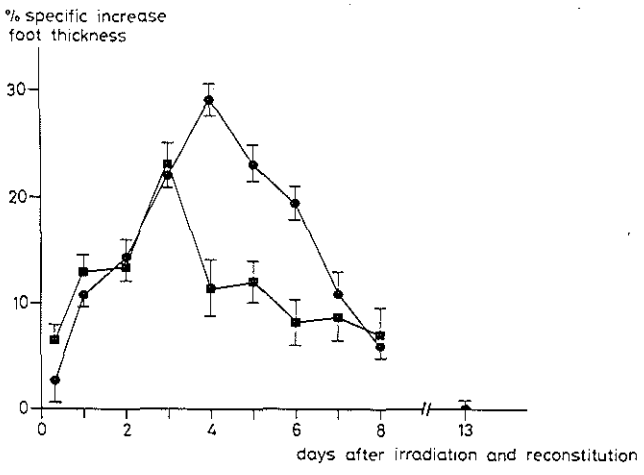

Figure 3.
Development of GvH-related DTH reactivity in the spleen of (C57BL/Rij x CBA/Rij) F1 mice inoculated with 5 x 10^7 C57BL/Rij spleen cells. Each experimental point represents the arithmetic mean ± 1 SEM of three different experiments.
●, 24 hr DTH responses; ■, 48 hr DTH responses (n = 15).

following days, the DTH reactivity steadily increased until a maximal 24 hr DTH response was found on the 4th day. Thereafter, the reactivity declined until day 13 after irradiation and reconstitution, at which time DTH reactivity could no longer be detected.

Measurement of the DTH response of the same mice 48 hr after challenge showed a somewhat different pattern. In the first 3 days after irradiation and reconstitution, the 48 hr response equalled the 24 hr response, but at days 4, 5 and 6 the 48 hr response was markedly decreased.

The lymph node cells of similarly treated (C57BL/Rij x CBA/Rij) F1 recipient mice were also tested for DTH reactivity using the above transfer system. Therefore, pooled inguinal, axillary, and mesenteric lymph node cells of the individual recipient mice were transferred into C57BL/Rij recipients. In these secondary recipients a significant DTH response was detectable 3 days after initiation of the GvH response (Fig. 4). Peak DTH reactivity in the lymph nodes was found to occur on the 5th day, thus 1 day later than in the spleen. As was found for the spleen, on the first days of the reactivity (days 3 and 4), the DTH response at 48 hr after challenge was higher than that at 24 hr. Later on (days 5 and 6) the 48 hr response was smaller.

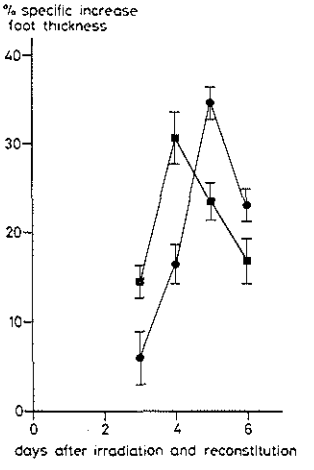

Figure 4.
Development of GvH-related DTH responsiveness in the lymph nodes of (C57BL/R CBA/Rij)F1 mice inoculated with 5 x 10^7 C57BL/Rij spleen cells. Each experimental point represents the arithmetic mean ± 1 SEM of two different experiments. ●, 24 hr DTH responses; ■, 48 hr DTH responses (n = 10).

Characteristics of the transferred DTH reactivity

Time course. A characteristic of DTH reactivity is the occurrence of the peak response at 24 hr or later after challenge. To evaluate whether this also holds for the GvH-related DTH reactivity measured in the system presented here, the time course of a typical 4-day DTH response was followed. Therefore, at 4 days after irradiation and reconstitution of (C57BL/Rij x CBA/Rij)F1 mice with 5 x 10^7 C57BL/Rij spleen cells, one spleen equivalent was transferred into C57BL/Rij recipient mice. At different intervals after challenge of these secondary recipients with 2 x 10^7 CBA/Rij spleen cells, the specific foot swelling was measured. At 5 hr after challenge, a slight specific increase (±8%) in foot thickness was found (Fig. 5). Another 5 hr later, no significant foot swelling could be detected any more. Thereafter a steady increase was measurable with a peak at 24 hr after challenge. After 24 hr the response declined. This result suggests that the reactivity transferred is of the classical (13) delayed type and that the immediate type component in the reaction is only detectable at 5 hr after challenge.

Dose-response relationship. The relationship between the number of spleen cells used for secondary transfer and the height of the DTH response was studied by means of spleen cells from mice irradiated and reconstituted with 5 x 10^7 C57BL/Rij spleen cells 4 days previously. Therefore, 0.5, 0.75, 1.0, and 1.5 spleen equivalents were transferred into C57BL/Rij mice, which

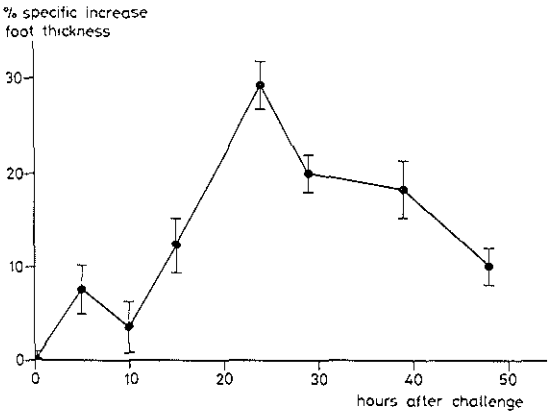

Figure 5.
Time course of the foot swelling of C57BL/Rij mice challenged with CBA/Rij spleen cells and inoculated with the spleen cells from (C57BL/Rij x CBA/Rij)F1 mice, which were lethally irradiated and injected with 5 x 10^7 C57BL/Rij spleen cells 4 days previously. Vertical bars represent 1 SEM (n = 5).

were challenged with CBA/Rij spleen cells. The 24 hr DTH responses of these mice were measured and the linear relationship was calculated. As can be seen in Fig. 6, transfer of graded numbers of spleen cells influences the response in a linear way.

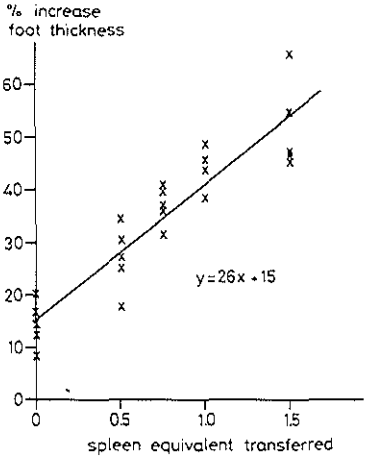

Figure 6.
Dose-response relationship of the number of spleen cells used for secondary transfer. Spleen cells were obtained from (C57BL/Rij x CBA/Rij)F1 mice, lethally irradiated and inoculated with 5 x 10^7 C57BL/Rij spleen cells 4 days previously. The individual responses are plotted. Values are not corrected for background DTH reactivity. The linear relationship was calculated from the individual responses (r^2 = 0.85; $P < 0.02$).

Specificity. In order to test the specificity of the GvH-related DTH reactivity for host H antigens, spleen cells from irradiated and semiallogeneically reconstituted (C57BL/Rij x CBA/Rij)F1 mice were transferred into C57BL/Rij recipients 4 days after reconstitution. These secondary recipients were challenged with 2 x 10^7 spleen cells from various mouse strains: CBA/Rij, DBA/2, Swiss and C57BL/Rij. These strains have different H-2 haplotypes. As shown in Table 1, a DTH response was elicited only by CBA/Rij spleen cells. Thus, the DTH responsiveness is specific for the H antigens of the host that initiated the GvH reaction.

Dependence of GvH-related DTH reactivity on Thy-1.2$^+$ cells. Both GvH (14) and DTH (15) reactivity are known to be dependent on T cells. We investigated whether the GvH-related DTH reactivity to H antigens measured in the system presented here is similarly dependent on T cells. This was done for both the initiation and the transfer of the DTH reactivity. Therefore, three groups of (C57BL/Rij x CBA/Rij)F1 mice were lethally

TABLE 1

SPECIFICITY OF GvH-RELATED DTH REACTIVITY FOR HOST HISTOCOMPATIBILITY ANTIGENS

challenging antigen haplotype[a]	recipient	response[b]
CBA/Rij (H-2^q)	C57BL	29.2 ± 3.2
DBA/2 (H-2^d)	C57BL	0.4 ± 1.7
Swiss (H-2^s)	C57BL	-1.8 ± 1.5
C57BL/Rij (H-2^b)	C57BL	-0.7 ± 2.3

a. (C57BL/Rij x CBA/Rij)F1 mice were lethally irradiated and injected with 5 x 10^7 C57BL/Rij spleen cells in order to elicit GvH reactivity. Four days later spleens of recipient mice were removed and the cells transferred into normal C57BL/Rij recipients. These recipient mice were challenged with 2 x 10^7 spleen cells from various mouse strains immediately after cell transfer.

b. Numbers represent the arithmetic mean of the specific increase of foot thickness ± 1 SEM of five mice. DTH reactions were measured 24 hr after challenge.

irradiated and reconstituted with C57BL/Rij spleen cells pretreated with either anti-Thy-1.2 serum and complement or normal mouse serum and complement. Four days after irradiation and reconstitution, recipient spleen cells were treated with either anti-Thy-1.2 serum and complement or normal mouse serum and complement, according to the scheme in Fig. 7. After this treatment one spleen equivalent was transferred into C57BL/Rij recipients, which were challenged immediately thereafter with 2×10^7 CBA/Rij spleen cells.

Treatment of C57BL/Rij spleen cells with anti-Thy-1.2 serum and complement before transfer into (C57BL/Rij x CBA/Rij)F1 recipients totally prevented the development of DTH-reactive cells, as can be seen in Table 2. Furthermore, pretreatment of host spleen cells with anti-Thy-1.2 serum and complement just before secondary transfer abolished the DTH reactivity of these cells. Treatment with normal mouse serum resulted in normal DTH responsiveness. These results suggest that T cells are responsible for both the development of anti-H DTH reactivity and the transfer of the state of DTH.

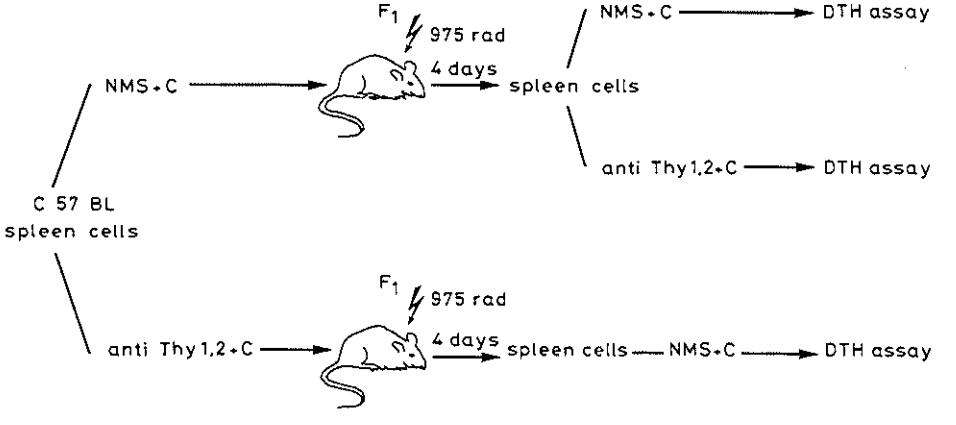

Figure 7.
Scheme of the experimental system used to determine the dependence of the initiation and transfer of GvH-related DTH responsiveness on Thy-1.2+ cells.

TABLE 2

DEPENDENCE OF INITIATION AND TRANSFER OF GvH-RELATED DTH REACTIVITY ON THY-1.2$^+$ CELLS

Treatment before initiation of GvH[a]	Spleen cells recovered on day 4	Treatment before transfer of spleen cells[b]	DTH response[c]
NMS[d] + C	5.8×10^7	NMS + C	23.3 ± 2.3
Anti-Thy-1.2 + C	1.1×10^7	NMS + C	-1.1 ± 2.8
NMS + C	5.7×10^7	Anti-Thy-1.2 + C	0.7 ± 2.5

a. GvH reactions were elicited by injection of 5×10^7 C57BL/Rij spleen cells into lethally irradiated (C57BL/Rij x CBA/Rij)F1 mice. Before transfer the spleens were treated with either NMS and C or anti-Thy-1.2 and C.

b. Four days after initiation of the GvH reaction, the spleens were harvested and treated with either NMS and C or anti-Thy-1.2 and C. Subsequently, the cells were transferred into normal C57BL/Rij mice. These recipient mice were challenged with 2×10^7 CBA/Rij spleen cells immediately after cell transfer.

c. Numbers represent the arithmetic mean of the specific increase of foot thickness $\pm$ 1 SEM of five mice. DTH reactions were measured 24 hr after challenge.

d. NMS, normal mouse serum; C, guinea pig complement.

DISCUSSION

This study shows that during an acute GvH reaction in spleen and lymph node cells of the host, a DTH responsiveness against host histocompatibility antigens appears. This hypersensitivity of delayed onset is antigen-specific and relies on Thy-1.2$^+$ cells. The DTH reactivity could be demonstrated during the first week after irradiaton of the host and semiallogeneic spleen cell transplantation, with a peak reactivity on the 4th and 5th days for spleen and lymph nodes, respectively (Figs. 3 and 4). This is somewhat earlier than the appearance of the characteristic GvH symptoms and long before the occurrence of deaths. The latter appears from the cumulative mortality curve (Fig. 1), showing a 50% mortality on days 15 to 16. This suggests that the development of DTH reactivity during the early phase of a GvH reaction is a temporary phenomenon associated with the initiation of the immune reaction leading to the characteristic symptoms of GvH disease, and finally to death.

The rate of appearance of GvH-related DTH reactivity in the spleen (Fig. 3) may indicate that proliferation of the transplanted allogeneic T cells is not a prerequisite for its appearance. This is consistent with the study of Phillips et al. (8) on the generation of macrophage inhibition factor during a MLC. In their studies the macrophage inhibition factor production could be demonstrated within 24 hr, and sc injection of

MLC supernatant, containing macrophage inhibition factor, could evoke a typical DTH response *in vivo*. On the other hand, the appearance of peak DTH reactivity in the spleen on day 4, and in the lymph nodes on day 5, correlates well with the DNA synthetic activity of adoptively transferred thymocytes (16) and the increase of the number of T cells after irradiation and allogeneic spleen cell transplantation (16a). This suggests that the increase of DTH reactivity during the first 4 to 5 days is attributable to a clonal expansion of the population of DTH reactive cells. The earlier decline of the curve showing 48 hr DTH responses compared with 24 hr DTH responses (Figs. 3 and 4) suggests that this clonal expansion of reactive T cells is limited to about the 4th and 5th days after irradiation and reconstitution, yielding mature DTH reactive cells with a short functional life span. The decrease of DTH reactivity after the 4th and 5th days is probably because of the transformation of the potentially lymphokine-producing lymphoblasts (17) into small lymphocytes (18), since it has been shown that only lymphoblasts can migrate *in vivo* into inflammatory sites (19). However, the decline in DTH reactivity can also be attributed to (A) a possible emigration of the DTH reactive cells from spleen and lymph nodes to other sites of the body, where they cause the symptoms of GvH disease (18); (B) the generation of DTH suppressing antibodies (20), blocking factors (21,22), or T suppressor cells (23,24); (C) an exhaustion of host antigens inducing the DTH reactivity (25); or (D) an exhaustion of the population of DTH reactive T cells because of extensive proliferation. All of these possible causes for the decline of cell-mediated immunity during a GvH reaction have been reviewed recently by Grebe and Streilein (26).

The DTH reactive T cells which temporarily occur during an acute GvH reaction are not likely to be part of the differentiation pathway to cytotoxic T lymphocytes, wich can also occur during a GvH reaction (27). This appears for example from the work of Dennert and Hatlen (28) and Huber et al. (29). The first authors showed that cells which mediate contact sensitivity to the trinitrophenyl group failed to display cytotoxicity against trinitrophenyl-coupled target cells. On the other hand, cytotoxic T lymphocytes could not transfer contact sensitivity to trinitrophenyl. Since antigen recognition in both reactions was similar, Dennert and Hatlen (28) concluded that delayed hypersensitivity and antibody-independent cell-mediated cytotoxicity are caused by two distinct T cell populations. This conclusion is supported by the experiments of Huber et al. (29), who used two different populations of T cells with the Ly-1^+ and Ly-$2,3^+$ phenotype, respectively. These au-

thors showed that DTH to sheep red blood cells is based on Ly-1^+ T cells and not on Ly-$2,3^+$ T cells, whereas cell-mediated cytotoxicity is mediated by Ly-$2,3^+$ T cells only.

In conclusion, this paper shows that the initiation of GvH disease in mice is associated with a DTH reactivity specific for the host H antigens. This DTH reactivity occurs before the appearance of the characteristic symptoms of GvH disease.

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APPENDIX PAPER II

IMMUNOBIOLOGY OF THE GRAFT-VERSUS-HOST REACTION II. THE ROLE OF PROLIFERATION IN THE DEVELOPMENT OF SPECIFIC ANTIHOST IMMUNE RESPONSIVENESS

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SUMMARY

The development of acute graft-versus-host (GvH) disease in (C57BL/Rij x CBA/Rij)F1 hybrid mice inoculated with C57BL/Rij spleen cells is associated with a specific antihost delayed-type hypersensitivity (DTH) in the lymphoid organs of these mice. In this paper the role of proliferation in the development of this DTH response was studied in relation to the cellular changes in spleen and lymph nodes during GvH disease. Blocking of DNA synthesis of the C57BL/Rij spleen cells by incubation with 25 µg of mitomycin C per ml before inoculation into the irradiated recipients did not prevent the antihost DTH responsiveness in the spleen during the first 2 days, and in the lymph nodes at 3 days after reconstitution. Thereafter, the DTH responsiveness was greatly decreased by the mitomycin C treatment. Blocking of both DNA and RNA and a great part of protein synthesis by pretreatment with 100 µg of mitomycin C per ml could completely prevent the development of DTH responsiveness in the recipient mice.
Elimination of the DNA synthetizing cells from the recipient's spleen and lymph nodes by tritiated thymidine (^{3}H-TdR) suicide *in vitro* revealed that the appearance of antihost DTH responsiveness in both organs is associated with a local proliferation of the reactive T cells. In both the spleen and lymph nodes, elimination of the DNA synthetizing cells at the moment of peak DTH reactivity reduced the DTH response to about 50% of the normal value. This decrease was larger before reaching peak DTH reactivity, whereas thereafter ^{3}H-TdR could no longer affect the height of the antihost DTH response. These results suggest that full development of specific antihost DTH responsiveness during an acute GvH reaction is dependent on proliferation of the reactive T cells.

INTRODUCTION

Transplantation of allogeneic immunocompetent cells into immunologically incompetent recipients can result in a GvH reaction.

A widely used GvH assay is the Simonsen splenomegaly test (1), which is based upon proliferation of both lymphoid and hematopoietic cells. Proliferation of donor lymphocytes is an associative response in the development of GvH disease (2,3). This has been shown after injection of chromosomally marked (T6T6) CBA spleen cells, injected i.v. in non-irradiated (C57BL x CBA)F1 hybrid mice (4), and after injection in neonatal C57BL mice (5), combinations, which are both different in the major histocompatibility complex. The requirement of proliferative activity of transplanted parental spleen cells for the induction of splenomegaly in non-irradiated F1 hybrid recipients has been investigated by Nisbet and Simonsen (6) and by Scollay et al. (7). These authors found that splenomegaly can be induced in the absence of donor cell proliferation. Apparently, upon stimulation by alloantigens, nondividing donor cells may release factors that induce host cells to proliferate.

In measuring systems involving lethally irradiated recipients, organ weight or lymphoid cell number measurements more nearly approximate the proliferative activity of donor lymphocytes. However, Strong et al. (8) measured donor proliferative activity in lethally irradiated mice after allogeneic (in a combination, which is different in the major histocompatibility complex) and syngeneic spleen cell transplantation by ^{3}H-TdR incorporation. At 5 days after transplantation, he found the same proliferative activity in both combinations, probably because of a prevailing hematopoiesis. On the other hand, Hilgard (9) reported the absence of host splenomegaly in 500 rad X-irradiated mice transplanted with allogeneic spleen cells, despite the fact that GvH disease developed. Therefore, it is questionable whether there is a direct relationship between the proliferative activity of donor-derived lymphocytes reacting to the host histocompatibility antigens, and the proliferative activity in the recipient spleen (10). Furthermore, the possibility has to be considered that upon stimulation by histocompatibility antigens, donor cells release factors which stimulate donor lymphocytes not specific for the host histocompatibility antigens to proliferate (11).

Only in a few studies the responding cells were assayed functionally, e.g., in a cytotoxicity assay (12-15). However, the question arises whether the bulk of proliferative activity as measured in the above-mentioned systems has anything to do with the generation of these cytotoxic cells (16-18). In the preceding paper of this series (19), we demonstrated that initiation of GvH disease by C57BL/Rij spleen cell transplantation in lethally irradiated (C57BL/Rij x CBA/Rij)F1 mice is asso-

ciated with a DTH responsiveness of the animal's spleen and lymph node cells to the host histocompatibility antigens. This report describes experiments that were designed to investigate to what extent proliferation of specifically reacting donor-derived T lymphocytes (A) occurs during and (B) is required for GvH reactivity in this system.

