CELLULAR AND GENETIC REQUIREMENTS FOR DELAYED TYPE HYPERSENSITIVITY

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ABBREVIATIONS

ATS	Anti-thymocyte serum
ATx	Thymectomy of adult mice
B cell	Bone marrow-derived lymphocyte
BCG	Bacillus Calmette Guérin
CML	Cell-mediated lympholysis
CTL	Cytotoxic T lymphocytes
DTH	Delayed type hypersensitivity
FCA	Freund's complete adjuvant
GvH-reaction	Graft-versus-Host reaction
H-antigen	Histocompatibility antigen
H-2 complex	Major histocompatibility complex of the mouse
H-2A locus	Gene locus in the I-A subregion of the H-2 complex
H-2C locus	Gene locus in the I-C subregion of the H-2 complex
H-2D locus	Marker locus of the D region of the H-2 complex
H-2K locus	Marker locus of the K region of the H-2 complex
H-2T	Gene locus in the T region
HvG-reaction	Host-versus-Graft reaction
H-Y antigen	Male-specific histocompatibility antigen
Ia antigen	Serologically detected I region coded antigens
Ir gene	Immune regulatory gene
I.v.	Intravenously
LAD	Lymphocyte activating determinant
LD antigen	Lymphocyte defined antigen
MHC	Major histocompatibility complex
MIF	Migration inhibiting factor
MLR	Mixed lymphocyte reaction
Mls-locus	Minor lymphocyte stimulating locus
MPS	Mononuclear phagocyte system
PPD	Purified protein derivative (of tubercle bacilli)

S.c.	Subcutaneously
SD antigen	Serologically defined antigen
SRBC	Sheep red blood cells
T cell	Thymus-derived lymphocyte
T1 cell	"Immature" T lymphocyte, short-lived after ATx
T2 cell	"Mature" T lymphocyte, long-lived after ATx
TNP	Trinitrophenol
V_H	Variable part of the heavy chain of the immunoglobulin molecule
V_L	Variable part of the light chain of the immunoglobulin molecule
V genes	Genes coding for the variable parts of the immunoglobulin molecule

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GENERAL INTRODUCTION

1. AIM OF THE STUDY

All living organisms are continuously exposed to pathogenic agents from the surrounding milieu, e.g. viruses, bacteria, fungi and parasites. Skin and mucous membranes form efficient barriers to these agents, but sometimes this defense is overcome. If an infectious agent succeeds in penetrating into the "milieu intérieur", at first granulocytes and mononuclear phagocytes become involved in the elimination of the intruding microorganisms. This part of the defense system is largely aspecific. Apart from this, vertebrates have a well developed immune system which can mount specific immune responses to invading organisms and foreign substances. The specificity of the immune reaction is based on the presence of receptors on the individual lymphocytes which recognize specifically any one of many foreign substances. Only after the recognition of the immunogenic material is a lymphocyte activated to perform its function. Immunogenic substances or antigens are operationally defined by their capacity to induce an immune response. The recognition of a particular antigen by the receptors of an individual lymphocyte is predetermined, i.e. the diversity of lymphocytes, each specific for one of the numerous imaginable foreign substances is generated before encounter with immunogenic material. In addition to the specificity of lymphocytes for a particular antigen, lymphocytes must also discriminate between self and non-self. If they should fail to do so, an immune response to tissues of the individual's own body would arise, leading to autoimmune disease (Burnet, 1972).

Immunity can be mediated in two ways: humoral immunity and cellular or cell-mediated immunity. Humoral immunity is mediated by humoral products of plasmacells, i.e. antibodies, which are present in large amounts in the blood and can act at a long distance from the place of origin. Humoral immunity can be transferred to other individuals by means of serum. Cellular immunity is mediated by lymphocytes, or by factors secreted by them, which act at a short distance. This latter type of immunity can only be transferred to other individuals by means of cells (Mackaness and Blanden, 1967). Examples of cell-mediated immunity are resistance to several bacteria and viruses, graft rejection, and delayed type hypersensitivity (DTH). DTH has found a general application in the diagnosis of infection with tubercle bacilli. Thus, people who have been infected with tubercle bacilli show a positive Mantoux reaction upon injection into the skin of antigens, derived from tubercle bacilli (O'Grady, 1967).