MATERIALS AND METHODS

Animals. C57BL/Rij (H-2^b) mice, 10 to 12 weeks old, female CBA/Rij (H-2^q) mice, 30 to 40 weeks old, male and female (C57BL/Rij x CBA/Rij)F1 (H-2^b/H-2^q) mice, 10 to 20 weeks old, were used. The C57BL/Rij and CBA/Rij mice were purchased from the Medical Biological Laboratory TNO, Rijswijk, and from the Netherlands Energy Research Foundation (ECN), Petten. The (C57BL/Rij x CBA/Rij)F1 mice were bred at the Laboratory Animals Centre of the Erasmus University, Rotterdam, The Netherlands.

Preparation of cell suspensions. Mice were killed by carbon dioxide. Immediately after killing, the organs were prepared for single-cell suspensions as described previously (19). Nucleated cells were counted with a Coulter Counter Model B.

Irradiation. The recipient (C57BL/Rij x CBA/Rij)F1 mice received 950 rad of whole body irradiation, generated in a Philips Müller MG 300 X-ray machine as described in detail previously (19). Radiation control mice died in 10 to 16 days.

Acute GvH reaction. Acute GvH reactions were elicited by iv injection of 5 x 10^7 nucleated male C57BL/Rij spleen cells in lethally irradiated female (C57BL/Rij x CBA/Rij)F1 recipient mice within 4 hr after irradiation. In this experimental set up, 50% of the mice had died from acute GvH disease on days 15 and 16 after irradiation and reconstitution. Female (C57BL/Rij x CBA/Rij)F1 mice that were lethally irradiated and reconstituted with 5 x 10^7 syngeneic male spleen cells survived for at least 150 days.

Antithymocyte serum. Antithymocyte serum was prepared by immunizing New Zealand White rabbits twice with 5 x 10^8 BALB/c (H-2^d) thymocytes according to the method of Jooste et al. (20). Before absorption the immunoglobulin fraction was isolated by saturated $(NH_4)_2SO_4$ precipitation. The antiserum was absorbed three times with BALB/c IgG2b plasma cell tumor cells (MOPC 195; kindly provided by Dr. J.M.N. Willers, University of Utrecht, The

Netherlands). Thereafter, the antiserum was absorbed with spleen cells from (C57BL/Rij x CBA/Rij)F1 mice that were thymectomized, lethally irradiated, and reconstituted with 2 x 10^6 syngeneic fetal liver cells. The specificity of the absorbed antiserum for T lymphocytes was determined as described in a previous paper from our laboratory (21).

Immunofluorescence staining of T and B cells. Immunofluorescence staining of T and B lymphocytes was done as described in detail by Van der Ham et al. (21). In all slides the number of lymphocytes positive for both B and T cell markers was less than 2%.

Mitomycin C treatment. Treatment of spleen cells with mitomycin C (Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan) was carried out as described by Blomgren and Svedmyr (22). A number of 2 to 10 x 10^8 spleen cells was incubated for 30 min at 37C in 40 ml of a balanced salt solution (BSS) (19) containing either 25 µg or 100 µg of mitomycin C per ml. After treatment with mitomycin C, the cells were washed twice by centrifugation at 1,500 rpm for 10 min and resuspended in BSS.

^{3}H-TdR suicide technique. Pooled spleen or lymph node cells were obtained from six lethally irradiated (C57BL/Rij x CBA/Rij)F1 hybrid mice reconstituted with 5 x 10^7 C57BL/Rij spleen cells 3 to 6 days before. The cells were suspended in 6.0 ml of BSS containing 100 µc of ^{3}H-methyl-thymidine per ml (specific activity, 20 to 25 c/mmol; radioactive concentration 1.0 mc/ml in sterile, aqueous solution; The Radiochemical Centre, Amersham, England). The suspensions were incubated for 60 min at 37C with continuous agitation. Control suspensions were incubated with an equal amount of cold thymidine ^{1}H-TdR. After the incubation procedure the cells were washed twice with BSS. A number of nucleated spleen or lymph node cells equivalent with one whole spleen or the pooled inguinal, axillary, and mesenteric lymph nodes was injected iv per recipient C57BL/Rij mouse. The C57BL/Rij mice inoculated with cells treated with either ^{3}H-TdR or ^{1}H-TdR were challenged with CBA/Rij spleen cells 24 hr after cell transfer.

Assay for DTH. The DTH assay has been described in detail in a previous paper (19).

RESULTS

Cellular changes in spleen and lymph nodes during GvH reaction. The cellular changes in spleen and peripheral lymph nodes of lethally irradiated (C57BL/Rij x CBA/Rij)F1 mice inoculated with 5×10^7 C57BL/Rij spleen cells were studied. Therefore, the total cellularity and the numbers of T and B cells were determined at various intervals after irradiation and reconstitution. After a sharp decrease of the total number of nucleated cells because of irradiation damage, the recovery started about 3 days after reconstitution (data not shown). Mice reconstituted with C57BL/Rij spleen cells showed a faster increase of cell numbers than syngeneically reconstituted mice, probably because of the GvH reaction. The splenic cellularity of syngeneically reconstituted mice surpassed that of semiallogeneically reconstituted mice between 5 and 6 days after reconstitution, probably because of a faster recovery of hemopoiesis after syngeneic spleen cell transplantation (data not shown).

The numbers of T and B cells in spleen and lymph nodes were determined on days 0, 2, 4, 6 and 8 after irradation, and allogeneic or syngeneic spleen cell reconstitution by means of the immunofluorescence technique. In both spleen and lymph nodes, the peak percentage (Fig. 1) and absolute number (Fig. 2) of T

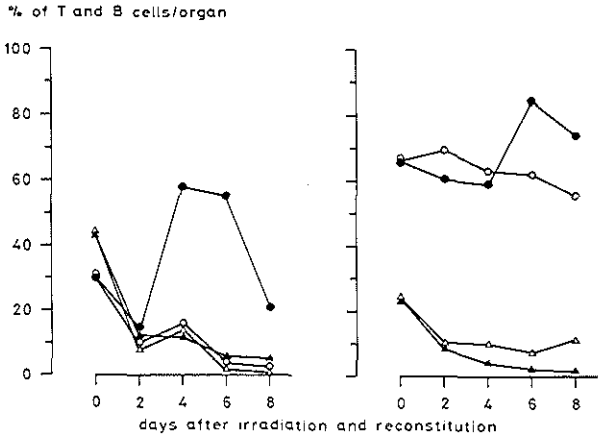

Figure 1
Percentages of T (○ , ●) and B (△ , ▲) cells in the spleen (left) and peripheral lymph nodes (right) of lethally irradiated (C57BL/Rij x CBA/Rij)F1 mice, reconstituted with either 5×10^7 C57BL/Rij spleen cells (● , ▲) or 5×10^7 syngeneic spleen cells (○ , △).
The figures representing the values 0 days after irradiation were obtained from nonirradiated control mice. Each experimental point represents the average of a group of at least five mice.

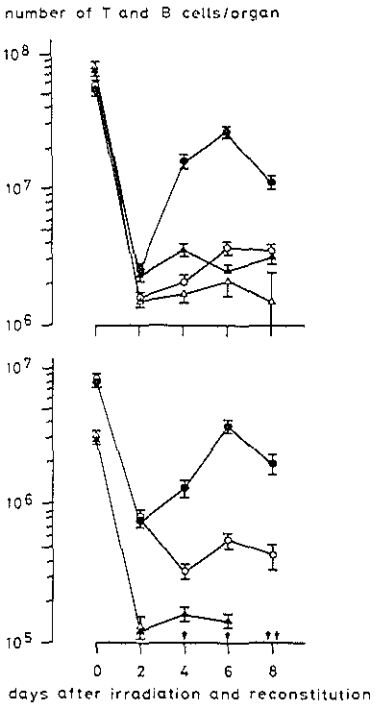

Figure 2
Recovery of the number of T (○ , ●) and B (Δ , ▲) cells in the spleen (upper) and lymph nodes (lower) of lethally irradiated (C57BL/Rij x CBA/Rij)F1 mice, reconstituted with either 5 x 10^7 C57BL/Rij spleen cells (● , ▲) or 5 x 10^7 syngeneic spleen cells (○ , Δ).
Arrowheads indicate cell numbers below the abscissa. The figures representing the values 0 days after irradiation were obtained from nonirradiated control mice. Each experimental point represents the average ± 1 SEM of a group of at least five mice.

cells were reached in the period between days 2 and 6 after allogeneic reconstitution. In the spleen the percentage of T cells increased between days 2 and 4, while in the lymph nodes this increase occurred between days 4 and 6. Such an increase of T cells was not observed after syngeneic reconstitution. Both after allogeneic and syngeneic reconstitution, no increase of B cell numbers could be detected in spleen and lymph nodes within 8 days after reconstitution.

Role of proliferation in the onset of GvH-related DTH.
After irradiation and reconstitution of (C57BL/Rij x CBA/Rij)F1 mice with 5 x 10^7 C57BL/Rij spleen cells, a specific antihost DTH reactivity can be transferred with spleen and lymph nodes cells from the primary recipient (19). In spleen and lymph nodes, this DTH reactivity steadily increases until days 4 and 5, respectively, and thereafter declines (19). The role of proliferat-

ion in the development of the transferable antihost DTH reactivity was studied by pretreatment of the C57BL/Rij donor spleen cells with mitomycin C (25 µg/ml) *in vitro*. This dose of mitomycin C blocks DNA synthesis and thereby prevents proliferation. Incubation of the donor spleen cells with 25 µg of mitomycin C per ml hardly affected the response of the recipient spleen cells during the first 2 days and the response of the recipient lymph nodes at 3 days after reconstitution (Fig. 3). However, the response evoked by mitomycin C-treated spleen cells was markedly decreased during the days thereafter as compared with the response evoked by nontreated spleen cells.
Blocking of DNA, RNA and a great part of protein synthesis by pretreatment of the C57BL/Rij spleen cells with 100 µg of mitomycin C per ml did not result in a detectable antihost DTH reactivity during the days that the reaction was followed (Fig. 3, left).

Role of proliferation in the progress of GvH-related DTH.
The proliferative activity in the compartment of antihost DTH effector cells was studied by means of ^{3}H-TdR suicide *in vitro*. Therefore, at 3 to 6 days after initiation of the GvH reaction, the host's spleen and lymph node cells were incubated separately with high specific activity ^{3}H-TdR for 60 min at 37C in order to kill the DNA synthetizing cells. After washing these cell suspensions, numbers of cells equivalent with one whole

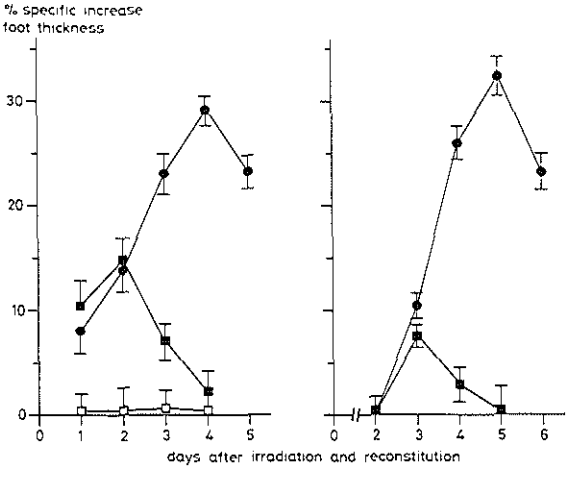

Figure 3
Development of GvH-related DTH responsiveness in the spleen (left) and lymph nodes (right) of lethally irradiated (C57BL/Rij x CBA/Rij)F1 mice inoculated with 5 x 10^{7} C57BL/Rij spleen cells, and treated with either 0 µg (●), 25 µg (■) or 100 µg (□) of mitomycin C per ml.
Each experimental point represents the average ± 1 SEM of a group of five mice.

spleen or the lymph nodes harvested from one animal were transferred into normal C57BL/Rij mice. After 24 hr these secondary recipient mice were challenged with CBA/Rij spleen cells and 24 hr later the DTH response was measured. Figure 4 (left) shows the responses evoked by spleen cells at 3, 4 and 5 days after irradiation and reconstitution. Comparison of these responses with those by ^{1}H-TdR-treated spleen cells, revealed that on day 3 after reconstitution the majority of the DTH-reactive cells in the spleen belongs to the proliferative compartment. On day 4 the size of this compartment was smaller, while on day 5 no effect of ^{3}H-TdR suicide on the DTH response could be detected.
Antihost DTH reactivity of the lymph node cells of the allogeneically reconstituted recipient mice was found to be similarly associated with proliferation. Elimination of the proliferating cells on day 4 resulted in a profound reduction of the DTH response (Fig 4, right). This effect was smaller on day 5, while on day 6 no significant contribution for proliferating DTH effector cells to the DTH response could be detected.

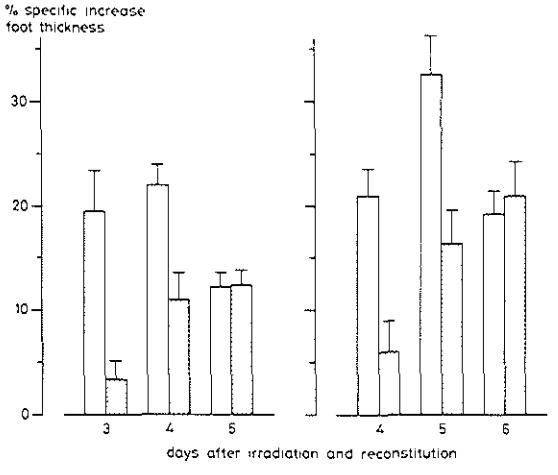

Figure 4
DTH responsiveness of spleen cells (left) and lymph node cells (right) of lethally irradiated (C57BL/Rij x CBA/Rij)F1 mice, reconstituted with 5×10^{7} C57BL/Rij spleen cells 3 to 6 days before cell transfer. After harvesting, the spleen and lymph node cells were treated in vitro with either ^{1}H-TdR (open columns) or ^{3}H-TdR (hatched columns) and subsequently inoculated into secondary C57BL/Rij recipients.
Each experimental group represents the average $\pm$ 1 SEM of a group of five mice.

DISCUSSION

This study shows that after semiallogeneic spleen cell transplantation into lethally irradiated mice, an antihost DTH responsiveness is built up in both spleen and lymph nodes. This DTH reactivity was previously shown to be mediated by T cells activated by and specific for the host histocompatibility antigens. The onset of DTH responsiveness in the lymph nodes is delayed as compared with the spleen (Fig. 3). This is not merely attributable to immigration into the lymph nodes of DTH effector cells generated in the spleen. This appears from the proliferative activity of the DTH effector cells in both organs. The proliferative activity is maximal in spleen and lymph nodes on days 3 and 4, respectively (Fig. 4). Then ^{3}H-TdR suicide *in vitro* did almost completely eliminate the antihost DTH reactivity from these organs. Previous studies dealing with cell cycle kinetics of antigen-activated B cells have shown that these cells have a generation time of 8.5 to 10 hr, with 74% of the cells being in S phase at any one moment (23). When the cycling time of antigen-activated T cells is of the same magnitude, a 1 hr pulse of highly active ^{3}H-TdR would account for elimination of most proliferating DTH-reactive cells.

A mitomycin C-mediated blockade of DNA synthetic activity could prevent the full development of antihost DTH reactivity in both spleen and lymph nodes (Fig. 3). In the spleen the inhibiting effect of mitomycin C was found on day 3 and later, while in the lymph nodes the effect of mitomycin C occurred on day 4 and later. This differential effect in spleen and lymph nodes suggests that the inhibition of the development of antihost DTH by mitomycin C is not merely because of a toxic effect, but indeed is related to proliferation. Lemmel and Good (24) have shown that the cross-linking of DNA by mitomycin C is not irreversible. The cells could repair this damage if not driven to proliferate immediately. The interval between treatment with mitomycin C and proliferation in our experiments appeared to be too short for such DNA repair.

Peak DTH reactivity in the spleen (day 4 after irradiaton and reconstitution) occurred before reaching maximal T cell numbers in this organ (Fig. 2). The underlying cause of this discrepancy is unclear. It is known that a specific immune response is generally accompanied by an aspecific immune response (25), possible because of the release of stimulatory products by the antigen-stimulated lymphocytes. These products can stimulate unrelated lymphocytes to proliferate (10). Therefore, the prolonged T cell proliferation after reaching peak DTH-

reactivity may be attributable to proliferation of T cells nonspecifically activated by the specifically activated DTH-reactive T cells. Alternatively, the increase of T cell numbers after reaching peak DTH reactivity might be because of the generation of other T cells, e.g., cytotoxic T cells (18,26). On the other hand, it is questionable whether the cytotoxic T cells proliferate during their generation (16).

The T cells which account for the early DTH reactivity after allogeneic spleen cell transplantation need not to proliferate, as appears after mitomycin C (25 µg/ml) inhibition of DNA synthesis (Fig. 3). This is in harmony with studies of Bennet and Bloom (27) and Phillips et al. (28) on the production of macrophage-inhibiting factor. These authors showed that sensitized lymphocytes can produce macrophage-inhibiting factor before proliferation. The relationship between mixed lymphocyte culture and DTH is apparent from studies of Phillips et al. (28), who showed that s.c. injection of mice with mixed lymphocytes culture supernatant, containing macrophage-inhibiting factor, can evoke a typical DTH response. The possibility that some cells escaped the antimitotic treatment seems unlikely, because pretreatment of cells with a dose of 1 µg of mitomycin C per ml can totally prevent proliferation (7). Incubation of spleen cells with 100 µg of mitomycin C per ml could completely prevent the development of a transferable antihost DTH reactivity in the recipient spleen (Fig. 3). A concentration of 100 µg of mitomycin C per ml not only blocks DNA synthesis, but also RNA synthesis and the larger part of protein synthesis (29). This suggests that RNA and protein synthesis are required for the appearance of the early, proliferation-independent antihost DTH reactivity in spleen and lymph nodes.

In conclusion, full development of specific antihost DTH responsiveness during an acute GvH reaction is dependent on proliferation of the reactive T cells. This holds for the immune response in both spleen and lymph nodes. Only during the first few days after induction of the GvH reaction does the antihost DTH response seem to be proliferation independent.

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APPENDIX PAPER III

FUNCTIONAL SEPARATION IN VIVO OF BOTH ANTIGENS ENCODED BY H-2 SUBREGION AND NON-H-2 LOCI

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Antigens coded for by the H-2K, H-2D and H-2I region of the major histocompatibility complex (MHC) of the mouse initiate different *in vitro* responses. I region-coded antigens activate mainly Lyt-1$^+$ T cells to produce a proliferative response in mixed lymphocyte cultures (MLC), whereas K and D region-coded antigens predominantly stimulate Lyt-2$^+$ T cells to become cytolytic effector cells (1). Proliferative responses in MLC and generation of cytolytic T cells *in vitro* can also be induced by minor histocompatibility antigens. For example, Mls-locus products can induce positive MLC responses, but not the generation of cytolytic T cells (2), whereas H-Y antigen does give rise to anti-H-Y cytotoxic T cell responses *in vitro*, following *in vivo* priming (3). Functional *in vivo* studies have shown that H-2K, H-2D and H-2I differences can account for graft rejection (4) and for mortality in Graft-versus-Host (GvH) reactions (5). Differences in only minor histocompatibility antigens are sufficient to cause graft rejection (6), and lethal GvH reaction after allogeneic bone marrow transplantation (7). Similarly, immunisation with only H-2 or only non-H-2 antigens can induce a state of delayed type hypersensitivity (DTH) to the immunising antigen, which can be measured with the footpad swelling test (8). So far, such *in vivo* experiments have shown little or no discrimination between responses to H-2 subregion antigens and responses to non-H-2 antigens. We have used a DTH assay to study the occurrence of T effector cells after *in vivo* immunisation with different histocompatibility antigens. We show here that DTH T-effector cells generated in GvH and Host-versus-Graft (HvG) reactions are specific for largely different sets of histocompatibility antigens, with selective stimulation by H-2I and Mls-locus antigens in GvH conditions.

We have developed a simple DTH assay which can be used to measure the development of T-effector cells induced by histocompatibility antigens during a GvH reaction (9,10). The assay is based on secondary transfer of lymphoid cells from animals undergoing a GvH reaction, and subsequent testing of the secondary

TABLE 1

ANTI-HOST IMMUNE REACTIVITY IN LETHALLY IRRADIATED RECIPIENTS AFTER SPLEEN CELL TRANSPLANTATION ACROSS H-2 SUBREGION DIFFERENCES

Inoculum	Recipient	H-2 subregion coding for immunising antigen[x]	Challenge	H-2 subregion coded antigens of cells used for challenge	DTH response
B10.A	B10.AQR	K	B10.AQR	K	3.2±1.6
B10.AQR	B10.A x B10.T(6R)	K+I	B10.A x B10.T(6R)	K+I	35.5±1.7
B10.AQR	B10.A x B10.T(6R)	K+I	B10.T(6R)	I	32.2±2.9
B10.AQR	B10.A x B10.T(6R)	K+I	B10.A	K	3.4±3.0
A.TH	A.TL	I(+Tla)	A.TL	I(+Tla)	31.4±2.6
A.TH	A.SW	D(+Tla)	A.SW	D(+Tla)	-0.?±1.4

GvH reactions were elicited by iv injection of 5×10^7 spleen cells (inoculum) into lethally irradiated recipient mice. Recipients were lethally irradiated (750 rad) within 4 h before reconstitution with the spleen cell inoculum; 5 d after reconstitution two-thirds of the total cell yield obtained from spleen, inguinal, axillary and mesenteric lymph nodes of a recipient mouse were transferred iv to a secondary recipient which was syngeneic to the original spleen cell donor. Lymphoid cells from all primary recipients of a particular combination were pooled before secondary transfer. Challenge was carried out with 2×10^7 (50 µl) spleen cells (treated with mitomycin C (Kyowa Hakko Kogyo) 100 µg ml^{-1} for 30 min at 37°C) injected sc into the instep of the right hind foot of the secondary recipients. At 24 h after challenge DTH responses were measured. Control mice only received a challenge dose. The swelling of these control mice was 12-20%. DTH responses are expressed as specific percentage increase in foot thickness, as described previously (9). Figures represent the arithmetic mean ± 1 SEM of five mice.

[x]H-2 subregion difference between spleen cell donor and irradiated recipient mice. The origin of the H-2 subregions (K, I-A, I-B, I-C, D) for the strains are as follows: A.TH, ssssd; A.SW, sssss; A.TL, skkkd; B10.A, kkkdd; B10.AQR, qkkdd; B10.T(6R), qqqqd.

recipients for DTH reactivity to the histocompatibility antigens which evoked the GvH reaction. Briefly, lymphoid cells from mice which have been irradiated and reconstituted with allogeneic spleen cells, are transferred intravenously (iv), at various intervals after reconstitution, into normal secondary recipients syngeneic to the original spleen cell donor. The secondary recipients are challenged in the right hind foot with 2×10^7 spleen cells syngeneic to the original irradiated recipients. The DTH response to this challenge is measured as the difference in thickness of the hind feet 24 h later. The specific increase in foot thickness is calculated as the relative increase in foot thickness of the immune mice minus the relative increase in foot thickness of control mice which have only received the challenge. The swelling of challenged control mice varies between 12 and 20%. Using this assay we studied the capacity of H-2 subregion-coded antigens and minor histocompatibility antigens to induce such T-effector cells after (semi-)allogeneic spleen cell transplantation in lethally irradiated mice. These results were correlated with the capacity of the same antigens to induce DTH-related T-effector cells during a HvG reaction.

In lethally irradiated congeneic mice, reconstituted with allogeneic spleen cells which were different at the whole or part of the H-2 complex, it could be demonstrated that the transferable anti-host DTH reactivity is directed exclusively to the I region of the H-2 complex; K and D subregion-coded antigens do not induce anti-host DTH T effector cells (Table 1).

H-2 compatible, non-H-2-incompatible spleen cell transplantation in lethally irradiated hosts revealed that antigens encoded by the Mls locus can also elicit the development of a transferable anti-host-directed DTH reactivity (Table 2). Mls^a and Mls^c coded antigens initiated both a positive MLC response and a distinct GvH-related DTH reactivity. Mls^b-locus antigens, on the other hand, were not able to initiate *in vitro* proliferation; this was associated with a marginal and short-lasting GvH-related DTH reactivity (Table 2).

Minor histocompatibility antigens other than Mls-locus products did not contribute to the expression of the anti-host DTH reactivity. This was shown by B10.D2 challenge of secondary BALB/c recipients of cells activated to DBA/2 non-H-2 antigens, and by B10.BR challenge of C3H/f and AKR recipients of cells activated to AKR and C3H/f, respectively. In this experiment the cells used for challenge had different Mls-locus antigens from the irradiated recipients. As there is extensive cross-reactivity in various mouse strains between non-H-2 alloantigens other than Mls-locus products (11), challenge with H-2 compatible, Mls locus-different spleen cells would demonstrate the contribution of these non-H-2 alloantigens in the expression of GvH-related DTH reactivity. No significant anti-host DTH reactivity could be elicited after challenge with B10.D2 or B10.BR spleen cells (Table 2). Therefore, non-H-2 alloantigens other than Mls locus-coded products probably do

TABLE 2

ANTI-HOST IMMUNE REACTIVITY IN LETHALLY IRRADIATED RECIPIENTS AFTER SPLEEN CELL TRANSPLANTATION ACROSS Mls-LOCUS DIFFERENCES

Inoculum	Recipient	Peak MLC response	Challenge	Mls locus-coded antigen of cells used for challenge	Peak DTH response
BALB/c	BALB/c x DBA/2	24,000±850 (4)	DBA/2	Mls^a	25.5±0.7(5)
BALB/c	BALB/c x DBA/2	-	B10.D2	Mls^b	0.5±1.2(5)
DBA/2	BALB/c x DBA/2	630±280 (3)	BALB/c	Mls^b	10.6±2.2(3)
AKR/FuRdA	C3Hf	22,300±1300(3)	C3H/f	Mls^c	25.8±1.8(7)
AKR/FuRdA	C3H/f	-	B10.BR	Mls^b	2.9±1.1(7)
C3H/f	AKR/FuRdA	38,000±2900(4)	AKR/FuRdA	Mls^a	26.7±2.0(7)
C3H/f	AKR/FuRdA	-	B10.BR	Mls^b	1.9±1.7(7)

GvH reactions were elicited by iv injection of 5 x 10^7 spleen cells (inoculum) into lethally irradiated recipient mice. The designation for H-2 haplotype and Mls locus for the strains are as follows: BALB/c, $H\text{-}2^d$, MLS^b; DBA/2, $H\text{-}2^d$, Mls^a; BALB/c x DBA/2, $H\text{-}2^{d/d}$, $Mls^{b/a}$; B10.D2, $H\text{-}2^d$, Mls^a; AKR/FuRdA, $H\text{-}2^k$, Mls^a; C3H/f, $H\text{-}2^k$, Mls^c; B10.BR, $H\text{-}2^k$, Mls^b. Recipients were lethally irradiated (850 rad) before reconstitution. At different intervals after reconstitution a number of spleen cells equivalent to one spleen was transferred iv into secondary recipients which were syngeneic to the original spleen cell donors. Peak one-way MLC responses are expressed as specific counts per min. Figures represent the arithmetic mean ± 1 SEM of a quadruplicate microculture. The day of peak MLC response is given in parentheses. Each culture consisted of 5 x 10^5 responder spleen cells and 5 x 10^5 stimulator (treated with 25 µg mitomycin C per ml) spleen cells. Activity of responder cells alone was 250-1,200 cpm and activity of mitomycin C-treated stimulator cells 50-300 cpm. Background activity was 35 cpm. Details of the technique will be published elsewhere (15). Challenge was carried out as described in Table 1 legend. Peak DTH responses are expressed as the specific percentage increase in foot thickness. Figures represent the arithmetic mean ± 1 SEM of five mice. The day of peak DTH response is given in parentheses.

TABLE 3

ANTI-GRAFT IMMUNE REACTIVITY AFTER IMMUNISATION WITH SPLEEN CELLS DIFFERING AT H-2 SUBREGIONS OR MINOR HISTOCOMPATIBILITY LOCI

Immunisation	Responder	Challenge	H-2 subregion or Mls locus-coded antigens of cells used for challenge	DTH response
B10.AQR	B10.A	B10.AQR	K	39.2±2.8
B10.AQR	B10.T(6R)	B10.AQR	I	38.0±3.2
A.TL	A.TH	A.TL	I(+Tla)	33.6±3.0
A.SW	A.TH	A.SW	D(+Tla)	26.6±2.3
BALB/c x DBA/2	BALB/c	DBA/2	Mls^a	39.2±2.2
BALB/c x DBA/2	BALB/c	B10.D2	Mls^b	28.4±3.0
BALB/c x DBA/2	DBA/2	BALB/c	Mls^b	44.4±2.8
AKR/FuRdA	C3H/f	AKR/FuRdA	Mls^a	31.0±2.2
AKR/FuRdA	C3H/f	B10.BR	Mls^b	22.1±2.0
C3H/f	AKR/FuRdA	C3H/f	Mls^c	34.6±2.4
C3H/f	AKR/FuRdA	B10.BR	Mls^b	28.8±1.5

Immunisation was carried out with 10^7 spleen cells sc, equally distributed over the inguinal areas by means of a 28-gauge needle. For the origin of H-2 subregions and Mls locus see legends to Tables 1 and 2. Challenge was carried out as described in the Table 1 legend. DTH responses were assayed 5 d after sc immunisation of the responder mice and expressed as the specific percentage increase in foot thickness. Figures represent the arithmetic mean ± 1 SEM of five mice.

not evoke anti-host DTH reactivity. Apparently, Mls-locus products and I-region antigens have a similar capacity for inducing anti-host-directed DTH T effector cells. K and D region antigens and non-H-2 antigens other than Mls-locus products are much less able to do so.

In contrast, in HvG reactions induced by subcutaneous (sc) injection of allogeneic spleen cells, K, D and I region-coded antigens as well as non-H-2 histocompatibility antigens could give rise to anti-graft DTH T-effector cells (Table 3). Also, minor histocompatibility antigens other than Mls-locus products contribute to the expression of the anti-graft DTH reactivity (Table 3). According to the work of Vadas et al. (12), there may be two subsets of T cells mediating DTH: Lyt-1^+ and Lyt-2^+ T cells, which are restricted by the I or the K and D regions of the MHC, respectively. Probably in GvH conditions only Lyt-1^+ DTH T-effector cells are activated (anti-H-2I and anti-Mls response) and in HvG conditions both Lyt-1^+ and Lyt-2^+ DTH T-effector cells are activated. This might be related to the frequency of T cells reactive to the various histocompatibility antigens (13,14).

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APPENDIX PAPER IV

DIFFERENTIAL RESPONSIVENESS TO H-2 SUBREGION CODED ANTIGENS IN GRAFT-VERSUS-HOST AND HOST-VERSUS-GRAFT REACTIONS

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SUMMARY

After transplantation of allogeneic lymphoid cells into lethally irradiated mice, the development of anti-host-directed T effector cells can be demonstrated by means of a delayed type hypersensitivity (DTH) assay. Using this assay we have shown that after transplantation of allogeneic spleen cells into lethally irradiated mice, the anti-host DTH reactivity is directed exclusively to the I region of the H-2 complex. H-2K and H-2D subregion coded antigens do not induce anti-host DTH T effector cells. In contrast, in the Host-versus-Graft (HvG)-DTH reaction, H-2I as well as H-2K and H-2D region coded antigens induce anti-graft DTH T effector cells.

Preimmunization of the donor mice with different H-2 subregion coded antigens does not influence the DTH responsiveness to these antigens under Graft-versus-Host (GvH) conditions. The same was found for anti-H-2I HvG-DTH responsiveness. However, the capacity to respond to H-2K and H-2D coded antigens in a HvG-DTH reaction is markedly enhanced by preimmunization. These results suggest that under GvH conditions only one subset of precursors of DTH T effector cells is activated (presumably anti-H-2I Lyt-1^+ T cells), whereas under HvG conditions two T cell subsets can give rise to DTH T effector cells (presumably anti-H-2I Lyt-1^+ T cells and anti-H-2K/D Lyt-2^+ T cells).

INTRODUCTION

The capacity of different H-2 subregion coded antigens to elicit Graft-versus-Host (GvH) reactions has been studied *in vivo* in various assay systems. Measurements of splenomegaly, lymph node enlargment and increased radioactive IUdR incorporation in spleen and lymph nodes following injection of histoincompatible immunocompetent cells, all revealed similar results (1,2,3). I-region coded antigens appeared to be very potent in eliciting GvH reactions, whereas K and D region coded antigens appeared to

be relatively weak. In contrast, in mortality studies of the GvH reaction, disparity for K, I and D caused similar mortality rates (4). A drawback of all these assays is that the specific anti-host immune response itself can not be measured. In Host-versus-Graft (HvG) reactions measurement of the specific anti-graft immune response is much more simple. In skin graft rejection studies (5), and for anti-graft delayed type hypersensitivity (DTH) after skin graft transplantation (6), similar responses were found to K, I and D subregion coded antigens, suggesting, but not proving, an equal immunogenicity of these antigens. In view of the lack of data on the specific anti-host immune response during GvH reactions it is not clear whether there is a discrepancy between the responses to the various H-2 subregion coded antigens under GvH and HvG conditions.

Recently, we have developed a DTH assay which can be used to measure the development of specific anti-host T effector cells, induced by histocompatibility (H) antigens during a GvH reaction (7). Using this assay we have studied the capacity of H-2 subregion coded antigens to induce such anti-host T effector cells after allogeneic and semi-allogeneic spleen cell transplantation into lethally irradiated mice. These results were correlated with the capacity of the same antigens to induce DTH-related T effector cells during a HvG reaction.

MATERIALS AND METHODS

Animals. C57BL/Rij ($H\text{-}2^b$) mice were purchased from the Medical Biological Laboratory TNO, Rijswijk (ZH), The Netherlands. B10.AQR ($H\text{-}2^{Y1}$), B10.T(6R) ($H\text{-}2^{Y2}$), B10.A ($H\text{-}2^a$), B10.G ($H\text{-}2^q$), A.SW ($H\text{-}2^s$), A.TL ($H\text{-}2^{t1}$) and A.TH ($H\text{-}2^{t2}$) mice were commercially obtained from OLAC Ltd., Bicester, United Kingdom. (B10.A x B10.T(6R))F1 ($H\text{-}2^{a/Y2}$) mice were bred at our own department. The origin of the H-2 subregions for the congenic strains used are shown in Table 1. The age of all mice varied between 12 and 20 weeks. When mice from either sex were used in one experiment, the combinations were always chosen in such a way that anti-H-Y responses were impossible.

Preparation of cell suspensions. Mice were killed by carbon dioxyde. Immediately after killing, the organs were prepared for single cell suspensions as described previously (7). Nucleated cells were counted with a Coulter Counter Model B.

Irradiation. For lethal irradiation of mice a dose of 700 rad whole body X-irradiation was applied, generated in a Philips Müller MG 300 X-ray machine as described previously (7).

Acute Graft-versus-Host reaction. Acute Graft-versus-Host (GvH) reactions were elicited by intravenous (iv) injection of 5×10^7 nucleated spleen cells into lethally irradiated allogeneic or semiallogeneic recipient mice within 4 hr after irradiation. The cells to be injected were suspended in a volume of 0.5 ml of a balanced salt solution (BSS).

Host-versus-Graft reaction. Host-versus-Graft (HvG) reactions were elicited by subcutaneous (sc) injection of 10^7 histoincompatible spleen cells in a volume of 0.1 ml of BSS, equally distributed over both inguinal areas.

Preimmunization for H-2 subregion coded antigens. In order to enlarge the pool of potentially reacting cells against H-2 subregion coded antigens, an *in vivo* cell transfer system was used. Thus, 15×10^7 spleen cells were iv injected into syngeneic recipients which were lethally irradiated 2 hr previously. Preimmunization was performed by intraperitoneal (ip) injection of these recipients with 5×10^7 spleen cells which were incompatible for a certain H-2 subregion with the irradiated recipient. After two weeks these recipients were tested for the capacity of GvH- and HvG-DTH reactivity. GvH reactivity of spleen cells from these preimmunized recipients was tested by transplantation into appropriate irradiated hosts.

TABLE 1

MHC ALLELES OF INBRED CONGENIC MOUSE STRAINS USED

Strain	MHC regions or subregions							
	K	I-A	I-B	I-J	I-E	I-C	S	D
A.TH	s	s	s	s	s	s	s	d
A.TL	s	k	k	k	k	k	k	d
A.SW	s	s	s	s	s	s	s	s
B10.A	k	k	k	k	k	d	d	d
B10.AQR	q	k	k	k	k	d	d	d
B10.T(6R)	q	q	q	q	q	q	q	d
B10.G	q	q	q	q	q	q	q	q

From Klein et al. (8).

Assay for delayed type hypersensitivity. The delayed type hypersensitivity (DTH) assay for measuring anti-host immune reactivity after allogeneic spleen cell transplantation has been described in detail in a previous paper (7). Briefly, a number of cells equivalent to the total cell yield obtained from spleen, inguinal, axillary and mesenteric lymph nodes from an irradiated and allogeneically reconstituted recipient mouse was transferred iv into a normal secondary recipient at different intervals after reconstitution. This secondary recipient was syngeneic to the original spleen cell donor mouse. The secondary recipient mice were challenged into the right hind foot with 2×10^7 spleen cells (treated with 100 µg mitomycin C per ml), syngeneic with the irradiated recipient. The DTH response to this challenge was measured as the difference in thickness of the hind feet 24 hr later. The specific increase in foot thickness was calculated as the relative increase in foot thickness of the immune mice minus the relative increase in foot thickness of control mice which only received the challenge. The swelling of challenged control mice varied between 12 and 20%.

RESULTS

Development of GvH-related DTH reactivity after irradiation and H-2 subregion incompatible spleen cell transplantation. The contribution of different H-2 subregion coded antigens in the development of specific anti-host immune reactivity was studied by transplantation of spleen cells into lethally irradiated recipients incompatible for defined subregions. For the H-2I subregion, 5×10^7 B10.AQR and A.TH spleen cells were transplanted into B10.T(6R) and A.TL recipient mice, respectively. At different intervals after reconstitution, a number of cells equivalent to the total cell yield obtained from spleen, inguinal, axillary and mesenteric lymph nodes from the recipient B10.T(6R) and A.TL mice was transferred into normal B10.AQR and A.TH recipients, respectively. These secondary recipients were challenged with 2×10^7 B10.T(6R) and A.TL spleen cells, respectively. As can be seen in Fig. 1, the H-2I subregion coded antigens evoked an anti-host DTH reactivity in both donor-recipient combinations. The maximum response occurred on day 5 after reconstitution. Thereafter the response decreased. By day 10 no significant DTH response could be detected any more.

Similarly, the capacity of H-2K and H-2D coded antigens to induce anti-host immune reactivity was studied. For H-2K, lethally irradiated B10.AQR mice were reconstituted with 5×10^7 B10.A

spleen cells, while for H-2D lethally irradiated A.TH mice were reconstituted with 5 x 10^7 A.SW spleen cells. In both combinations no or only marginal anti-host DTH responses were elicited throughout the experimental period.

The contribution of different H-2 subregion antigens in the development of specific anti-host immune reactivity was also studied after spleen cell transplantation across a combined H-2I and H-2K difference. Therefore lethally irradiated (B10.A x B10.T(6R))F1 hybrid mice were reconstituted with 5 x 10^7 B10.AQR spleen cells. At 5 days after reconstitution, pooled spleen, peripheral and mesenteric lymph node cells from these recipients were transferred iv into normal B10.AQR mice. Different groups of these secondary recipients were challenged with either (B10.A x B10.T(6R))F1, B10.T(6R) or B10.A spleen cells, in order to test the GvH reactivity against H-2K+I, H-2I and H-2K, respectively. As can be seen in Table 2, in a fully H-2 incompatible combination apparently only H-2I region coded antigens contribute to the expression of the anti-host DTH responsiveness.

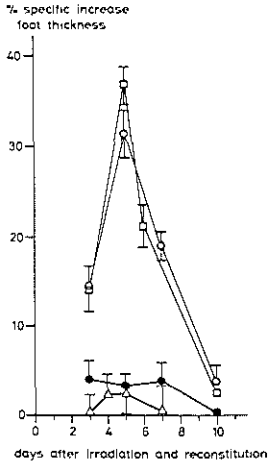

Figure 1
Development of anti-host DTH reactivity in the spleen of lethally irradiated B10.T(6R) mice inoculated with B10.AQR spleen cells (□), in the spleen of lethally irradiated A.TL mice inoculated with A.TH spleen cells (○), in the spleen of lethally irradiated B10.AQR mice reconstituted with B10.A spleen cells (●) and in the spleen of lethally irradiated A.TH mice reconstituted with A.SW spleen cells (Δ). All transplants consisted of 5 x 10^7 nucleated cells. Each experimental point represents the arithmetic mean ± 1 SEM of a group of at least 5 mice.

TABLE 2

RELATIVE CONTRIBUTION OF H-2I AND H-2K REGION CODED ANTIGENS IN THE EXPRESSION OF GVH- AND HVG-RELATED DTH

System	Inoculum	Recipient	Challenge	H-2 subregion coded antigens used for challenge	DTH response
GvH[a]	B10.AQR	B10.A x B10.T(6R)	B10.A x B10.T(6R)	K + I	35.5 ± 1.7[c]
	B10.AQR	B10.A x B10.T(6R)	B10.T(6R)	I	32.2 ± 2.9
	B10.AQR	B10.A x B10.T(6R)	B10.A	K	3.4 ± 3.0
System	Immunizing cells	Responder	Challenge	H-2 subregion coded antigens used for challenge	DTH response
HvG[b]	B10.A x B10.T(6R)	B10.AQR	B10.A x B10.T(6R)	K + I	46.9 ± 2.0
	B10.A x B10.T(6R)	B10.AQR	B10.T(6R)	I	36.1 ± 1.3
	B10.A x B10.T(6R)	B10.AQR	B10.A	K	39.7 ± 1.5

a. GvH reactions were elicited by iv injection of 5×10^7 spleen cells (inoculum) into lethally irradiated recipient mice. At 5 days after reconstitution a number of cells equivalent to the total cell yield obtained from spleen, peripheral and mesenteric lymph nodes of a recipient mouse was transferred iv to a non-irradiated secondary recipient, syngeneic to the original spleen cell donor. These secondary recipient mice were challenged with 2×10^7 spleen cells.

b. HvG reactions were elicited by sc injection of 10^7 spleen cells equally distributed over the inguinal areas. Five days later these mice were challenged with 2×10^7 spleen cells.

c. DTH responses are expressed as the specific percentual increase in foot thickness. Figures represent the arithmetic mean ± 1 SEM of 5 mice.

Primary DTH responsiveness to H-2 subregion incompatible spleen cells.