Stimulation of the immune system by an antigen not only leads to an active immune response to the antigen, but also to the development of immunological memory specific for this antigen. A state of memory causes a more vigorous and faster humoral or cellular immune response upon the second contact with the antigen. The effectiveness of vaccination against certain infectious diseases reflects the capacity for memory formation by the immune system. Immunological memory is carried by lymphocytes with a lifespan of many months (Miller and Mitchell, 1969).

The lymphocytes whose progeny produces antibodies are called B lymphocytes. The lymphocytes, which are capable of mediating cellular immunity are called T lymphocytes. B lymphocytes are not essential for cell-mediated immune responses (Allison, 1972). Both B and T lymphocytes must be considered as heterogeneous populations of cells, which can be subdivided into many subpopulations, according to their function, differentiation stage, lifespan, etc.

The experiments described in this thesis were intended to characterize the T lymphocytes involved in delayed type hypersensitivity, and to investigate some aspects of their differentiation pathway. The genetic requirements for induction and expression of DTH to a particular group of tissue antigens was also analysed. In this study, the mouse was chosen as the experimental animal since (1) this species has a number of technical advantages, which makes the mouse a protagonist in the study of immunology, (2) large numbers of genetically well defined inbred mouse strains are available and (3) DTH reactivity can easily be induced and measured in mice (Crowle, 1975).

2. *STRUCTURE OF THE IMMUNE SYSTEM OF THE MOUSE*

2.1. Central and peripheral lymphoid organs

In the central lymphoid organs the antigen-independent differentiation of haemopoietic stem cell derived precursor cells into immunocompetent lymphocytes occurs. Thus, the bone marrow is the major production site for immunocompetent, "virgin" B lymphocytes (Phillips et al., 1977) During the differentiation in the bone marrow B lymphocytes acquire immunoglobulins on their surface membrane (Osmond, 1975). These immunoglobulins serve as the antigen-specific receptor of the B lymphocytes. In the thymus, committed precursor cells mature and differentiate into immunocompetent T lymphocytes (Roelants et al., 1976; Cantor and Weissman, 1976). For the maturation and differentiation of the precursor cells into T lymphocytes the close proximity of thymic epithelial cells and the influence of thymic hormone are essential (Stutman, 1975; Kruisbeek, 1978). The T lymphocytes do not acquire surface immunoglobulins during their differentiation in the thymus, but some surface antigens (e.g. Thy-1, Lyt, and H-2) become fully expressed, and others (TL) may arise and disappear during further differentiation. Moreover, T cells acquire an antigen-specific receptor in the thymus. The nature of this receptor is not yet fully understood (see chapter 6).

The generation of immunocompetent lymphocytes in the central lymphoid organs is reflected by the relatively high proliferative activity in these organs, compared to the other, peripheral, lymphoid organs (Osmond and Nossal, 1974; Bryant, 1974). Upon maturation the "virgin" T and B lymphocytes leave the thymus and bone marrow, respectively, and migrate to the peripheral lymphoid organs, where a further, antigen-driven, differentiation may take place. The most important peripheral lymphoid organs are the lymph nodes, spleen, and the gut associated lymphoid tissues. Part of the lymphocytes can migrate from blood into peripheral lymphoid tissues, and re-enter the blood again via the thoracic duct lymph. This migration pattern is named recirculation, and results in a continuously changing distribution of the lymphocytes over the different lymphoid organs (Gowans and Knight, 1964; Goldschneider and McGregor, 1968).

The induction of a cellular or humoral immune response generally occurs in the peripheral lymphatic tissues. After antigenic stimulation B lymphocytes proliferate and transform into plasmacells, which remain localized in the peripheral lymphatic tissues. Similarly, T helper cells are also retained in the lymphatic tissue during their helper activity. On the other hand, T cells

mediating an effector function in cellular immune responses emigrate from the peripheral lymphoid organ after the induction phase and may perform their function anywhere in the body. The involvement of peripherally localized effector T cells manifests itself in the DTH reaction and graft rejection.

The distinction between central and peripheral lymphoid organs is an oversimplification, since exceptions to this compartmentalization of antigen-independent and antigen-driven differentiation exist:

1. The presence of the bone marrow is not obligatory for the production of immunocompetent B lymphocytes, since in the absence of functional bone marrow their generation can also occur in the spleen (Kincade et al., 1978; Rozing et al., 1978).
2. Immunocompetent lymphocytes can migrate back into the central lymphoid organs, where large numbers of effector cells for humoral and cellular immune responses can reside (Youdim et al., 1973; Benner and Haaijman, 1979).

2.2. Thymus-derived lymphocytes

Cells, belonging to the T cell lineage carry the Thy-1 surface antigen (Raff and Wortis, 1970). This antigen is not expressed on other blood cells, except on the so-called natural killer cells. This latter cell type is a lymphoid cell and might also belong to the T cell lineage (Herberman et al., 1978), but does not require differentiation in the thymus. The stem cell-derived precursor cells, committed for T cell differentiation, also carry Thy-1 antigen, though in small quantity (Roelants et al., 1975). Morphologically, T lymphocytes form a heterogeneous population, consisting of small, medium and large sized cells. The lifespan of T lymphocytes varies considerably. A large proportion of the recirculating small lymphocytes has a lifespan of several months (Sprent and Basten, 1973).

In the spleen and lymph nodes the T lymphocytes are largely found in the periarteriolar lymphoid sheath and paracortical areas, respectively (Waksman et al., 1962; Parrott et al., 1966). They are generally situated between the cytoplasmic extensions of the interdigitating cells, which constitute their microenvironment (Veldman, 1970; Van Ewijk et al., 1974; Veerman and Van Ewijk, 1975; Van Ewijk, 1977).

T lymphocytes are essential in cell-mediated immunity. As so called T helper cells they may cooperate with B cells in the humoral immune response. T lymphocytes perform their immunologi-

cal effector functions either by the release of humoral factors which act at a short distance (e.g. in T helper function and in T cell-mediated activation of macrophages in DTH) or by direct cell-cell contact. Cytotoxic T cells use this latter mechanism in the killing of target cells (e.g. in graft rejection). T helper function and cytotoxic T cell function are mediated by two separate subsets of T lymphocytes which can be distinguished from each other by the presence of certain surface antigens, belonging to the Lyt-system (Cantor and Boyse, 1975a; b). T cells of one subset cannot differentiate into cells of the other subset (Huber et al., 1976a).

2.3. Bone marrow-derived lymphocytes

Like T lymphocytes, bone marrow-derived lymphocytes (B cells) are a heterogeneous population of cells, consisting of small, medium-sized and large cells. In the peripheral lymphatic tissues B cells are largely found in the follicles, where they lie in close contact with the follicular dendritic cells. These follicular dendritic cells may retain immune complexes on their surface (Nossal et al., 1968) and are probably involved in antigen-dependent B cell differentiation (Chen et al., 1978).

Immunocompetent B cells are characterized by the presence of immunoglobulins on their cell surface. After appropriate antigenic stimulation B cells can differentiate into plasma cells. These cells perform the effector function of the B cell lineage by production and secretion of antibodies. Antibodies act systemically and can persist for several days. T lymphocytes are able to enhance as well as to suppress the response of the B lymphocytes to most antigens.

The population of B lymphocytes consists of both short-lived and long-lived cells (Elson et al., 1976). A considerable part of the long-lived B cells recirculate, and carry immunological memory (Strober, 1975; Fidler et al., 1977).

2.4. Mononuclear phagocytes

Mononuclear phagocytes are derived from blood borne monocytes (Van Furth and Cohn, 1968; Crofton et al., 1978). Macrophages constitute the basic elements of the mononuclear phagocyte system (MPS). Mononuclear phagocytes can occur free or tissue bound. Kupffer cells of the liver, macrophages in the lung, and presumably the interdigitating cells in the lymphoid organs belong to this MPS (Veerman and Van Ewijk, 1975). Macrophages are essen-

tial for the induction of humoral as well as cellular immune responses, since they are involved in the antigen-processing and the presentation of antigens in an immunogenic form as suggested by in-vitro and in-vivo experiments (Mosier and Coppleson, 1968; Unanue, 1972; Van Ewijk et al., 1977).

Substances having the capacity to enhance non-specifically the immune response to a simultaneously injected antigen are called adjuvants. The adjuvant used mostly in animal studies is Freund's complete adjuvant (FCA), which is composed of water in oil emulsion, containing tubercle bacilli. The mineral oil has a depot function, while a glycopeptide constituent of the cell wall of mycobacteria (muramyldipeptide) carries the adjuvant property (Merser et al., 1975; Hiu, 1977). Adjuvants initiate a long-lasting accumulation of lymphocytes within the draining lymph nodes. Macrophage function seems to be linked to the adjuvant-induced changes in lymphocyte migration (Frost and Lance, 1978). They become activated by the adjuvant and process the antigen in such a way that the presented antigen provides a good immunogenic stimulus for the T cell system in particular.