The capacity of different H-2 subregion coded antigens to induce a DTH response was also studied under Host-versus-Graft (HvG) conditions. For the H-2I subregion coded antigens, B10.T(6R) and B10.AQR mice were subcutaneously (sc) immunized with 10^7 B10.AQR and B10.T(6R) spleen cells, respectively. At varying intervals after immunization, separate groups of mice were challenged with similar spleen cells as used for immunization. In both these combinations the DTH reactivity started to increase at 3 days after immunization (Fig. 2). Maximum responses were observed on day 5 after immunization. Thereafter the primary DTH reactivity persisted on a plateau level. At 130 days after immunization of B10.T(6R) mice with H-2I incompatible B10.AQR cells, still a quite high level of DTH reactivity could be detected. The capacity of H-2K and H-2D subregion coded antigens to induce a HvG reaction was also tested in this DTH assay. For H-2K coded antigens, B10.A and B10.AQR mice were sc immunized with 10^7 B10.AQR and B10.A spleen cells, respectively. For H-2D the A.TH versus A.SW combination was used. In all three combinations the DTH reactivity started to increase on day 3 after immunization and reached the maximum value on day 5. Thereafter the DTH reac-

tivity steadily declined. By day 12 no significant DTH reactivity could be detected any more (Fig. 2). Immunization with H-2K region different spleen cells induced an equally high maximum DTH response as H-2I region different spleen cells. After immunization with H-2D region different spleen cells, the maximum response was lower (Fig. 2).

The relative contribution of H-2K and H-2I region coded antigens to the development of primary DTH responsiveness was studied after immunization with spleen cells differing at both H-2I and H-2K. Therefore, B10.AQR mice were sc immunized with 10^7 (B10.A x B10.T(6R))F1 spleen cells. At 5 days after immunization different groups of mice were challenged with either (B10.A x B10.T (6R))F1, B10.T(6R) or B10.A spleen cells, in order to test the HvG reactivity against H-2K+I, H-2I and H-2K, respectively. As can be seen in Table 2, H-2I and H-2K coded antigens elicited an equally high DTH response after immunization across a H-2I+K barrier. Challenge with spleen cells incompatible for both the H-2K and H-2I caused only a small increase of the DTH response as compared to challenge with H-2I or H-2K only. Probably, in HvG reactions H-2I and H-2K region coded antigens elicit DTH effector cells with the same vigour. This stands in contrast to the development of GvH-related DTH reactivity (Table 2).

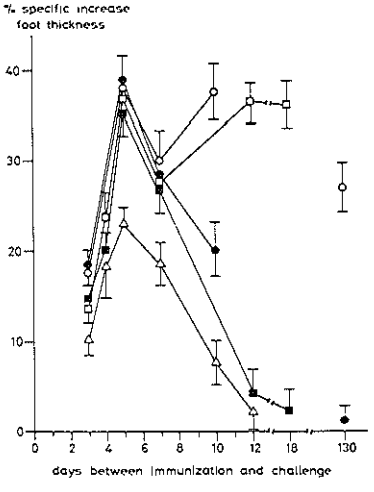

Figure 2
Primary DTH responsiveness of B10.T(6R) mice against B10.AQR cells (○), B10.AQR mice against B10.T(6R) cells (□), B10.A mice against B10.AQR cells (●), B10.AQR mice against B10.A cells (■) and A.TH mice against A.SW cells (△). All immunizations were done by sc injection of 10^7 spleen cells. Each experimental point represents the arithmetic mean ± 1 SEM of a group of at least 5 mice.

Influence of preimmunization against H-2 subregions on the development of GvH- and HvG-related DTH reactivity.
The question arises as to whether the absence of anti-host DTH reactivity after spleen cell transplantation across a H-2K or H-2D difference (Fig. 1) is due to an inability of the H-2K/D antigens to induce GvH-related DTH, or is due to a shortage of H-2K/D reactive T cells in the allogeneic spleen cell inoculum. In order to discriminate between these two possibilities we selectively enlarged the pool of H-2K and H-2D reactive T cells by preimmunization of the donor mice. This was done as described in the Materials and Methods section. A number of 5×10^7 spleen cells from A.SW mice preimmunized against A.TH, and of B10.AQR mice preimmunized against B10.A, were transplanted into lethally irradiated A.TH and B10.A mice, respectively. The anti-host DTH reactivity was tested 5 days after transplantation. As can be seen in Table 3, preimmunization against H-2K or H-2D region coded antigens did not enhance the capacity of anti-host immune reactivity. Preimmunization against H-2I coded antigens in a B10.AQR versus B10.T(6R) combination also did not increase the anti-host immune reactivity.

TABLE 3

INFLUENCE OF PREIMMUNIZATION AGAINST H-2 SUBREGIONS ON THE DEVELOPMENT OF GvH- AND HvG-RELATED DTH

System	Inoculum	Preimmunization	Recipient	H-2 subregion coding for immunizing antigen	DTH response
	A.SW	-	A.TH	D	2.2 ± 2.4[d]
	A.SW	A.TH[c]	A.TH	D	0.6 ± 1.8
	B10.AQR	-	B10.T(6R)	I	37.0 ± 2.6
GvH[a]	B10.AQR	B10.T(6R)	B10.T(6R)	I	26.8 ± 4.4
	B10.AQR	-	B10.A	K	4.0 ± 2.1
	B10.AQR	B10.A	B10.A	K	2.4 ± 1.4
System	**Responder**	**Preimmunization**	**Immunizing cells**	**H-2 subregion coding for immunizing antigen**	**DTH response**
	A.SW	-	A.TH	D	10.3 ± 1.8
	A.SW	A.TH	A.TH	D	40.8 ± 3.0
	B10.AQR	-	B10.T(6R)	I	17.2 ± 1.6
HvG[b]	B10.AQR	B10.T(6R)	B10.T(6R)	I	17.6 ± 2.0
	B10.AQR	-	B10.A	K	20.4 ± 1.8
	B10.AQR	B10.A	B10.A	K	45.0 ± 3.1

a. GvH reactions were elicited by iv injection of 5×10^7 spleen cells from normal or preimmunized donor mice into lethally irradiated recipient mice. At 5 days after reconstitution the anti-host DTH responsiveness was determined as described in the legend of Table 2.

b. HvG reactions were elicited by sc injection of 10^7 spleen cells into normal or preimmunized responder mice. These mice were challenged with 2×10^7 spleen cells 3 days after immunization.

c. Preimmunization of spleen cell donor mice in the GvH system, and of responder mice in the HvG system was performed as described in the Materials and Methods section.

d. DTH responses are expressed as the specific percentual increase in foot thickness. Figures represent the arithmetic mean ± 1 SEM of 5 mice.

Under HvG conditions, preimmunization against H-2K and H-2D region coded antigens did increase the DTH response. This is apparent from the secondary response of A.SW mice against A.TH cells, and of B10.AQR mice against B10.A cells. When the responder mice were challenged with the homologous cells as early as 3 days after the booster injection, the primed mice showed a clearly enhanced DTH response as compared to the response by non-primed mice (Table 3). In contrast, preimmunization against H-2I region coded antigens did not enhance the DTH responsiveness. In order to compare the results obtained in the GvH and HvG systems, the capacity of spleen cells from primed mice to give rise to an enhanced HvG-DTH reactivity was also tested in an adoptive transfer system. After transfer of spleen cells from mice primed towards H-2K antigens into lethally irradiated syngeneic recipient mice, similar results were obtained after booster and challenge of these recipient mice as after booster and challenge of the primed mice themselves (data not shown).

DISCUSSION

This study shows that there is a difference between H-2I and H-2K/D region coded antigens in their capacity to elicit anti-host directed DTH reactivity after allogeneic spleen cell transplantation. On the other hand, both types of antigens are able to evoke anti-graft DTH responses. After transplantation of spleen cells into lethally irradiated hosts differing at the H-2I subregion, a clear anti-host DTH response was found with a maximum at 5 days after irradiation and reconstitution (Fig. 1). H-2K and H-2D region coded antigens, however, did not induce anti-host DTH T effector cells. These results were confirmed by the finding that after spleen cell transplantation across a H-2K plus H-2I barrier only the proliferation-inducing (9) H-2I region coded antigens did elicit the expression of the anti-host immune reactivity as a DTH response (Table 2). Similar results were obtained after H-2 compatible, non-H-2 incompatible spleen cell transplantation into lethally irradiated hosts. There we found that the anti-host DTH T effector cells were directed almost exclusively to other proliferation-inducing (10) histocompatibility antigens, namely those encoded for by the Mls locus (11). Non-H-2 alloantigens other than Mls locus coded products did hardly or not evoke anti-host DTH reactivity (11). Thus, the inability of H-2K/D region products (9) and non-H-2 alloantigens other than Mls locus products (10) to stimulate efficiently T cell proliferation *in vitro* coincides with the inability to evoke DTH T effector cells during a GvH reaction (11; and Fig. 1). These findings about GvH-related DTH reactivity correlate

well with the fact that primarily a disparity for the I-region leads to the production of chemotactic activity in the supernatant of a mouse MLC (12). Such chemotactic agents are needed for the effector phase of DTH (13). In contrast, disparities for H-2K and H-2D, but not for the H-2I or Mls locus, lead to the production of interferon in mouse MLC (14).

Sc immunization of mice with spleen cells differing at both H-2K and H-2I induced an anti-I and anti-K DTH response of the same strength (Table 2). These results suggest that under HvG conditions, two subsets of T cells mediating DTH are activated, namely anti-H-2I Lyt-1^+ T cells and anti-H-2K/D Lyt-2^+ T cells. Evidence for this supposition comes from the work of Smith and Miller (15). They demonstrated that both Lyt-1^+ and Lyt-2^+ T cells were responsible for DTH reactivity in mice sc immunized with fully histoincompatible spleen cells. Furthermore, T cells mediating DTH to dinitrofluorobenzene (DNFB) recognize DNFB in mice which are only compatible for either K, I or D (16), suggesting that there are two subsets of T cells which can mediate DTH, namely Lyt-1^+ and Lyt-2^+ cells which are restricted by the H-2I and H-2K/D regions, respectively. Our finding that H-2K and H-2D region coded antigens did not give rise to any measurable anti-host immune responsiveness, might be ascribed to the relatively small pool of anti-H-2K or anti-H-2D reactive T cells. After enlargement of the pool of potentially anti-H-2K/D reactive cells, also no measurable anti-host immune responsiveness could be obtained (Table 3). In contrast, under HvG conditions preimmunization of responder mice against H-2K or H-2D region coded antigens did result in a clear secondary type DTH response. This suggests that under GvH conditions not a quantitative but a qualitative incapability exists to induce anti-host DTH reactivity against H-2K and H-2D region coded antigens. Probably, under GvH conditions may be only Lyt-1^+ DTH T effector cells are activated (anti-H-2I response). Remarkable, both in GvH and in HvG conditions preimmunization against H-2I did neither potentiate the anti-host, nor the anti-graft DTH responsiveness (Table 3). Probably the frequency of specifically anti-H-2I reactive T cells is already so high in nonsensitized mice that they hardly increase in frequency.

Recently it was shown that the only cells which can proliferate and generate cytotoxic effector cells in a murine MLC across a disparity at the H-2K or H-2D region are Lyt-123^+ cells (17,18). Possibly, under GvH conditions elicited by transplantation of H-2K or H-2D different, but H-2I identical spleen cells in irradiated hosts, Lyt-123^+ cells are driven by the excess of antigen to become cytotoxic active cells, whereas under the limited

antigenic pressure in HvG conditions these Lyt-123$^+$ cells become DTH reactive cells. This would be in harmony with the work of Kaufman et al. (19) who showed that Lyt-123$^+$ cells could mediate DTH under HvG conditions.

The kinetics of the HvG-DTH response was found to be highly dependent on the H-2 subregion incompatibility between graft and recipient. After sc immunization with spleen cells differing at the H-2I subregion, anti-graft DTH responsiveness persisted for at least 130 days. This stands in clear contrast to the response to sc administered spleen cells differing at H-2K or H-2D subregions. In this latter case anti-graft DTH reactivity could no longer be detected already at 12 days. Probably sc administered spleen cells differing at H-2I region can persist for long periods in a normal host, thereby continuously activating new H-2I reactive T cells for DTH. This stands in contrast to H-2K and H-2D different spleen cells. Such cells probably elicit cytotoxic reactivity in the host; and by this cytotoxic reactivity the transplanted cells are eliminated after some time.

In conclusion, this paper shows that stimulation by H-2I region coded antigens is essential for the development of anti-host DTH effector cells after transplantation of spleen cells into lethally irradiated, H-2 incompatible recipients. In such GvH reactions H-2K and H-2D region coded antigens do not give rise to DTH T effector cells. In contrast, in HvG reactions both H-2I and H-2K/D region coded antigens are fully capable of inducing the generation of DTH T effector cells.

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APPENDIX PAPER V

DIFFERENTIAL RESPONSIVENESS TO MLS LOCUS ANTIGENS IN GRAFT-VERSUS-HOST AND HOST-VERSUS-GRAFT REACTIONS

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SUMMARY

After (semi-)allogeneic transplantation of lymphoid cells into lethally irradiated mice, the development of anti-host directed T effector cells can be demonstrated by means of a simple delayed type hypersensitivity (DTH) assay. Using this assay we have shown that in H-2 compatible combinations Mls locus antigens can induce the generation of such T effector cells during a graft-versus-host (GvH) reaction. Other non H-2 alloantigens are probably of minor importance. The capacity of Mls locus antigens to induce distinct anti-host DTH reactivity correlated with the capacity to induce a one-way mixed lymphocyte culture (MLC) response. Mls^a and Mls^c locus antigens initiated both a positive MLC response as well as distinct GvH-related DTH reactivity. On the other hand, in the combination DBA/2 *versus* (BALB/c x DBA/2)F1, the Mls^b locus antigen was not able to initiate *in vitro* proliferation, a lack of response which coincided with a marginal and short-lasting GvH-related DTH reactivity.

In contrast, the host-versus-graft (HvG) DTH reaction of BALB/c and DBA/2 mice to subcutaneously injected (BALB/c x DBA/2)F1 spleen cells was equally strong. Here antigens other than those coded for by the Mls locus were mainly responsible for the anti-graft DTH response. These results suggest that T effector cells generated in GvH and HvG reactions are specific for largely different sets of minor histocompatibility antigens, with a selective stimulation by Mls locus antigens under GvH conditions.

INTRODUCTION

Not only the major histocompatibility complex (MHC) of the mouse encodes for antigens that stimulate T lymphocytes to strong proliferative responses in a mixed lymphocyte culture (MLC) test, but also the products of the Mls locus, which is neither linked to nor part of the MHC, can strongly stimulate in a MLC test (1).

Injection of lymphocytes into Mls locus incompatible neonatal F1 recipients did not give rise to splenomegaly (2), probably due to non-expression of Mls locus determinants on neonatal cells (3). Testing of MHC compatible, Mls locus incompatible lymphocytes in a popliteal lymph node assay, showed a graft-versus-host (GvH) reactivity (2,4).

In the host-versus-graft (HvG) popliteal lymph node assay CBA/H x (CBA/H x DBA/2)F1 backcross derived cells, injected into parental CBA/H mice, induced an equally large proliferative response to H-2 identical Mls locus compatible and to H-2 identical, Mls locus incompatible donor cells (2). In a comparable approach, Festenstein et al. (5) have shown that parental mice rejected skin grafts from Mls locus identical backcross mice in about the same time as skin grafts from Mls locus different backcross mice. Eichwald and Weissman (6) parabiosed the MHC identical, but Mls locus different BALB/c and DBA/2 mice. After some time the DBA/2 partner, but not the BALB/c, showed symptoms of severe parabiotic intoxication. Nisbet and Edwards (7), however, parabiosed BALB/c parents to BALB/c x (BALB/c x DBA/2) F1 backcross mice, both in Mls locus compatible and in Mls locus incompatible combinations. These authors found an equal mortality in both groups, indicating that differences at Mls loci are not implicated in mortality. Thus, the role of incompatibilities outside the MHC in the development of T effector cells for GvH and HvG reactivity is still unclear.

Recently we developed a simple delayed type hypersensitivity (DTH) assay which is appropriate for measuring the development of histocompatibility antigen-induced T effector cells during a GvH reaction (8,9). The assay is based upon secondary transfer of lymphoid cells from animals undergoing a GvH reaction, and subsequent testing of the secondary recipients for DTH reactivity to the histocompatibility antigens which evoked the GvH reaction. Initiation and transfer of the GvH-related DTH reactivity is highly dependent on Thy-1.2^{+} cells (8). Using this assay we then studied the capacity of Mls locus antigens to induce such DTH T effector cells after (semi-)allogeneic spleen cell transplantation in lethally irradiated mice. The results are correlated with the capacity of the same antigens to induce DTH related T effector cells during a HvG reaction.

MATERIALS AND METHODS

Animals. BALB/c ($H\text{-}2^d$, Mls^b) female mice and DBA/2 ($H\text{-}2^d$, Mls^a) male mice were purchased from the Medical Biological Laboratory TNO, Rijswijk (ZH), The Netherlands. (BALB/c x DBA/2)F1 ($H\text{-}2^d$, Mls^b/Mls^a) female mice were obtained from the Radiobiological Institute TNO, Rijswijk (ZH), The Netherlands. B10.D2 ($H\text{-}2^d$, Mls^b) and B10.BR ($H\text{-}2^k$, Mls^b) male mice were kindly provided by the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands. C3H/f ($H\text{-}2^k$, Mls^c) male mice and (C57BL/Rij x CBA/Rij)F1 ($H\text{-}2^b/H\text{-}2^q$) female mice were bred at the Laboratory Animals Centre of the Erasmus University, Rotterdam, The Netherlands. (BALB/c x C3H/f)F1 ($H\text{-}2^d/H\text{-}2^k$, Mls^b/Mls^c) female and AKR/FuRdA ($H\text{-}2^k$, Mls^a) male mice were bred at our own department. The age of the mice varied between 15 and 30 weeks. When mice of either sex were used in one experiment, the combinations were always chosen in such a way that anti H-Y responses were impossible.

Preparation of cell suspensions. Mice were killed by carbon dioxide. Immediately after killing the mice, the spleens were removed and brought into a balanced salt solution (BSS). This solution was prepared according to Mishell and Dutton (10). The spleens were minced with scissors and squeezed through a nylon or stainless steel filter to give a single cell suspension. Nucleated cells were counted with a Coulter Counter Model B.

Irradiation. The recipient mice received 850 rad whole body irradiation, generated in a Philips Müller MG 300 X-ray machine as described previously (8).

Acute GvH reaction. Acute GvH reactions were elicited by intravenous (iv) injection of 5×10^7 nucleated spleen cells into lethally irradiated recipient mice within 4 hr after irradiation. The cells to be injected were suspended in a volume of 0.5 ml of BSS.

Immunization. Primary immunization with histocompatibility antigens was performed with 10^7 spleen cells in a volume of 0.1 ml of BSS. The cells were injected subcutaneously (sc), and equally distributed over both inguinal areas by means of a 28-gauge needle.

Mitomycin C treatment. Treatment of spleen cells with 25 or 100 µg mitomycin C (Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan) per ml was done as described previously (8).

Estimation of cell proliferation in vitro. Washed cell suspensions were filtered through sterile nylon wool in order to remove clumps and tissue debris, and cultured in RPMI 1640 supplemented with Penicillin (100 U/ml), Streptomycin (100 µg/ml), L-glutamine (2 mM) and 10% fresh normal mouse serum. Cultures were set up in Costar 3596 flat bottom wells (Cambridge, Mass., U.S.A.). A number of 5×10^5 (100 µl) responder cells (R) and 5×10^5 (100 µl) stimulator cells (S) (treated with 25 µg mitomycin C per ml) were dispersed into each well, so that quadruplicate cultures were plated for each combination. Control cultures consisted of 10^6 responder cells or 10^6 stimulator cells alone. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Seventeen hours prior to termination, 1 µCi of ^{3}H-methyl-thymidine (spec. act. 5Ci/mM; The Radiochemical Centre, Amersham, England) was added to each well. The cells were harvested from the culture wells with a semi-automatic sample precipitator (Cryoson, Midden Beemster, The Netherlands) and counted in a liquid scintillation counter (Packard, Model 3375). Specific counts per minute (cpm) were calculated as:

$$\text{spec.cpm} = \text{cpm}(5\times10^5\ R + 5\times10^5\ S) - \frac{\text{cpm}\ (10^6\ R) + \text{cpm}\ (10^6\ S)}{2}$$

The activity of 10^6 R cells ranged from 500 to 2500 cpm, and the activity of 10^6 S cells ranged from 100 to 600 cpm in the various experiments. Background activity was 35 cpm.

Estimation of cell proliferation in vivo. The method described by North et al. (11) was used. Briefly, 4 days after sc immunization with 10^7 allogeneic or syngeneic spleen cells (treated with 25 µg mitomycin C per ml), the mice were injected iv with a dose of 20 µCi ^{3}H-methyl-thymidine, spec. act. 5Ci/mM. Thirty min later their inguinal lymph nodes were taken out and homogenized in 5% ice-cold trichloracetic acid (TCA). Each homogenate was extracted twice for 1 hr with 20 ml cold 5% TCA. Thereafter the homogenate was extracted in 6 ml of 5% TCA at 90°C for 1 hr. After cooling, 1 ml of the supernatant was added to 9 ml scintillant consisting of 3 ml Triton X-100 and 6 ml toluene containing 4 mg PPO per liter and counted in a liquid scintillation counter (Packard, Model 3375). Radioactivity was corrected for background and quenching and expressed as cpm. Background activity was 35 cpm.

Assay for delayed type hypersensitivity. The delayed type hypersensitivity (DTH) assay for measuring anti-host immune reactivity after allogeneic spleen cell transplantation has been described in detail in a previous paper (8). Briefly, a number of cells equivalent to one whole spleen from an irradiated and

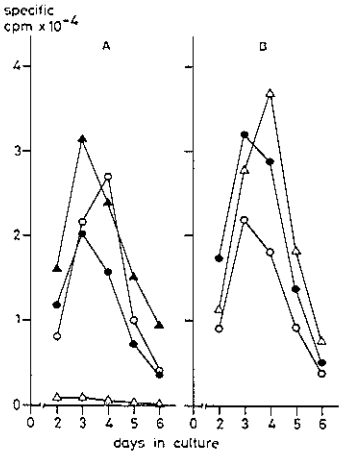

Figure 1
Kinetics of ^{3}H-thymidine incorporation in one way MLC after stimulation with Mls antigens. Responder (R) and stimulator (S) cells were always spleen cells. S cells were treated with 25 µg mitomycin C per ml. (A) BALB/c (R)→(BALB/c x DBA/2) F1 (S) (O); DBA/2 (R)→(BALB/c x DBA/2) F1 (S) (Δ). For comparison the response of BALB/c and DBA/2 cells in an H-2 incompatible combination is shown: BALB/c (R)→(C57BL/Rij x CBA/Rij) F1 (S) (●); DBA/2 (R)→(C57BL/Rij x CBA/Rij) F1 (S) (▲). (B) AKR/FuRdA (R)→C3H/f (S) (O); C3H/f (R)→AKR/FuRdA (S) (Δ). For comparison the response of AKR/FuRdA cells in an H-2 incompatible combination is shown: AKR/FuRdA (R)→(BALB/c x C3H/f) F1 (S) (●). Each experimental point represents the arithmetic mean of three separate experiments. Values are corrected for background (35 cpm).

allogeneically reconstituted recipient mouse was transferred iv into a normal secondary recipient at different intervals after reconstitution. This secondary recipient was syngeneic to the original spleen cell donor mouse. The secondary recipient mice were challenged into the right hind foot with 2×10^{7} spleen cells (treated with 100 µg mitomycin C per ml), syngeneic with the original recipient. The DTH response to this challenge was measured as the difference in thickness of the hind feet 24 hr later. Measurements were done with a footpad meter with an accuracy of 0.05 mm. The specific increase in foot thickness was calculated as the relative increase in foot thickness of the immune mice minus the relative increase in foot thickness of control mice which only received the challenge. The swelling of challenged control mice varied between 12 and 22%, which is about 0.20-0.40 mm.

RESULTS

Kinetics of MLC responses to Mls locus antigens.

The capacity of Mls locus antigens of our mice to induce proliferation was tested in a one-way mixed lymphocyte culture (MLC)

reaction. The following combinations were tested: BALB/c (H-2^d, Mls^b) and DBA/2 (H-2^d, Mls^a) *versus* (BALB/c x DBA/2)F1, and AKR/FuRdA (H-2^k, Mls^a) *versus* C3H/f (H-2^k, Mls^c) and *vice versa*. Stimulator cells were treated with 25 µg mitomycin C per ml and not irradiated with 3000 rad, because this latter treatment reduced the response to Mls locus antigens to a large extent (data not shown). Stimulation with spleen cells from mice, irradiated *in vivo* with 850 rad reduced the responsiveness as well, although to a smaller extent (data not shown).

As can be seen in Fig. 1A, BALB/c spleen cells could strongly proliferate in a MLC test upon stimulation with (BALB/c x DBA/2) F1 spleen cells, with a maximum response on day 4 of culture. In contrast, DBA/2 spleen cells were not stimulated to proliferate by (BALB/c x DBA/2)F1 spleen cells. Both BALB/c and DBA/2 spleen cells did proliferate upon stimulation with H-2 incompatible (C57BL/Rij x CBA/Rij)F1 spleen cells, with a maximum response on day 3. These results show that Mls^a locus antigen strongly stimulates proliferation of Mls^b locus positive cells, but not the other way around.

Fig. 1B shows that C3H/f spleen cells are strongly stimulated to proliferate by AKR spleen cells with a maximum response at day 4. In the opposite direction, AKR spleen cells were equally well stimulated by C3H/f spleen cells until about day 2. However, the maximum response, reached on day 3, was lower.

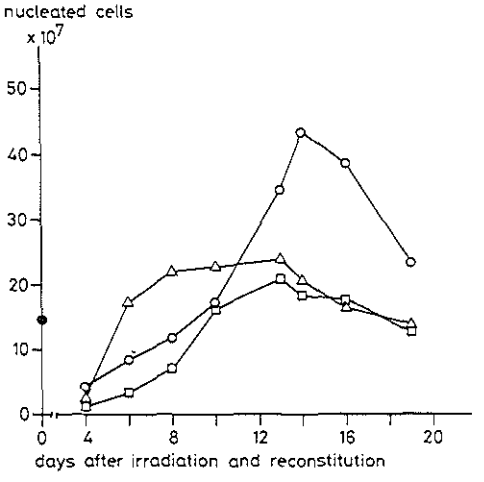

Figure 2
Recovery of the number of nucleated cells in the spleen of lethally irradiated (BALB/c x DBA/2) F1 mice, reconstituted with either 5 x 10^7 BALB/c (O), DBA/2 (Δ) or (BALB/c x DBA/2) F1 (□) spleen cells. The figure representing the value 0 days after irradiation was obtained from non-irradiated (BALB/c x DBA/2) F1 control mice. Each experimental point represents the arithmetic mean of 4 mice.

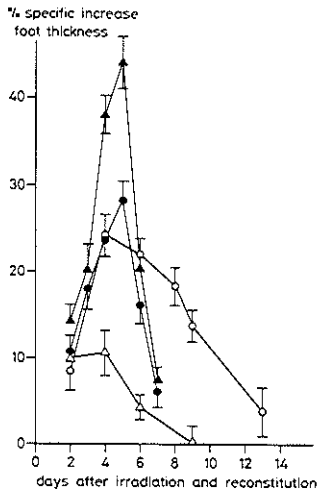

Figure 3
Development of anti-host DTH reactivity in the spleen of lethally irradiated (BALB/c x DBA/2) F1 mice, inoculated with 5 x 10^7 BALB/c (O) or DBA/2 (Δ) spleen cells and in the spleen of lethally irradiated (C57BL/Rij x CBA/Rij) F1 mice, inoculated with 5 x 10^7 BALB/c (●) or DBA/2 (▲) spleen cells. Each experimental point represents the arithmetic mean ± 1 SEM of two different experiments (n=10).

In a fully histoincompatible combination AKR spleen cells appeared to be fully capable of proliferating with a maximum response on day 3. These results show that during the first 2-3 days of culture both Mls^a and Mls^c locus antigens strongly stimulate proliferation. However, at continued cultivation Mls^a locus antigen can induce a higher level of proliferation than Mls^c.

Cellular changes in the spleen after Mls locus different spleen cell transplantation.
The cellular changes in the spleen of lethally irradiated (BALB/c x DBA/2)F1 mice inoculated with either 5 x 10^7 BALB/c, DBA/2 or (BALB/c x DBA/2)F1 spleen cells were studied. After reconstitution with BALB/c and especially with DBA/2 spleen cells, an earlier increase of cellularity was found as compared with that in syngeneically reconstituted mice (Fig. 2). At 12-16 days after irradiation and BALB/c spleen cell reconstitution, a pronounced splenomegaly was observed, which was not found after DBA/2 and syngeneic spleen cell reconstitution.

Development of GvH-related DTH reactivity after irradiation and Mls locus incompatible spleen cell transplantation.
At various intervals after irradiation of (BALB/c x DBA/2)F1 mice and reconstitution with 5 x 10^7 BALB/c or DBA/2 spleen

cells, the recipient spleens were transferred into normal BALB/c and DBA/2 mice respectively. Immediately therafter, these secondary recipients were challenged with 2 x 10^7 DBA/2 or BALB/c spleen cells respectively. Similarly, the capacity of BALB/c and DBA/2 spleen cells to mount an anti-host DTH reactivity in an allogeneic H-2 incompatible combination was tested. Therefore, (C57BL/Rij x CBA/Rij)F1 mice were chosen as recipients. Fig. 3 shows that BALB/c spleen cells were capable of giving rise to an anti-host DTH reactivity in the lethally irradiated H-2 compatible, Mls locus different (BALB/c x DBA/2)F1 host. The maximum response occurred on day 4-6 after reconstitution. Thereafter the response gradually decreased. The anti-host DTH reactivity appeared to be directed toward the Mls^a locus antigen, since after challenge with B10.D2 ($H\text{-}2^d$, Mls^b) spleen cells no significant DTH response was found (Table 1, upper part), although extensive cross-reactivity exists between other non-H-2 alloantigens of DBA/2 and B10.D2 mice (12). DBA/2 spleen cells, however, did not cause such a strong antihost DTH response in the Mls locus different (BALB/c x DBA/2) F1 host. Only during the first 4 days could a low response be detected. In contrast, in an allogeneic, H-2 incompatible combination both BALB/c and DBA/2 spleen cells were capable of strong anti-host DTH reactivity, with a maximum at 5 days after reconstitution, and a sharp decrease thereafter.

As a second Mls locus different, H-2 compatible combination AKR/FuRdA and C3H/f mice were tested. Therefore the transfer-

TABLE 1
ROLE OF OTHER NON-H-2 ALLOANTIGENS THAN MLS LOCUS PRODUCTS AS TARGETS OF GVH- AND HVG-RELATED DTH REACTIVITY

System	Inoculum	Recipient	Challenge	Response
	BALB/c	(BALB/c x DBA/2)F1	B10.D2	0.5±1.2[c]
GvH[a]	AKR/FuRdA	C3H/f	B10.BR	2.9±1.1
	C3H/f	AKR/FuRdA	B10.BR	1.9±1.7
System	**Immunizing cells**	**Responder**	**Challenge**	**Response**
	(BALB/c x DBA/2)F1	BALB/c	B10.D2	28.4±3.0
HvG[b]	C3H/f	AKR/FuRdA	B10.BR	28.8±1.5
	AKR/FuRdA	C3H/f	B10.BR	22.1±2.0

a. GvH reactions were elicited by iv injection of 5 x 10^7 spleen cells (inoculum) in lethally irradiated recipient mice. After 7 days the recipient spleen cells were transferred into secondary recipients which were syngeneic to the original spleen cell donors. These secondary recipients were challenged with 2 x 10^7 spleen cells.

b. HvG reactions were elicited by sc injection of 10^7 spleen cells equally distributed over the inguinal areas. Five days later these mice were challenged with 2 x 10^7 spleen cells.

c. DTH responses are expressed as the specific percentual increase in foot thickness. Figures represent the arithmetic mean ± 1 SEM of 5 mice.

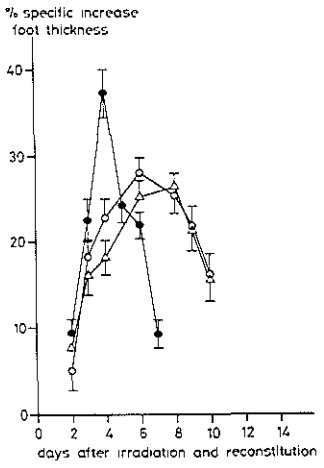

Figure 4
Development of anti-host DTH reactivity in the spleen of lethally irradiated C3H/f mice inoculated with 5 x 10^7 AKR/FuRdA spleen cells (○), in the spleen of lethally irradiated AKR/FuRdA mice inoculated with 5 x 10^7 C3H/f spleen cells (Δ), and in the spleen of lethally irradiated (BALB/c x C3H/f) F1 mice, inoculated with 5 x 10^7 AKR/FuRdA spleen cells (●). Each experimental point represents the arithmetic mean ± 1 SEM of two different experiments (n=10).

able anti-host DTH reactivity was determined at different intervals after irradiation and reconstitution of AKR and C3H/f mice with 5 x 10^7 C3H/f or AKR spleen cells, respectively. As shown in Fig. 4 both AKR and C3H/f spleen cells were capable of giving an anti-host DTH reactivity, with a maximum response at about day 6-8 after reconstition. Thereafter the reactivity gradually declined. The anti-host DTH reactivity appeared to be directed toward the Mls^a or Mls^c locus antigens, since after challenge with B10.BR ($H\text{-}2^k$, Mls^b) spleen cells no significant DTH response was found (Table 1, upper part). Also the anti-host DTH reactivity of AKR spleen cells in H-2 incompatible, Mls locus incompatible (BALB/c x C3H/f)F1 mice was studied (Fig. 4). In this combination a strong anti-host DTH response could be seen with a maximum at 4 days after reconstitution, followed by a sharp decline thereafter. Apparently, in an H-2 incompatible combination the Mls locus difference cannot cause a maintenance of the anti-host DTH reactivity on a high level, as is the case in H-2 compatible combinations.

Primary DTH responsiveness to Mls locus incompatible spleen cells. In order to test the contribution of Mls locus incompatibilities to HvG reactions, BALB/c and DBA/2 mice were sc immunized with 10^7 (BALB/c x DBA/2)F1 spleen cells and AKR and C3H/f mice were sc immunized with 10^7 C3H/f and AKR spleen cells, respectively. At varying intervals after immunization, these groups of mice received a challenge dose of DBA/2, BALB/c, C3H/f and AKR spleen cells, respectively. In Fig. 5 the primary DTH reactivity of the

various combinations is shown. At 2-3 days after immunization the DTH reactivity started to increase rapidly. In all combinations the peak reactivity was observed 5 days after immunization. Thereafter the reactivity declined. The DTH reactivity that developed during this HvG reaction was mainly directed toward non-H-2 histocompatibility antigens other than Mls locus products. This appeared from the relatively high DTH response of BALB/c (Mls^b) mice, immunized with (BALB/c x DBA/2)F1 (Mls^b, Mls^a) spleen cells, challenged with B10.D2 (Mls^b) spleen cells and the DTH response of AKR (Mls^a) and C3H/f (Mls^c) mice, immunized with C3H/f and AKR spleen cells respectively, challenged with B10.BR (Mls^b) spleen cells (Table 1, lower part).

In order to compare the reactivity in HvG reactions with the responsiveness in GvH reactions, in which the hosts were lethally irradiated, in a subsequent experiment the immunizing (BALB/c x DBA/2)F1 spleen cells were irradiated (850 rad) *in vivo* before harvesting. As can be seen in Fig. 5A, also in this case a clear primary DTH reactivity developed with an equally high maximum on day 5 for both combinations. Only the height of the maximum response was lower than that after immunization with non-irradiated spleen cells.

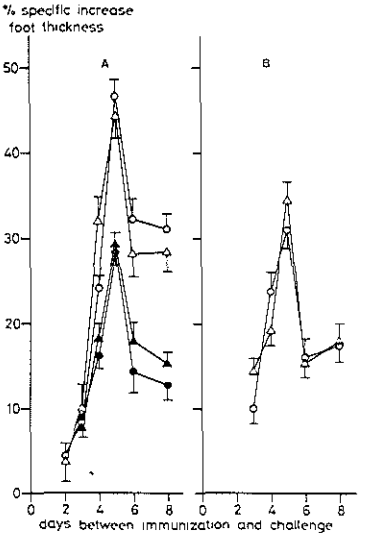

Figure 5
(A) Primary DTH responsiveness of BALB/c (O) and DBA/2 (Δ) mice sc immunized with 10^7 spleen cells from (BALB/c x DBA/2) F1 mice; or BALB/c (●) and DBA/2 (▲) mice sc immunized with 10^7 spleen cells from (BALB/c x DBA/2) F1 mice which were lethally irradiated (850 rad) previously.

(B) Primary DTH responsiveness of C3H/f (O) mice sc immunized with 10^7 AKR/FuRdA spleen cells, and DTH responsiveness of AKR/FuRdA (Δ) mice sc immunized with 10^7 C3H/f spleen cells. Each experimental point represents the arithmetic mean ± 1 SEM of a group of at least 5 mice.

TABLE 2

PROLIFERATIVE ACTIVITY DURING THE DEVELOPMENT OF ANTI-GRAFT DTH REACTIVITY TO MLS INCOMPATIBLE, H-2 COMPATIBLE SPLEEN CELLS

Immunization[a]	Recipient	Response[b]	Stimulation index[c]
(BALB/c x DBA/2)F1	BALB/c	3202±212	5.8±0.4
(BALB/c x DBA/2)F1	DBA/2	999±54	3.8±0.4
BALB/c	BALB/c	552±44	-
DBA/2	DBA/2	266±20	-

a. Immunization was performed sc with 10^7 spleen cells, treated before with 25 µg mitomycin C per ml in order to prevent proliferation of the injected cells.

b. Figures represent the mean response in cpm ± 1 SEM of 5 mice, measured 4 days after immunization. Values are corrected for background and quenching. Background activity was 35 cpm.

c. Stimulation index was calculated as proliferative capacity (in cpm) of the immune mice divided by the proliferative activity (in cpm) of the corresponding control mice.

Proliferative activity in the development of anti-graft immune reactivity.

The *in vivo* proliferative activity in the development of anti-graft DTH reactivity after sc immunization of BALB/c and DBA/2 mice with 10^7 (BALB/c x DBA/2)F1 spleen cells was determined *in vivo* in the inguinal lymph nodes. Both in BALB/c and DBA/2 mice an increased proliferative activity was observed at 4 days after immunization (Table 2). In BALB/c mice, however, the proliferative activity was higher than in DBA/2 mice, probably due to stimulation by the Mls^a locus antigen.

DISCUSSION

This study shows that antigens encoded for by the Mls locus can elicit the development of a transferable anti-host directed DTH reactivity after transplantation of H-2 compatible, but non-H-2 incompatible spleen cells in lethally irradiated hosts. The capacity of Mls locus antigens to induce distinct anti-host DTH reactivity fully correlated with the capacity to induce an one way MLC response. Thus, in the BALB/c *versus* (BALB/c x DBA/2)F1 combination, and in the C3H/f *versus* AKR combination, Mls^a locus antigen initiated a positive MLC response (Fig. 1) as well as distinct GvH-related DTH reactivity (Figs. 3 and 4). The same result was found for Mls^c in the AKR *versus* C3H/f combination. On the other hand, in the combination DBA/2 *versus* (BALB/c x DBA/2)F1, the Mls^b locus antigen was not able to initiate *in vitro* proliferation (Fig. 1), and splenomegaly (Fig. 2), a

failure which coincided with a marginal and short-lasting GvH-related DTH reactivity (Fig. 3). This finding correlates well with our previous study on the role of proliferation in the development of specific anti-host immune responsiveness in a fully histoincompatible combination (9). There it was found that blocking of the proliferative capacity of donor spleen cells with mitomycin C (25 µg per ml) did not prevent the anti-host DTH responsiveness during the first 2 days after transplantation, but did so thereafter. Elimination of the proliferative compartment of reacting lymphocytes with the ^{3}H-thymidine suicide technique, showed that the maximum proliferative activity preceded the maximal anti-host DTH reactivity (9). Maybe also in Mls locus different, H-2 identical combinations only the early proliferative activity is important for the full development of anti-host DTH responsiveness. This might explain why the kinetics of the GvH-related DTH reactivity in the AKR *versus* C3H/f and C3H/f *versus* AKR combinations is the same, in spite of a prolonged MLC stimulating capacity of the Mls^{a} positive AKR cells after the third day of culture.

So far, Mls locus antigens have been reported to induce only T cell proliferation, and no cytotoxic T cells (13,14). However, at this moment the results are obscured by the occurrence of numbers of non-H-2 incompatibilities still present in the first generation of Mls locus typed backcross mice (15). It is possible that these minor histoincompatibilities other than Mls locus antigens are also essential in the development of anti-host immune reactivity. The role of proliferation upon stimulation with Mls locus differences could be a strengthening of the developing anti-host immune reactivity. Röllinghoff and Wagner (16) showed in their studies an amplification effect of Mls locus induced T cell proliferation upon the induction of anti-H-2 cytotoxic T lymphocytes, similar to the proposal of Schendel and Bach in which LD induced T cell proliferation potentiates anti-SD cytotoxic responses (17). The question arises whether the transferable anti-host DTH reactivity in our system is directed against Mls locus products themselves or against other non-H-2 alloantigens. Our experiments with cross-reacting (12) non-H-2 alloantigens revealed that the anti-host T effector cells in DTH were directed mainly toward the Mls locus antigen and that probably non-H-2 alloantigens other than Mls locus coded products did not evoke anti-host DTH reactivity (Table 1). In studies with congenic mice (18) differing at the H-2 complex or part of the H-2 complex, we found a remarkable parallel. There we could demonstrate that the anti-host DTH reactivity is directed almost exclusively toward I-region antigens, and not toward K and D region antigens. Just like Mls pro-

ducts, I-region antigens stimulate T cell proliferation in MLC (19). The inability of K and D products to stimulate efficiently T cell proliferation *in vitro* (19) again coincided with the inability to evoke DTH T effector cells during a GvH reaction. Klein et al. (20) were able to demonstrate cytotoxic effector cells against I-region antigens; so the possibility has to be considered that the Mls system is a complex system with linked genes which could provide targets for effector cells (14).

The role of Mls locus products in the development of host anti-graft directed T effector cells was completely different from that in the GvH situation. In the HvG reaction the role of Mls locus differences is of minor importance (Fig. 5 and Table 1). Other non-H-2 differences are probably responsible for most of the DTH reactivity after immunization with H-2 identical spleen cells (Table 1). Although T effector cells specific for Mls locus products could not be detected, a distinct proliferation in the inguinal lymph nodes 4 days after sc immunization with H-2 identical non-H-2 different spleen cells was found (Table 2). Thus, non-H-2 alloantigens other than Mls locus products can also induce a primary proliferative response in HvG reactions.