In cell-mediated immunity macrophages often perform an aspecific effector function. Humoral substances produced by antigen-specific T lymphocytes can "switch on" macrophages to phagocytize and eliminate the antigen more vigorously (Waksman, 1979). Activated T lymphocytes can also secrete antigen-specific cytophilic factors, which adhere to macrophages, and "arm" them for specific killing of target cells. This factor was called specific macrophage arming factor (Evans and Alexander, 1970). Cytophilic antibodies can adhere to the macrophage surface as well, and thus enhance phagocytosis of the specific antigen (Boyden, 1963).

In conclusion it is apparent that at all stages of the immune response cells of the MPS play an important role.

3. *HISTOCOMPATIBILITY ANTIGENS*

3.1. *General outline*

Depending on the way antigens are presented to the immune system, two types can be distinguished, viz:
1. Conventional antigens
2. Tissue antigens

Most conventional antigens require processing and presentation by macrophages in order to become immunogenic for both B and T lymphocytes (Unanue, 1972). Conventional antigens are either particulate (e.g. bacteria, sheep red blood cells) or soluble (e.g. proteins, polysaccharides, hapten-carrier complexes). An optimal humoral response to part of these antigens requires the cooperation of T and B cells. These antigens are called thymus-dependent antigens. A number of conventional antigens exists, which do not require macrophage processing, but are potent activators of B cells without T cell help (Feldman, 1972a; b). The latter antigens (e.g. polyvinylpyrrolidone) are called thymus-independent antigens.

Tissue antigens often do not require presentation or processing by macrophages. Their immunogenicity is mainly due to their presence on the surface of viable tissue cells (Sørenson, 1972). The tissue antigens which account for the phenomenon of graft rejection, the so-called histocompatibility (H) antigens, can be subdivided into two main groups, according to the strength with which they lead to graft rejection (Counce et al., 1956):
1. major histocompatibility antigens, which cause a rapid, acute graft rejection.
2. minor histocompatibility antigens, which account for comparatively slow and more chronic graft rejections.

In the mouse the major H-antigens are coded for by a cluster of genes, lying on chromosome 17. This cluster of genes became known as the major histocompatibility complex (MHC) or in the mouse, the H-2 complex. In other vertebrates (e.g. frog, dog, rat, cattle, human) the presence of a MHC has also been demonstrated. It is obvious that study of the MHC in man (the HLA complex) is of the utmost importance in improving the clinical results of transplantations. On the other hand, the minor H-antigens which are coded for by genes spread all over the genome also influence the success of organ transplantations.

Another group of "tissue" antigens are the surface antigens, which appear, for example, due to viral infection or when spontaneous tumours arise. Virally induced surface antigens and tumour associated antigens may be considered as antigens which

cause a modification of the antigenic structure of the cell (Zinkernagel and Doherty, 1977). Antigens of this kind can also be produced artificially by chemical treatment of lymphoid cells with trinitrophenol (TNP). TNP-altered cells provide a model to study the immune response to this type of antigens. Both minor H-antigens and the type of tissue antigens described in this paragraph are usually associated with MHC-coded antigens and are sometimes referred to as modified syngeneic tissue antigens (Paul and Benacerraf, 1977).

3.2. The H-2 complex

3.2.A. Genetics

In 1946 Snell developed a breeding scheme to produce strains of inbred mice which differed from each other by a single H-antigen, or a cluster of H-antigens. As the criterion for H-antigen difference between two strains he used the survival time of skin grafts exchanged between the two mouse strains (Snell, 1948). Mouse strains differing from each other in a single gene coding for an H-antigen, are termed congenic mouse strains. Nowadays a large number of congenic mouse lines have been established, defining H-2 as well as non H-2 genes (Klein, 1975).