In conclusion, this paper shows that stimulation by Mls locus products is essential for the development of anti-host DTH T effector cells after transplantation of allogeneic, H-2 compatible, non- H-2 incompatible spleen cells into lethally irradiated recipients. In such GvH reactions other non-H-2 alloantigens do not give rise to DTH T effector cells. In contrast, in HvG reactions non-H-2 alloantigens other than Mls locus products are fully capable of inducing the generation of DTH T effector cells.

ACKNOWLEDGMENTS

We are indebted to Prof.Dr. O. Vos for his helpful advice during this study. We thank Mrs. Cary Meijerink-Clerkx for excellent secretarial assistance. This investigation is part of a project program on the regulation of hemopoiesis, subsidized by the Netherlands Foundation for Medical Research (FUNGO).

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APPENDIX PAPER VI

DIFFERENT H-2 SUBREGION CODED ANTIGENS AS TARGETS FOR T CELL SUBSETS SYNERGIZING IN GRAFT-VERSUS-HOST REACTION

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SUMMARY

After allogeneic transplantation of lymphoid cells into lethally irradiated mice, specific anti-host-directed T effector cells are generated. This can be demonstrated by means of a delayed type hypersensitivity (DTH) assay. In donor-recipient combinations differing at the major histocompatibility complex (MHC) or parts of the MHC, it could be demonstrated that anti-host directed DTH T effector cells are the progeny of long-lived, recirculating T2 cells, activated by antigens encoded by the I region of the MHC. This response can be amplified by short-lived sessile T1 cells. The amplifier activity can only be detected in case of a K region difference next to an I region difference, indicating that T1 amplifier cells are activated by H-2K region coded antigens. T1 cells themselves are incapable to display anti-host DTH reactivity.

INTRODUCTION

Cantor and Asofsky (1) demonstrated a synergistic effect of thymus cells and peripheral lymph node cells or peripheral blood cells in the production of splenomegaly in neonatal F1 recipients. According to this observation Raff and Cantor (2) proposed to subdivide T lymphocytes into two subpopulations, namely, T1 cells which are short-lived, sessile cells, sensitive to adult thymectomy (ATx), and T2 cells which are long-lived, recirculating cells, sensitive to anti-thymocyte serum (ATS). Further studies of Cantor and Asofsky (3) were interpreted as to indicate that T1 cells were the precursors of the effector cells, and that T2 cells amplified their response.

Tittor et al. (4) demonstrated a T1-T2 cooperation in an one-way mixed lymphocyte culture (MLC) reaction, but their studies did not touch directly upon the question of which T cell subpopulation consists of precursors of proliferating cells and which one consists of amplifying cells. Recent studies of Wright et al. (5) revealed that irradiated thymus cells retained the capa-

city for synergy in mixtures of rat thymus and lymph node cells in the proliferative response to alloantigens, whereas irradiated lymph node cells did not. They showed that thymus cells have the capacity of amplifying the proliferative response of lymph node cells in an one-way MLC. This capacity is radioresistant, but requires *de novo* protein synthesis. Lymph node cells lack this capacity of amplifier activity of thymus cells. These experiments suggest that T1 cells can amplify the proliferative response by T2 cells.

The question arises as to whether there is a discrepancy between the initial *in vivo* studies and the later *in vitro* experiments with regard to the subpopulations of T cells that give rise to T effector and T amplifier activity.

In this report we have studied *in vivo* the role of T1 and T2 cells in the development of specific anti-host immune reactivity by allogeneic lymphoid cells in lethally irradiated hosts. Furthermore the genetic requirements for optimal activation of both these T cell subsets was investigated. We used a delayed type hypersensitivity (DTH) assay which is appropriate to measure the development of specific anti-host T effector cells induced by histocompatibility antigens during a GvH reaction.

MATERIALS AND METHODS

Animals. (C57BL/Rij x CBA/Rij)F1 (H-$2^{b/q}$) female mice were bred at the Laboratory Animals Centre of the Erasmus University, Rotterdam, The Netherlands. DBA/2 (H-2^{d}) male mice and C57BL/Rij (H-2^{b}) male and female mice were purchased from the Medical Biological Laboratory TNO, Rijswijk (ZH), The Netherlands. B10.AQR (H-2^{y1}) male and female mice, B10.T(6R) (H-2^{y2}) female mice, B10.G (H-2^{q}) female mice, A.TL (H-2^{t1}) female mice and A.TH (H-2^{t2}) female mice were commercially obtained from OLAC Ltd., Bicester, United Kingdom. (B10.A x B10.T(6R)) F1 (H-$2^{a/y2}$) female mice were bred at our own department. The origin of the H-2 subregions for the congenic strains used, are shown in Table 1. The age of all mice varied between 12 and 30 weeks. When mice from either sex were used in one experiment, the combinations were always chosen in such a way that anti H-Y responses were impossible.

Preparation of cell suspensions. Mice were killed by carbon dioxyde. Immediately after killing, the organs were prepared for single cell suspensions as described previously (7). Nu-

TABLE 1

MHC HAPLOTYPES OF INBRED CONGENIC MOUSE STRAINS USED

Strain	MHC regions							
	H-2K	I-A	I-B	I-J	I-E	I-C	S	H-2D
A.TH	s	s	s	s	s	s	s	d
A.TL	s	k	k	k	k	k	k	d
B10.AQR	q	k	k	k	k	d	d	d
B10.G	q	q	q	q	q	q	q	q
(B10.A x B10.T(6R)F1	k q	k q	k q	k q	k q	d q	d q	d q

From Klein et al. (6).

cleated cells were counted in Zaponin-treated samples with a Coulter Counter model B (Coulter Electronics, Dunstable, Bedfordshire, England).

Irradiation. The recipient mice received 700 rad whole body irradiation, generated in a Philips Müller MG 300 X-ray machine as described previously (7).

Adult thymectomy. Adult thymectomy (ATx) and sham-thymectomy (ShTx) were always performed when the mice were 6-week-old. The surgery was performed as described by Miller (8). The mice were anaesthetized with Nembutal (Abbott S.A., Saint-Rémy-sur-Avre, France; 70 mg/kg body weight). The organs of the ATx and ShTx mice were used 6-8 weeks after surgery. All ATx mice were examined thoroughly for thymic remnants before using their organs. Spleen cells from ATx and ShTx mice are referred to in the text as ATx spleen cells and ShTx spleen cells, respectively.

Selective elimination of Thy-1.2 positive cells. Monoclonal IgM anti-Thy-1.2 serum (clone F7D5) was purchased from OLAC Ltd., Bicester, United Kingdom. Cell suspensions were treated for 30 min at 4°C with anti-Thy-1.2 serum. The amount of anti-Thy-1.2 serum used was three times more than required to kill at least 95% of corticosteroid-resistant thymocytes (9). After incubation, the cells were centrifuged, resuspended in balanced salt solution

(BSS), and incubated with guinea pig complement (Flow Laboratories, Irvine, Scotland) for 15 min at 37°C. Thereafter the cells were washed three times and resuspended in BSS.

Anti-thymocyte serum. Anti-thymocyte serum (ATS) was prepared and absorbed as described previously (9). For elimination of T2 cells the mice received two subcutaneous (sc) injections of 0.2 ml ATS, or normal rabbit serum (NRS) as control. This amount was equally distributed over the inguinal and axillary areas. These injections were given 5 and 2 days before the organs were used. Spleen cells from ATS and NRS treated mice are referred to in the text as ATS spleen cells and NRS spleen cells, respectively.

Lymph node seeking cells. Isolation *in vivo* of recirculating, lymph node seeking T2 cells was done according to the method of Tigelaar and Asofsky (10) with some minor modifications. The experimental design is shown in Fig. 1. A number of 15×10^7 pooled cells from spleen, peripheral and mesenteric lymph nodes was iv injected into syngeneic recipients which were lethally irradiated 2 hr previously. At 24 hr after inoculation the peripheral lymph nodes were harvested from these recipients. Such peripheral lymph nodes contained about 4×10^6 more nucleated cells than lymph nodes from lethally irradiated mice which were not infused with spleen and lymph node cells. The cell yield from all peripheral lymph nodes of a single irradiated recipient was called one lymph node seeking equivalent (1 LNS eq.).

Acute GvH reactions. Acute GvH reactions were elicited by iv injection of the appropriate inoculum into lethally irradiated recipient mice within 4 hr after irradiation. The cells to be injected were suspended in a volume of 0.5 ml BSS.

Assay for delayed type hypersensitivity. The delayed type hypersensitivity (DTH) assay for measuring anti-host immune reactivity after allogeneic lymphoid cell transplantation has

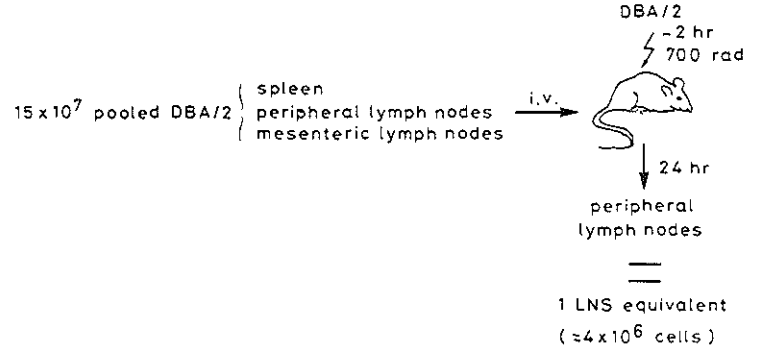

Figure 1
Experimental design for isolation of recirculating, lymph node seeking T2 cells.

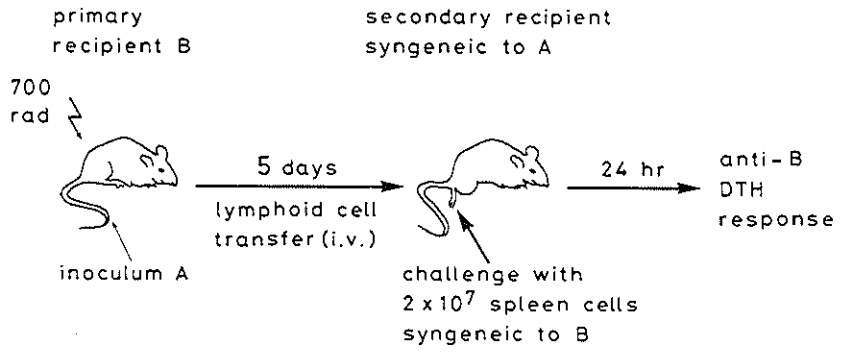

Figure 2
Scheme of the experimental set up used to demonstrate GvH-related DTH reactivity against host histocompatibility antigens.

been described in detail in a previous paper (7). Briefly, a number of cells equivalent to one whole spleen or the total cell yield obtained from spleen, inguinal, axillary and mesenteric lymph nodes from an irradiated and allogeneically reconstituted recipient mouse was transferred iv into a normal secondary recipient at different intervals after reconstitution. This secondary recipient was syngeneic to the original lymphoid cell donor. Lymphoid cells from all primary recipients of a particular experimental group were pooled before secondary transfer. The secondary recipient mice were challenged into the right hind foot with 2×10^7 spleen cells (treated with 100 µg mitomycin C per ml), syngeneic with the irradiated recipient. The DTH response to this challenge was measured as the difference in thickness of the hind feet 24 hr later. A scheme of the experimental system is shown in Fig. 2. Measurements were done with a footpad meter with an accuracy of 0.05 mm. The specific increase in foot thickness was calculated as the relative increase in foot thickness of the immune mice minus the relative increase in foot thickness of control mice which only received the challenge. The swelling of challenged control mice varied between 12 and 22%, which is about 0.2-0.4 mm.

RESULTS

Dependence of GvH-related DTH reactivity on T1 and T2 cells. The development of GvH-related DTH reactivity was investigated after transplantation of either 10^7 DBA/2 or 5×10^7 C57BL spleen cells into lethally irradiated (C57BL x CBA)F1 mice. At different intervals after irradiation and reconstitution the recipient spleens were transferred into normal DBA/2 and C57BL mice, respectively. Immediately thereafter, these secondary

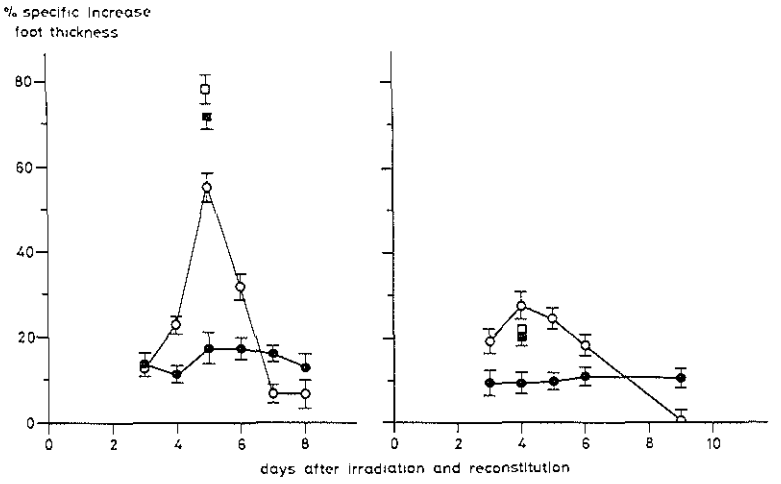

Figure 3
Development of GvH-related DTH responsiveness in the spleen of lethally irradiated (C57BL x CBA) F1 mice inoculated with 10^7 ShTx (O), ATx (●) spleen cells or 10^7 ShTx (□), ATx (■) lymph node cells from DBA/2 mice (left), and of lethally irradiated (C57BL x CBA) F1 mice inoculated with 5×10^7 ShTx (O), ATx (●) spleen cells or 10^7 ShTx (□), ATx (■) lymph node cells from C57BL mice (right). Each experimental point represents the arithmetic mean ± 1 SEM of a group of at least five mice.

recipients were challenged with 2×10^7 (C57BL x CBA)F1 spleen cells in order to test the anti-host immune response. Peak anti-host DTH reactivity was attained on day 5 for the DBA/2 to (C57BL x CBA)F1 transplantation, and on day 4 for the C57BL to (C57BL x CBA)F1 transfer (Fig. 3).

In order to test whether both T1 and T2 cells contribute to the development of GvH-related DTH reactivity, similar experiments were done with spleen cells from donors which were thymectomized at adult age (ATx). These spleen cells were harvested 6-8 weeks after ATx. Such spleens are depleted of short-lived, sessile T1 cells, but not of long-lived, recirculating T2 cells. After transfer of spleen cells from ATx DBA/2 and ATx C57BL mice only a marginal anti-host immune response developed. Apparently, T1 cells are necessary for optimal anti-host immune reactivity in case of spleen cell transplantation across major and minor histocompatibility (H) differences. Such a contribution of T1 cells could not be shown in case of transplantation of DBA/2 and C57BL lymph node cells (Fig. 3). On the other hand, elimination of T2 cells, by *in vivo* treatment with ATS (11) completely prevented the capacity of GvH-related DTH reactivity in both spleen and lymph nodes (Table 2).

Cellular cooperation between T1 and T2 cells in the development of anti-host immune reactivity.
To investigate whether putative T1 and T2 cells have an additive or synergistic activity in GvH-related DTH reactivity, DBA/2 spleen cell suspensions containing T1 cells (ATS spleen cells) and T2 cells (ATx spleen cells) were either alone or together transferred into lethally irradiated (C57BL x CBA)F1 recipients.

In the upper part of Fig. 4 it can be seen that 3×10^7 DBA/2 ATx spleen cells gave a significant anti-host DTH response, whereas 3×10^7 DBA/2 ATS spleen cells did not. Inoculation of lethally irradiated (C57BL x CBA)F1 mice with both 3×10^7 DBA/2 ATS spleen cells and 3×10^7 DBA/2 ATx spleen cells gave a somewhat higher anti-host DTH response than the sum of the responses by both inoculations alone (Fig. 4, upper part).

TABLE 2

DEPENDENCE OF GvH-RELATED DTH REACTIVITY ON T2 CELLS

Inoculum	DTH response[a)]
10^7 DBA/2 NRS SPL	45.2 ± 1.1
10^7 DBA/2 ATS SPL	2.5 ± 1.4
10^7 DBA/2 NRS LN	60.0 ± 2.4
10^7 DBA/2 ATS LN	3.2 ± 1.6
5×10^7 C57BL NRS SPL	32.4 ± 1.2
5×10^7 C57BL ATS SPL	1.1 ± 2.0
10^7 C57BL NRS LN	24.9 ± 0.6
10^7 C57BL ATS LN	0.6 ± 1.6

GvH reactions were elicited in lethally irradiated (C57BL x CBA)F1 mice by i.v. injection of 10^7 spleen (SPL) or peripheral lymph node (LN) cells from ATS or NRS-treated DBA/2 mice, and by 5×10^7 SPL or 10^7 LN cells from ATS or NRS-treated C57BL mice. Anti-host DTH reactivity due to DBA/2 lymphoid cell transplantation was tested on day 5; anti-host DTH reactivity due to C57BL lymphoid cell transplantation was tested on day 4.

a) Numbers represent the arithmetic mean of the specific increase of foot thickness ± 1 SEM of 5 mice. DTH reactions were measured 24 hr after challenge.

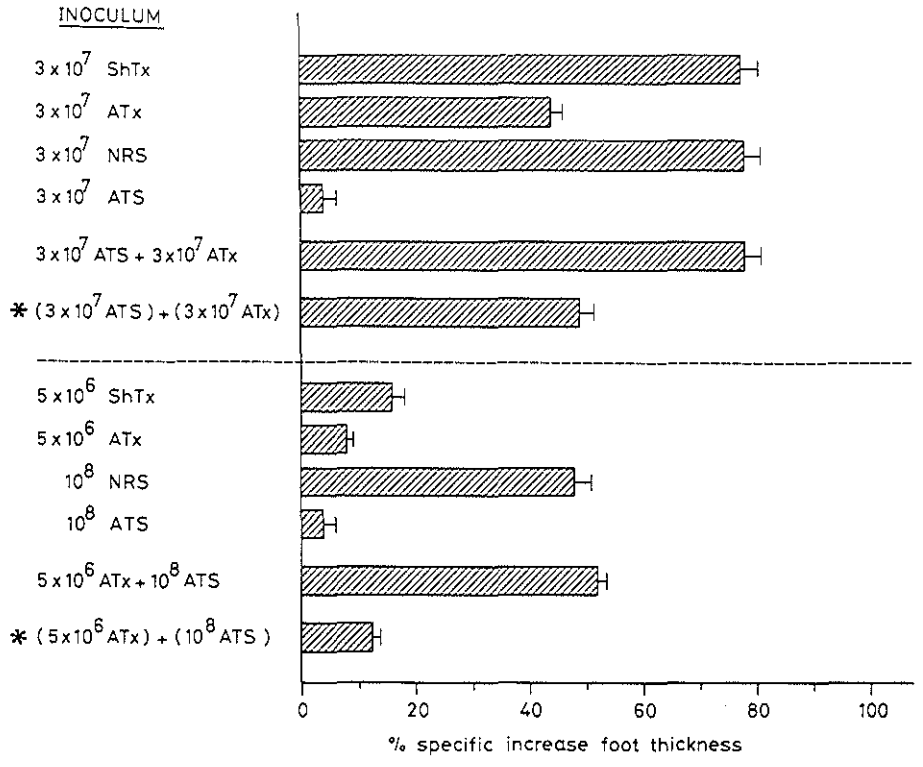

Figure 4
GvH-related DTH responsiveness of spleen cells of lethally irradiated (C57BL x CBA) F1 mice, reconstituted with spleen cells from ATS- or NRS-treated DBA/2 mice and ATx or ShTx DBA/2 mice. Addition of an asterix (*) means that both inocula were separately injected in different lethally irradiated (C57BL x CBA) F1 recipient mice. In that case the spleen cells from both types of primary recipients were transferred into secondary recipients. These recipients were tested for anti-host DTH reactivity. Horizontal bars represent 1 SEM (n=5).

Such a synergistic response was not found when a whole spleen from a primary irradiated recipient inoculated with 3×10^7 ATx spleen cells, was transferred together with a whole spleen from a primary recipient inoculated with 3×10^7 ATS spleen cells. Thus, the putative T1 and T2 cells have to cooperate during the induction phase of the anti-host immune response.

Synergistic activity of T1 and T2 cells in the development of GvH-related DTH reactivity was even more evident when optimal numbers of T1 and T2 cells were transferred. These data are shown in the lower part of Fig. 4. Here, 10^8 ATS spleen cells were used as a source of T1 cells and 5×10^6 ATx spleen cells as a source of T2 cells.

Synergistic activity between putative T1 and T2 cells in GVH-related DTH reactivity can also be shown by using lymph node seeking lymphocytes as a source of T2 cells. Fig. 5 shows that inoculation of lethally irradiated (C57BL x CBA)F1 mice with both 4×10^7 ATS spleen cells and one lymph node seeking equivalent (1 LNS eq.) resulted in a much higher anti-host DTH response than the sum of the responses generated by both cell populations alone.

When 2×10^7, 4×10^7 or 10^8 DBA/2 thymocytes were used as a source of putative T1 cells, and transferred together with 1 LNS equivalent from DBA/2 as a source of T2 cells, no or only marginal synergistic activity could be detected in the development of GvH-related DTH reactivity (Fig. 6). Inoculation of 10^8 thymocytes alone resulted in a good anti-host DTH reactivity, suggesting the occurrence of T2 cells in the thymocyte inoculum. Inoculation of 4×10^7 thymocytes together with 10^8 ATS spleen cells as a source of T1 cells resulted in a clear synergistic GvH-related DTH response (Fig. 6). Apparently, T2 cells from the thymus can cooperate with T1 cells in the ATS spleen cell inoculum.