In heterozygous mice genetic recombination during meiosis can occur. After backcross of such a heterozygous mouse to a parental strain, recombinant mice may arise. These recombinants have a chromosome which is constituted of genetic material derived partly from the one and partly from the other original parental strain. Serological analysis of the H-2 complex coded antigens, originally started by Gorer (1947), made it possible to discover intra H-2 complex recombinants. The availability of inbred congenic recombinant mice and the possibility of serological analysis of H-2 complex coded antigens finally led to the complicated model of the mouse MHC, as shown in Fig. 1.

3.2.B. The map of the H-2 complex

The H-2 complex of the mouse is situated on chromosome number 17 and can be divided into five regions, the K, I, S, G and D region. The I-region consists of at least 5 subregions I-A, I-B, I-J, I-E and I-C. Each region or subregion contains minimally one marker gene locus, or cluster of loci (Fig. 1). Recently, at the D-end, a new H-2 locus, the H-2L locus, has been reported, and at least ten loci are now known in the H-2 complex (Klein, 1978).

The H-2K and H-2D loci code for complex surface antigens. It is possible to raise antibodies against parts of these large mole-

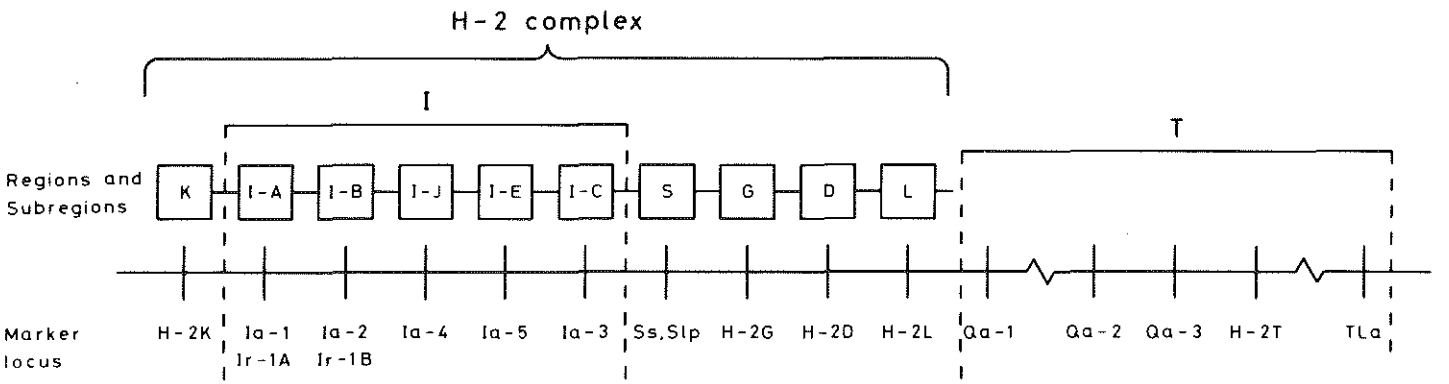

Fig. 1
The genetic map of the H-2 complex and the T-region. The loci in the T-region are tentatively ordered, since their relative positions have not yet been established.

cular structures (molecular weight of approximately 50,000 daltons). Therefore these antigens are also referred to as serologically-defined (SD) antigens. Against some antigenic determinants of the large molecules constituting the SD antigens antisera can be raised which "cross-react" with SD antigens of one or more unrelated mouse strains. These antigens were therefore called public antigens. Other antisera were found which only reacted to antigenic determinants, which were specific for a mouse strain with a particular allele, and no cross-reactivity was found with unrelated strains. These antigens are named private antigens, and they can distinguish the different unrelated H-2 alleles of the K or D locus.

The surface antigens coded for by the I-region were initially not detectable serologically. The existence of this MHC-region was demonstrated in experiments on the responsiveness of different mouse strains to one and the same antigen. It was found that the strength of the immune response to such an antigen was regulated by genes situated between the K and D region (McDevitt et al., 1972). These genes were termed immune regulatory genes (Ir genes). At the same time it became apparent that some genes in the I-region code for cell surface antigens, which in vitro can induce proliferation of allogeneic T lymphocytes (Bach et al., 1972). Following this observation, these I-region coded antigens were also known as lymphocyte defined (LD) antigens, or lymphocyte activating determinants (LAD). Recently, it became possible to detect I-region coded antigens serologically, and the serologically detected surface antigens are now known as immune response antigens or Ia antigens (Shreffler et al., 1974). It is not clear as yet whether the Ir genes, the genes coding for LAD and those coding for Ia antigens are identical.