Evidence that putative T1 and T2 cells indeed are T cells

In order to investigate whether the putative T1 and T2 cells indeed were T cells, anti-Thy-1.2 treatment *in vitro* was applied for their selective elimination. Pretreatment of spleen cells from NRS-treated mice, from ATS-treated mice, and from mice which had been subjected to ATx 6-8 weeks previously, completely prevented the anti-host DTH responsiveness by these cell populations (Fig. 7). This shows that the responses normally mediated by untreated cells rely on T cells. Treatment of either ATS

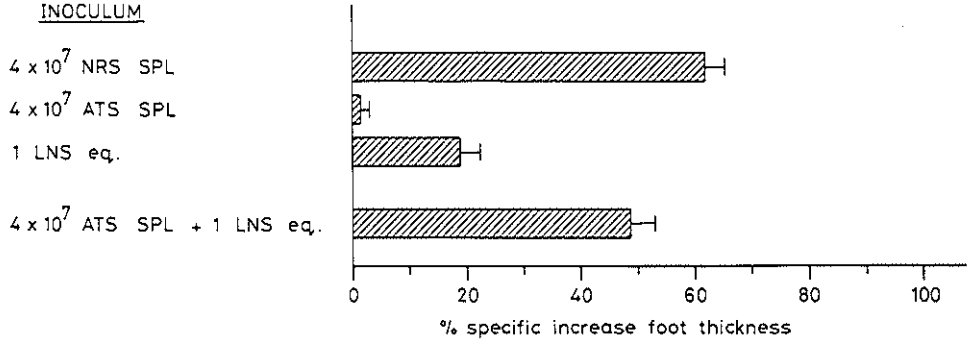

Figure 5
GvH-related DTH responsiveness of spleen cells of lethally irradiated (C57BL x CBA) F1 mice, reconstituted with spleen (SPL) cells from NRS- or ATS-treated DBA/2 mice and one lymph node seeking equivalent (1 LNS eq). Horizontal bars represent 1 SEM (n=5).

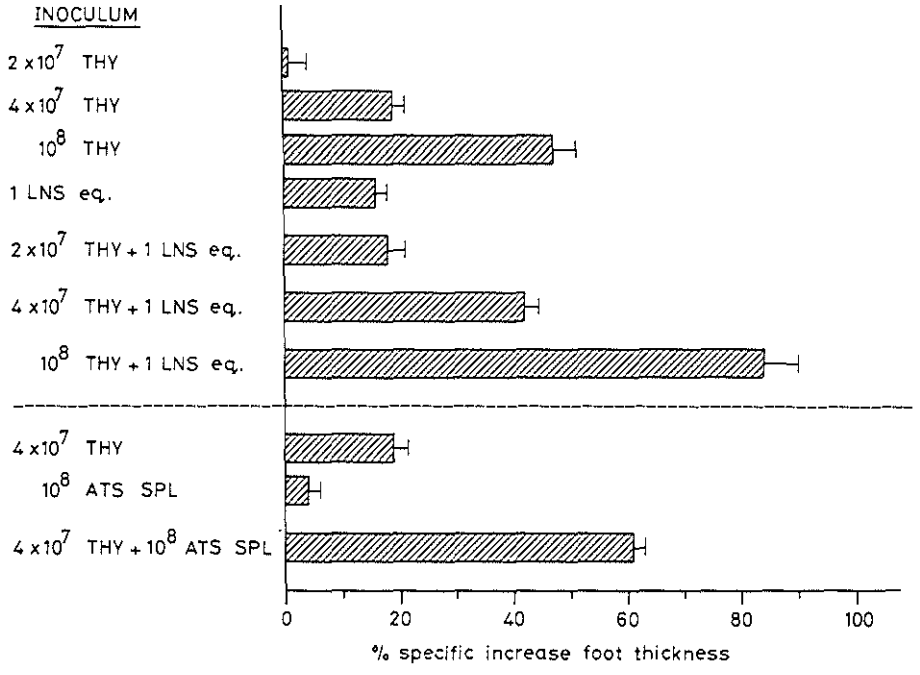

Figure 6
GvH-related DTH responsiveness of spleen cells of lethally irradiated (C57BL x CBA) F1 mice, reconstituted with either DBA/2 thymocytes (THY) and one lymph node seeking equivalent (1 LNS eq.); or reconstituted with DBA/2 thymocytes and spleen cells from ATS-treated DBA/2 mice. Horizontal bars represent 1 SEM (n=5).

spleen cells or ATx spleen cells, and subsequent transfer in combination with ATx spleen cells and ATS spleen cells, respectively, also prevented a synergistic anti-host DTH response by both cell populations (Fig. 7). Therefore, both putative T1 and T2 cells are real T cells.

H-2 subregion coded antigens as targets for T1 and T2 cells. In the aforementioned experiments T1-T2 cell synergism after spleen cell transplantation across a combination of major and minor H-antigens was demonstrated. Preceding experiments revealed that spleen cell transplantation across H-2K or H-2D differences did not result in a detectable anti-host DTH reactivity (12). However, after spleen cell transplantation across a H-2I difference a clear anti-host DTH response developed (12).

Here we studied whether T1-T2 cell synergism can also occur after spleen cell transplantation across an H-2I barrier alone. Therefore, lethally irradiated B10.T(6R) mice were inoculated with either 10^8 B10.AQR ATS spleen cells or 1 LNS equivalent from

B10.AQR, or both. As can be seen in Table 3, 1 LNS equivalent from B10.AQR gave rise to an anti-host DTH response after transplantation into irradiated B10.T(6R) mice, whereas 10^8 B10.AQR ATS spleen cells did not. Simultaneous transplantation of both inocula into the same recipient did not increase the anti-host DTH response (Tabel 3). The same result was found in a similar experiment using A.TH and A.TL mice as another H-2I incompatible combination. Apparently, in a H-2I incompatible combination only T2 cells react against the H-2I region coded antigens. In a combination differing at both H-2I and H-2K (i.e., B10.AQR into (B10.A x B10.T(6R))F1) and in case of a whole H-2 difference (i.e., C57BL into B10.G) T1-T2 synergism did occur. In both combinations the response by the lymph node seeking (T2) cells was evidently potentiated by ATS spleen (T1) cells (Table 3). Most likely H-2K region coded antigens stimulate T1 cells to amplify the response of T2 cells against H-2I coded antigens.

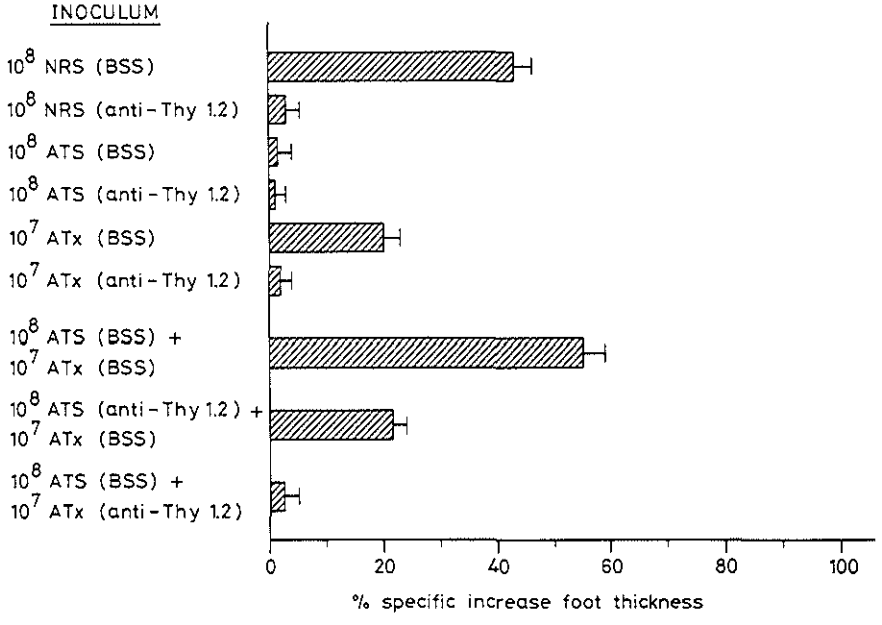

Figure 7
GvH-related DTH responsiveness of spleen cells of lethally irradiated (C57BL x CBA) F1 mice, reconstituted with spleen cells from ATS- or NRS-treated DBA/2 mice and spleen cells from ShTx or ATx DBA/2 mice. Between brackets the in vitro pretreatment of the inoculum. Horizontal bars represent 1 SEM (n=5).

TABLE 3

H-2 SUBREGION CODED ANTIGENS AS TARGETS FOR T1 AND T2 CELLS

Inoculum	Recipient	H-2 subregion coding for immunizing antigen	DTH response
10^8 B10.AQR NRS spleen	B10.T(6R)	I	34.4 ± 3.3
10^8 B10.AQR ATS spleen (T1)	B10.T(6R)	I	-0.9 ± 1.8
1 LNS eq. B10.AQR (T2)	B10.T(6R)	I	19.6 ± 2.9
10^8 B10.AQR ATS spleen (T1)+ 1 LNS eq. B10.AQR (T2)	B10.T(6R)	I	20.4 ± 1.6
10^8 A.TL NRS spleen	A.TH	I	31.2 ± 2.1
10^8 A.TL ATS spleen (T1)	A.TH	I	0.1 ± 2.1
1 LNS eq. A.TL (T2)	A.TH	I	16.5 ± 1.9
10^8 A.TL ATS spleen (T1)+ 1 LNS eq. A.TL (T2)	A.TH	I	15.1 ± 2.0
10^8 B10.AQR NRS spleen	B10.A x B10.T(6R)	K+I	33.4 ± 2.6
10^8 B10.AQR ATS spleen (T1)	B10.A x B10.T(6R)	K+I	3.5 ± 2.6
1 LNS eq. B10.AQR (T2)	B10.A x B10.T(6R)	K+I	11.4 ± 2.0
10^8 B10.AQR ATS spleen (T1)+ 1 LNS eq. B10.AQR (T2)	B10.A x B10.T(6R)	K+I	26.8 ± 1.2
10^8 C57BL NRS spleen	B10.G	K+I+D	25.9 ± 1.4
10^8 C57BL ATS spleen (T1)	B10.G	K+I+D	-1.8 ± 3.2
1 LNS eq. C57BL (T2)	B10.G	K+I+D	8.1 ± 1.4
10^8 C57BL ATS spleen (T1)+ 1 LNS eq. C57BL (T2)	B10.G	K+I+D	25.2 ± 2.3

GvH reactions were elicited by iv injection of the inoculum into lethally irradiated recipient mice.

At 5 days after reconstitution a number of cells equivalent to the total cell yield obtained from spleen, inguinal, axillary and mesenteric lymph nodes of a recipient mouse was transferred iv to a secondary recipient syngeneic to the original spleen cell donor. Lymphoid cells from all primary recipients of a particular combination were pooled before secondary transfer. These secondary recipient mice were challenged with 2×10^7 spleen cells, syngeneic to the irradiated recipients, immediately after cell transfer. DTH responses are expressed as the specific percentual increase in foot thickness. Figures represent the arithmetic mean ± 1 SEM of 5 mice.

DISCUSSION

This study shows that for optimal development of GvH-related DTH reactivity both short-lived, sessile T1 cells and long-lived, recirculating T2 cells are required (Fig. 4). Cellular cooperation between both T cell subpopulations results in a much stronger anti-host response than the sum of the responses by both cell populations separately. This synergism occurs during the induction phase of the anti-host immune response (Fig. 4), and is dependent on recognition of different H-2 subregion coded antigens by T1 and T2 cells.

Elimination of T2 cells by *in vivo* treatment with ATS could completely prevent the development of anti-host immune reactivity, even when large numbers of T1 cells were used for transplantation. Previously we have observed the same in a delayed GvH reaction. When semi-allogeneic bone marrow cells were transplanted into lethally irradiated recipients, elimination of recirculating T2 cells by ATS treatment of the donor mice could almost completely prevent the development of anti-host immune reactivity (9). Comparison of the anti-host immune responses by semi-allogeneic bone marrow cells in lethally irradiated ShTx and ATx mice, revealed that newly formed T cells influence the height of the anti-host DTH response. These newly formed T cells, possibly equivalent to T1 cells, accelerated and enhanced the anti-host immune response, which was elicited by mature, recirculating T2 cells residing in the bone marrow (9). These results suggest that recirculating T2 cells give rise to DTH-effector cells in the development of GvH-related DTH reactivity, and that sessile T1 cells have an amplifier function, in particular when suboptimal numbers of T2 cells are present in the transplant (c.f. the upper and lower part of Fig. 4).

When thymocytes were used as a source of T1 cells, no or only marginal synergistic responses could be detected (Fig. 6). Feldmann and Erb (13) showed in their studies that mixtures of thymus and lymph node cells were occasionally synergistic, but mixtures of anti-lymphocyte serum (ALS)-treated spleen cells and ATx spleen cells were always clearly synergistic in the *in vitro* induction of T helper activity for keyhole limpet hemocyanin. No helper function could be induced in ALS spleen cells. Thus, the T helper cell precursors resided in the ATx spleen and T amplifier cells in the ALS spleen. Using the same system, Feldmann et al. (14) showed that T amplifier cells belong to the pool of Lyt-123$^+$ cells in the spleen which are ATx sensitive (T1 cells). These cells amplified Lyt-1$^+$, ATx resistant, ATS sensitive precursors (T2 cells) in their differentiation to T helper effector cells. This suggests

that both in GvH-related DTH and in induction of T helper activity more differentiated T cells than those residing in the thymus are needed for optimal amplifier activity. These amplifier cells probably belong to the population of post-thymic precursor cells described by Stutman (15).

In our GvH system generation of T2-derived DTH effector cells can only be initiated by H-2I region coded antigens (12; table 3) and Mls locus coded antigens (12). H-2K and H-2D region coded antigens are unable to elicit an anti-host DTH response (12). I-region differences alone do not result in any amplifier activity of T1 cells (Table 3). On the other hand, a K and D region difference, next to an I-region difference, does induce amplifier activity in T1 cells (Table 3). Apparently in GvH-related DTH the collaborating T1 and T2 cells are activated by different H-2 subregion coded antigens. Thus, both T cell subpopulations must have different receptors, and belong to different T cell differentiation pathways.

Observations of Scollay et al. (16) and Mathieson et al. (17) suggest that there exist two separate lines of intrathymic differentiation, one leading to Lyt-1$^+$ cells and the other to Lyt-123$^+$ cells. The latter cells might further differentiate into Lyt-23$^+$ cells, after their migration as Lyt-123$^+$ cells to the periphery (16,18). A part of this postthymic Lyt-123$^+$ population is ATx sensitive and thus fulfills the criteria of T1 cells (19). It is this subpopulation which can amplify T helper activity in humoral responses (14,20). We suggest that the same subpopulation of postthymic Lyt-123$^+$ cells amplifies the generation of anti-host DTH effector cells from (probably Lyt-1$^+$) T2 cells (Fig. 8). These T2-derived DTH effector cells can release humoral factors which lead to the expression of GvH-related DTH upon transfer (this paper), to differentiation of Lyt-123$^+$ cells into allo-

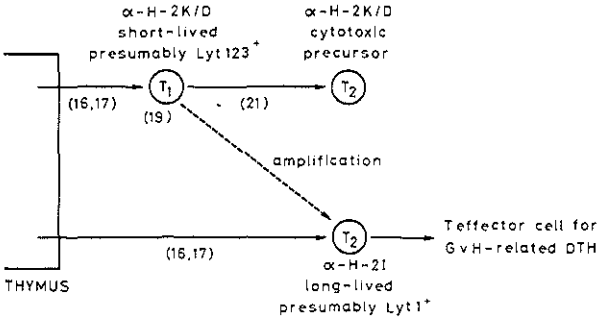

Figure 8
Model for T1 cell amplification of T2 cell-derived GvH-related DTH reactivity.

reactive Lyt-23$^+$ precursors of cytotoxic T lymphocytes (21) and stimulation of differentiation of Lyt-23$^+$ precursors into Lyt-23$^+$ cytotoxic effector cells (22). The analogy between T2-derived DTH effector cells and T2-derived T helper effector cells leads us to speculate that in case of enhancement of T2 helper cell activity by T1 amplifier cells (14,20) both subpopulations are restricted by different H-2 subregions, i.e., T helper cells by H-2I and T amplifier cells by H-2K/D.

In conclusion, this paper shows that after allogeneic spleen cell transplantation into lethally irradiated hosts T1-T2 cell cooperation is required for optimal development of anti-host DTH reactivity. Anti-host DTH T effector cells are the progeny of the pool of recirculating T2 cells, which are mainly activated by H-2I region coded antigens. T amplifier cells belong to the pool of sessile T1 cells and are probably activated only by H-2K and/or H-2D region coded antigens. Studies on the Lyt phenotype of these T cell subpopulations are in progress.

Abbreviations used in this paper:
ATS, anti-thymocyte serum; ATx, adult thymectomy; BSS, balanced salt solution; DTH, delayed type hypersensitivity; GvH, graft-versus-host; 1 LNS eq., one lymph node-seeking equivalent; MHC, major histocompatibility complex; MLC, mixed lymphocyte culture; ShTx, sham-thymectomy.

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APPENDIX PAPER VII

ANTI-HOST IMMUNE REACTIVITY AFTER ALLOGENEIC BONE MARROW TRANSPLANTATION

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SUMMARY

During initiation of a delayed Graft-versus-Host (GvH) reaction by injection of C57BL/Rij bone marrow cells into lethally irradiated (C57BL/Rij x CBA/Rij)F1 hybrid mice, a state of delayed type hypersensitivity (DTH) against host histocompatibility antigens occurs. This DTH reactivity was maximal 12 days after bone marrow transplantation and could no longer be demonstrated at day 20. In adult thymectomized (ATx) recipients this DTH reactivity appeared about 16 days after transplantation and reached a maximum at about 20 days. The maximum DTH responses of ATx mice were lower than the maximum responses of non-thymectomized mice. These findings were studied in relation to cellular changes in the thymus, spleen and lymph nodes. Pretreatment of the C57BL/Rij bone marrow cells with anti-Thy-1.2 serum and complement *in vitro*, and treatment of the C57BL/Rij donor mice *in vivo* with anti-thymocyte serum before harvesting the bone marrow cells, could completely or almost completely prevent GvH-related DTH reactivity.

These results suggest that mature T cells, residing in the bone marrow inoculum, play a major functional role in the development of anti-host DTH reactivity and that T cells newly formed under influence of the recipient's thymus can accelerate and enhance this GvH-related DTH responsiveness.

INTRODUCTION

Transplantation of allogeneic bone marrow cells into lethally irradiated mice may produce radiation chimeras who suffer from secondary disease. The pathogenesis of this disease is primarily caused by a Graft-versus-Host (GvH) reaction. Such a GvH reaction may be caused by immunocompetent T lymphocytes which reside in the bone marrow inoculum. Evidence for this supposition comes from experiments involving velocity sedimentation and density separation of bone marrow cells (1,5,23). These studies revealed maximal GvH activity in the lymphocyte-rich

fractions (23) and in the fractions with the greatest *in vitro* proliferative responsiveness to irradiated allogeneic cells (1). Treatment of donor mice with anti lymphocyte serum (ALS) could completely aboslish the GvH inducing capacity of spleen and lymph node cells (2) and greatly reduce the GvH eliciting capacity of bone marrow cells (9), suggesting that bone marrow T cells belong to the pool of potentially recirculating cells (8).

On the other hand, the possibility has to be considered that T cells, newly formed under influence of the recipient's thymus, are also involved. Goedbloed and Vos (6) studied the influence of thymectomy of the host before irradiation and allogeneic bone marrow transplantation on the incidence of secondary disease. Van Putten (21) studied this effect in a xenogeneic combination. In both these studies it was found that thymectomy of the recipients prior to the bone marrow transplantation can cause a delay in mortality. However, the authors could not distinguish between death caused by secondary disease and death caused by a wasting syndrome due to thymectomy. Chen et al. (4) also found that the thymus influences the rate of secondary disease, but not the final cumulative mortality. On the other hand, Simmons (15) could not detect any difference in survival pattern of sham-operated and thymectomized, allogeneically reconstituted mice. An important drawback in all these studies is the fact that no quantitative assay for the GvH reaction in irradiated recipients is available.

In previous studies (22) we have found that after transplantation of C57BL/Rij spleen cells into lethally irradiated (C57BL/Rij x CBA/Rij)F1 mice a specific delayed type hypersensitivity (DTH) to the host histocompatibility antigens developes in spleen and lymph nodes of the recipient mice. This DTH reactivity can be demonstrated by transfer of the recipient spleen and lymph node cells into normal C57BL/Rij mice. Challenge of these secondary recipients with CBA/Rij spleen cells then evokes an easily measurable DTH response. Using this new assay we now studied the development of anti-host immune reactivity after semi-allogeneic bone marrow transplantation in lethally irradiated thymectomized and sham-operated control mice.

MATERIALS AND METHODS

Animals. C57BL/Rij ($H\text{-}2^b$) male mice, 10-15 weeks old, CBA/Rij ($H\text{-}2^q$) female mice, 30-40 weeks old, (C57BL/Rij x CBA/Rij)F1 ($H\text{-}2^{b/q}$) male and female mice, 10-20 weeks old, were used. They were purchased from the Medical Biological Laboratory TNO, Rijswijk (ZH), and the Laboratory Animals Center of the Erasmus University, Rotterdam, The Netherlands.

Preparation of cell suspensions. Cell suspensions were prepared in a balanced salt solution (BSS) as described previously (22). Bone marrow cells were collected by flushing femurs and tibiae with BSS. For reconstitution 10^7 bone marrow cells, suspended in a volume of 0.5 ml BSS, were injected intravenously (iv). The bone marrow cells were always obtained from 10 weeks old C57BL/Rij donor mice. Nucleated cells were counted with a Coulter Counter Model B.

Irradiation. The recipient (C57BL/Rij x CBA/Rij)F1 mice received 925 rads whole body irradiation, generated in a Philips Müller MG 300 X-ray machine as described in detail previously (22). Radiation control mice died in 10-16 days.

Adult thymectomy. Adult thymectomy (ATx) and sham-thymectomy (ShTx) was always performed at 6 weeks of age. The surgery was done as described by Miller (12). The mice were anaesthetized by 70 mg per kg body weight Nembutal (Abbott S.A., Saint-Rémy-sur-Avre, France). The ATx mice were rested a minimum of 10 weeks before experimental use. All ATx mice were examined for thymic remnants at the end of each experiment.

Selective elimination of Thy-1.2 positive cells. The production of anti Thy-1.2 sera and their use for selective elimination of T cells have been described previously (22). Bone marrow cells were treated for 30 min at 4^oC with anti Thy-1.2 serum. The amount of anti Thy-1.2 serum used was 2 or 3 times more than was needed to kill at least 95% of corticosteroid resistant thymocytes (CRT). These CRT were obtained by ip injection of 30 mg per kg body weight dexamethasone sodium phosphate (Merck & Co., Rahway, N.J.) 2 days before harvest. After incubation the cells were centrifuged, resuspended in BSS and incubated with guinea pig complement for 15 min at 37^oC. Thereafter, the cells were washed 3 times and resuspended in BSS.

Anti thymocyte serum. Anti thymocyte serum (ATS) was prepared in New Zealand White rabbits with two iv injections of 5×10^8 BALB/c thymocytes, according to the method of Jooste et al. (7).

Before use in the *in vivo* experiments ATS and normal rabbit serum (NRS) were absorbed once with an equal volume of mouse red blood cells. C57BL/Rij bone marrow donors were injected subcutaneously (sc) with 0.2 ml ATS or NRS, equally distributed over the inguinal and axillary areas. These injections were given 5 and 2 days before isolating the bone marrow.

For use in the immunofluorescence staining technique, the immunoglobulin fraction of the ATS was isolated by saturated $(NH_4)_2SO_4$ precipitation. This fraction was absorbed three times with BALB/c IgG2b plasma cell tumor cells, and thereafter once with spleen cells from (C57BL/Rij x CBA/Rij)F1 mice which were thymectomized, lethally irradiated and reconstituted with 2 x 10^6 syngeneic fetal liver cells. The specificity of the absorbed antiserum for T lymphocytes was determined as described in a previous paper of our laboratory (20).

Immunofluorescence staining of T and B cells. Before reacting with antisera, the cells were washed with 5% bovine albumine (Poviet, Amsterdam, The Netherlands) in 0.01 Mol phosphate buffered saline (5% BA-PBS; pH 7.8). Thereafter 10^6 cells in 25 µl were incubated for 30 min at 4°C with 25 µl of an appropriate dilution of ATS, washed 2 times with 1% BA-PBS, resuspended in the final volume of 25 µl and mixed with 25 µl FITC-goat anti rabbit-immunoglobulin conjugate and 25 µl TRITC-goat anti mouse-immunoglobulin conjugate (both from Nordic, Tilburg, The Netherlands). After incubating for another 30 min the cells were washed 3 times with 1% BA-PBS and mounted on a glass slide in an equal volume of buffered glycerol (9 parts glycerol and 1 part PBS). The slides were examined with a Zeiss standard microscope equipped with a vertical illuminator IV/F and an Osram HBO 50 mercury lamp. Per slide 300 cells were scored.

Assay for delayed type hypersensitivity. The delayed type hypersensitivity (DTH) assay has been described in detail in a previous paper (22). Briefly, a number of cells equivalent to one whole spleen from one irradiated and reconstituted (C57BL/Rij x CBA/Rij)F1 recipient mouse was transferred iv into male C57BL/Rij recipient mice. The DTH reactivity of these recipient mice was determined by measuring the difference in thickness of the hind feet 24 hr after sc injection of 2 x 10^7 CBA/Rij spleen cells into the instep of the right hind foot. The specific increase in foot thickness was calculated as the relative increase in foot thickness of the immune mice minus the relative increase in foot thickness of the control mice. The swelling of challenged normal C57BL/Rij control mice varied between 15 and 22%.

RESULTS

Cellular changes in thymus, spleen and lymph nodes after irradiation and bone marrow transplantation.
The cellular changes in thymus, spleen and peripheral lymph nodes (inguinal and axillary) of lethally irradiated (C57BL/Rij x CBA/Rij)F1 mice inoculated with 10^7 C57BL/Rij bone marrow cells, were studied.

After a sharp decrease of the total number of nucleated cells in the thymus due to irradiation damage, the recovery started around day 7. This was found both after semiallogeneic and after syngeneic reconstitution (Fig. 1). Syngeneically reconstituted mice showed a faster increase and reached higher numbers of thymocytes than mice reconstituted with C57BL/Rij bone marrow cells.

The numbers of T and B cells in spleen and peripheral lymph nodes were determined by means of the membrane immunofluorescence technique. These numbers were assayed during the first 40 days after irradiation and semiallogeneic bone marrow transplantation in sham-thymectomized (ShTx) and thymectomized (ATx) recipients. These figures were compared with the recovery of T and B cells in the spleen and lymph nodes of non-thymectomized recipients, which were irradiated and syngeneically

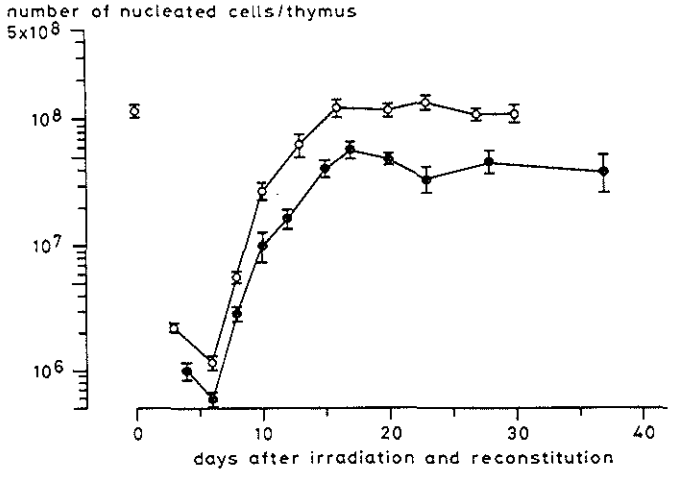

Figure 1
Recovery of the number of nucleated cells in the thymus of lethally irradiated (C57BL/Rij x CBA/Rij)F1 mice, reconstituted with either 10^7 C57BL/Rij bone marrow cells (●) or 10^7 syngeneic bone marrow cells (O). The figures representing the values 0 days after irradiation were obtained from non-irradiated control mice. Each experimental point represents the average $\pm$ 1 SEM of 5 mice.

reconstituted. In the spleen of ShTx mice a temporary increase and subsequent decrease of T cell numbers was found between day 10 and 15 after semiallogeneic bone marrow transplantation (Fig. 2). Maximum T cell numbers in these ShTx recipients were higher than in similarly reconstituted ATx recipients by day 12; after day 23, T cell numbers in ShTx recipient mice started to increase again. In syngeneically reconstituted mice the increase of T cell numbers in the spleen started as late as 2 weeks after transplantation. B cell recovery, on the other hand, was much faster in these syngeneically reconstituted mice (Fig. 2). No difference in B cell recovery could be detected between semiallogeneically reconstituted ShTx and ATx recipient mice.

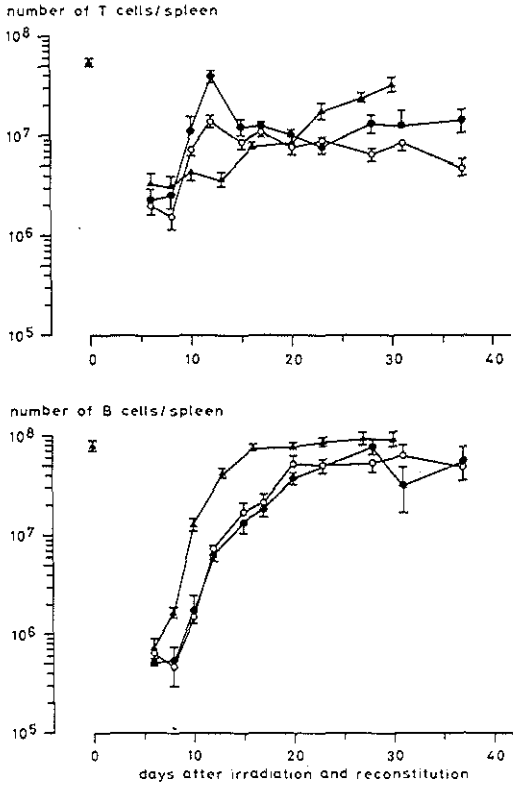

Figure 2
Recovery of the number of T cells (upper part) and B cells (lower part) in the spleen of lethally irradiated ShTx (●) and ATx (○) (C57BL/Rij x CBA/Rij) F1 mice reconstituted with 10^7 C57BL/Rij bone marrow cells, and of similarly, but syngeneically reconstituted (C57BL/Rij x CBA/Rij)F1 mice (▲). The figures representing the values 0 days after irradiation were obtained from non-irradiated control mice. Each experimental point represents the average ± 1 SEM of 5 mice.

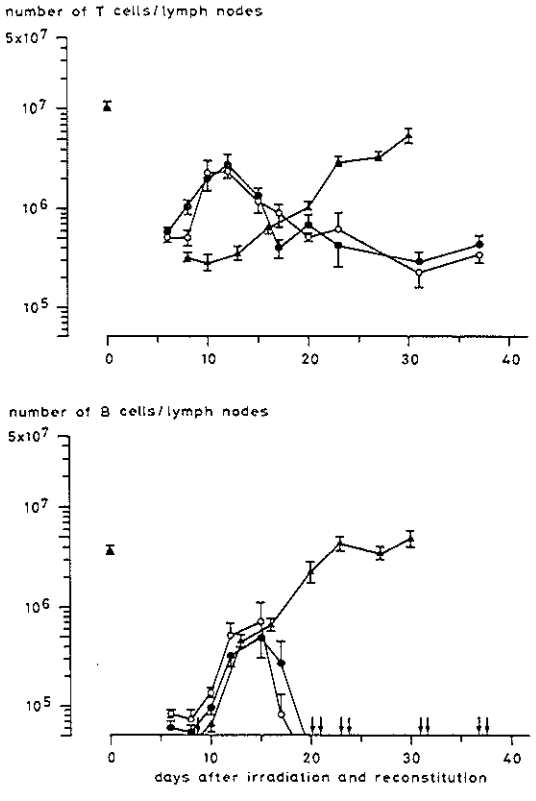

Figure 3
Recovery of the number of T cells (upper part) and B cells (lower part) in the peripheral lymph nodes of lethally irradiated ShTx (●) and ATx (O) (C57BL/Rij x CBA/Rij)F1 mice reconstituted with 10^7 C57BL/Rij bone marrow cells, and of similarly, but syngeneically reconstituted (C57BL/Rij x CBA/Rij)F1 mice (▲). Arrows indicate cell numbers below the abscissa. The figures representing the values 0 days after irradiation were obtained from non-irradiated control mice. Each experimental point represents the average ± 1 SEM of 5 mice.

In the peripheral lymph nodes of both ShTx and ATx semiallogeneically reconstituted mice an increase and subsequent decrease of T cell numbers was found between day 8 and day 15 (Fig. 3). Again, in syngeneically reconstituted mice this early increase of T cell numbers was not observed. In these mice T cell recovery started to increase gradually after about 15 days. No significant difference in T cell numbers in the lymph nodes of ShTx and ATx recipients could be detected within the period of observation of 40 days. B cell recovery in the lymph nodes started about 10 days after irradiation and allogeneic or syngeneic bone marrow transplantation (Fig. 3). However, at day 16-17 after allogeneic reconstitution, the B cell numbers in the lymph nodes decreased sharply, probably due to a developing GvH reaction.

Development of GvH-related DTH reactivity in the spleen of ShTx and ATx mice after irradiation and semiallogeneic bone marrow transplantation.

At various intervals after irradiation and reconstitution of (C57BL/Rij x CBA/Rij)F1 mice with 10^7 C57BL/Rij bone marrow cells, the recipient spleens were transferred into normal C57BL/Rij mice. Immediately thereafter, these C57BL/Rij mice were challenged with 2 x 10^7 CBA/Rij spleen cells. At 24 hr after challenge the DTH reaction was measured. The upper part of Fig. 4 shows the development of DTH reactivity in ShTx recipient mice. Already 8 days after transplantation a DTH reactivity could be demonstrated. Maximum DTH reactivity was found to occur at about day 12. Thereafter the reactivity declined until no significant DTH reactivity could be detected any more on day 20. In ATx recipient mice, on the other hand, (Fig. 4, lower part) DTH reactivity started to increase

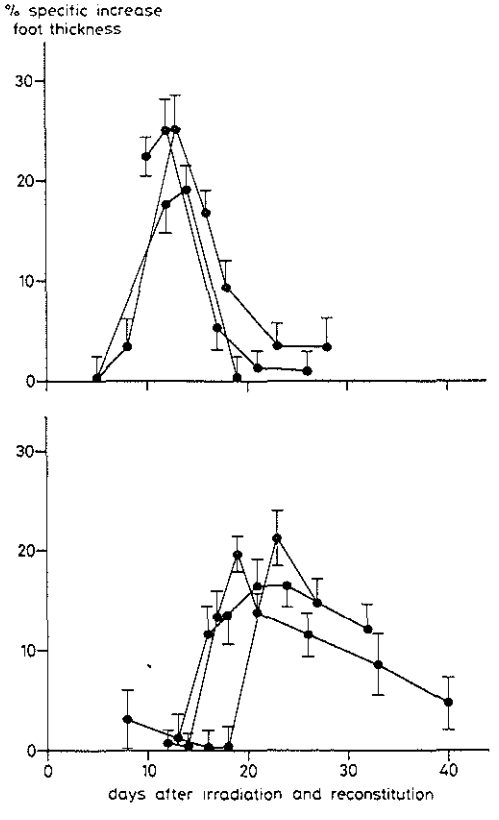

Figure 4
Development of GvH-related DTH responsiveness in the spleen of lethally irradiated ShTx (upper part) and ATx (lower part) (C57BL/Rij x CBA/Rij)F1 mice, transplanted with 10^7 C57BL/Rij bone marrow cells. Each experimental point represents the average $\pm$ 1 SEM of a group of at least 5 mice. Three different experiments are shown.

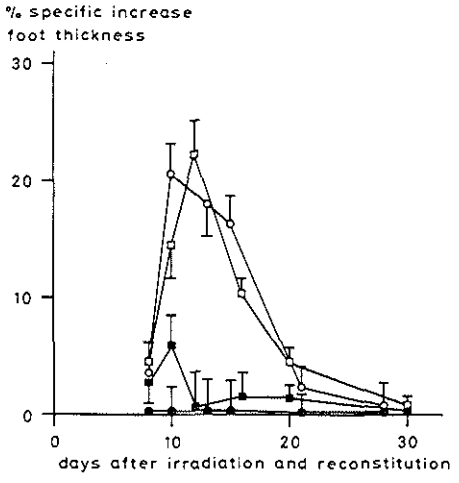

Figure 5
Development of GvH-related DTH responsivenss in the spleen of lethally irradiated (C57BL/Rij x CBA/Rij)F1 mice, reconstituted with 10^7 C57BL/Rij bone marrow cells, which were treated in vitro with either anti-Thy-1.2 serum (●) and C or NMS (○) and C before transplantation; or reconstituted with 10^7 bone marrow cells from C57BL/Rij donors, which were treated in vivo with ATS (■) or NRS (□) before harvesting the bone marrow cells. Each experimental point represents the average ± 1 SEM of a group of at least 5 mice.

around day 15, reached maximum values around day 20 and steadily decreased thereafter. The course of the DTH reactivity in ATx recipient mice in different experiments was more variable than in ShTx recipients.

Dependence of GvH-related DTH reactivity on T cells residing in the bone marrow.

In order to test whether the anti-host DTH reactivity rely on Thy-1.2 positive cells which reside in the bone marrow inoculum, the C57BL/Rij bone marrow cells were treated *in vitro* with anti Thy-1.2 serum and complement (C) or normal mouse serum (NMS) and C before transplantation into irradiated (C57BL/Rij x CBA/Rij)F1 recipient mice. As can be seen in Fig. 5, treatment of the bone marrow cells with anti Thy-1.2 serum and C before transplantation completely prevented the development of anti-host DTH reactivity during the observation period of 30 days. The question of whether these bone marrow T cells belong to the pool of mature recirculating T cells was studied by pretreatment of the C57BL/Rij donor mice with anti-thymocyte serum (ATS) *in vivo* 5 and 2 days before harvesting the bone marrow cells. Control donor mice were treated with normal rabbit serum (NRS). As can be seen in Fig. 5, ATS treatment greatly reduced the capacity of the bone marrow cells to elicit an anti-host DTH response.

DISCUSSION

The present study shows that after semiallogeneic bone marrow transplantation into lethally irradiated mice, an anti-host DTH responsiveness is built up in the spleen of the recipient mice. This DTH reactivity starts to increase 8 days after reconstitution, reaches maximum values on day 12-13 and decreases sharply thereafter. By day 20 no significant response could be detected anymore (Fig. 4). This correlates well with the increase and subsequent decrease of number of T cells in the spleen (Fig. 2) and lymph nodes (Fig. 3) of the ShTx recipient mice, and the time course of the anti-host response in irradiated CBA mice inoculated with C57BL bone marrow cells, as measured with the Simonsen splenomegaly assay by Van Bekkum et al. (19). These authors found an anti-host response by day 10 and 15 after reconstitution. Thereafter they could no longer detect anti-host reactivity.

In contrast to this early anti-host immune reactivity, the symptoms of secondary disease in this C57BL/Rij → (C57BL/Rij x CBA/Rij)F1 combination appear about 30 days after irradiation and reconstitution. The severity of this disease varies considerable. Some of the chimeras die, other recover after about 3 months, and some of the animals have no signs of the disease at all. This variation does not occur in the anti-host immune response, measured in our DTH system. This response develops in all animals, at the same time, and to about the same extent.

An old, but still inconclusively answered question is whether delayed GvH disease in mice is caused by mature T cells residing in the transplanted inoculum, or is due to newly formed T cells, which arise from primitive precursor cells under influence of the recipient thymus (18). Treatment of the bone marrow inoculum with anti Thy-1.2 serum and complement *in vitro* before transplantation, eliminated its ability to induce GvH mortality in lethally irradiated (semi)allogeneic recipients (13,16). As can be seen in Fig. 5, after pretreatment of the bone marrow cells with anti Thy-1.2 serum and complement *in vitro* also no anti-host DTH reactivity could be detected. Similarly, elimination of the mature recirculating T2 cells from the bone marrow by ATS *in vivo* (8) could almost completely prevent the development of anti-host DTH reactivity (Fig. 5). Ledney and Van Bekkum (9) could enhance the survival rate of the recipient mice from 10% to 50% by treatment of the allogeneic bone marrow donors with purified ALS. But as stated by Thierfielder and Rodt (17), in the mouse the parent → F1 combination is an "easy system" with regard to GvH sup-

pression. The above mentioned results suggest that mature T cells residing in the bone marrow inoculum have a predominant role in the development of secondary disease in the parent→F1 combination studied.

Löwenberg (10) described a delayed form of GvH disease after transplantation of allogeneic fetal liver cells in the C57BL/Rij → CBA/Rij combination. Since the fetal liver is considered to contain virtually no immunocompetent T cells at the chosen moment of harvest, this study and work of Lydyard and Ivanyi (11) suggest that newly formed T cells contribute to the development of GvH disease. In earlier studies of Goedbloed and Vos (6) and Van Putten (21) the contribution of T cells newly formed under influence of the recipient's thymus also became apparent, since thymectomy of the recipients before allogeneic and xenogeneic bone marrow transplantation could delay mortality. Osmond and Nakatsui (14) showed that thymectomy diminishes the recovery of GvH reactivity in the spleen of syngeneically bone marrow reconstituted rats, when assayed 3 weeks after reconstitution. In our experiments thymectomy was found to delay the onset of GvH-related DTH reactivity. Furthermore, after thymectomy the maximum DTH response was somewhat diminished. These results suggest that mature T cells elicit the anti-host DTH reactivity, whereas T cells newly formed under influence of the thymus, which starts to recover before the onset of GvH-related DTH reactivity (Fig. 1), accelerate and enhance this response. In contrast to ShTx recipients the occurrence of anti-host DTH reactivity in the spleen of ATx recipients did not coincide with a temporary increase of the number of splenic T cells, suggesting that lymphocyte numbers are not a reliable measure for the anti-host immune reactivity in this semiallogeneic combination.

In the splenomegaly GvH assay, synergy occurs between newly formed T1 cells and long-lived T2 cells (3). In that system the eliciting cell is the T1 cell, while the T2 cell amplifies the response of these short-lived cells (3). Obviously this is the reverse as compared with our anti-host DTH assay. Although this difference might be attributed to the use of irradiated, adult recipients versus non-irradiated, newborn ones, the possibility has to be considered that it is due to the different parameter studied.

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