The S-region contains two marker loci, the serum substance (Ss) gene and the sex-limited protein (Slp) gene. Both genes control the levels of some serum substances (a.o. the fourth component of complement), and they divide the H-2 complex into a K-end and a D-end. Furthermore, the G-region contains the H-2G locus, which controls surface determinants on red blood cells.

Distal to the H-2 complex the T-region is situated. The T-region was originally defined by the Tla locus, which codes for the TL surface antigen, present on thymic leukemia cells and on thymocytes of some mouse strains. TL antigens are serologically defined. Recently it was found that antisera against antigens coded for by the Tla locus were specific for certain peripheral lymphocytes. Since TL antigens only occur on thymocytes, it was concluded that this antiserum defined a distinct antigenic system,

and this group of antigens was termed Qa. Now, a total of three Qa loci have been defined serologically (Flaherty et al., 1978). The T region also codes for a number of histocompatibility antigens, which can be involved in graft rejection (e.g. H-2T, Fig. 1).

According to convention, alleles of a single locus of the MHC are designated by small superscript letters, indicating their genetic origin (e.g. H-$2K^k$, H-$2D^b$). The combinations of allelic forms of the loci within the H-2 complex are called haplotypes and they are also designated by small letter superscripts (e.g. H-2^b, H-2^d). The H-2 loci occur in many variant alleles, and the total number of identified H-2 haplotypes now known is 109, including those from wild mice (Klein et al., 1978). This large number indicates the polymorphic nature of the H-2 complex. In Table 1 the H-2 haplotypes and the alleles of the different loci have been presented for a number of frequently used congenic mice.

TABLE 1

H-2 HAPLOTYPE AND MAJOR HISTOCOMPATIBILITY COMPLEX ALLELES OF FREQUENTLY USED CONGENIC MOUSE STRAINS

Strain	H-2 haplotype	MHC region or subregion K	I-A	I-B	I-J	I-E	I-C	S	D
B10.ScSn; BALB.B	b	b	b	b	b	b	b	b	b
BALB/c; DBA/2; B10.D2	d	d	d	d	d	d	d	d	d
AKR; C3Hf; B10.Br	k	k	k	k	k	k	k	k	k
A; B10.A	a	k	k	k	k	k	d	d	d
SWISS; A.SW	s	s	s	s	s	s	s	s	s
B10.AQR	y1	q	k	k	k	k	d	d	d
B10.T(6R)	y2	q	q	q	q	q	q	q	d
A.TL	t1	s	k	k	k	k	k	k	d
A.TH	t2	s	s	s	s	s	s	s	d

3.2.C. Lymphocyte-defined antigens versus serologically-defined antigens of the H-2 complex

The fundamental distinction made between the K- and D-region coded antigens on the one hand, and the I-region coded antigens on the other hand, was initially based on the possibility of serological detection only of the SD antigens and the ability to induce T cell proliferation exclusively by the LD antigens. This view was strengthened by the observation that cytotoxic T cells were directed only against SD antigens on the surface of target cells, but not to I-region coded antigens (Alter et al., 1973). Later it became apparent that this distinction between LD and SD antigens was somewhat artificial, since K-region coded antigens could also stimulate T cell proliferation (Klein, 1978): thus a locus of the K-region presumably codes for a LAD. Furthermore, it was found that both the I-A and the I-C subregion contained a locus coding for a histocompatibility antigen, accounting for skin graft rejection (Klein et al., 1976). These loci were termed H-2A and H-2C locus, respectively. The antigens they code for can also serve as a target for cytotoxic T cells (Klein, 1978). Again, it is not known whether the H-2A and H-2C locus are identical to the Ia-1 and Ia-3 locus, respectively.

TABLE 2

THE DISTRIBUTION OF Ia ANTIGENS

Cell types	I-A	I-B	I-J	I-E	I-C
B lymphocytes	+	-	-	+	-
T lymphocytes	+	-	+	+	±
Macrophages	+	-	-	+	+
Epidermal cells (Langerhans cells)	+	-	-	+	±
Serum	+	-	-	+	-

The presence of SD antigens on cells is ubiquitous, but Ia antigens can only be found on a limited number of cell types, most notably on B lymphocytes, spermatocytes, some subpopulations of T lymphocytes and macrophages,and on epithelial cells (Hämmerling, 1975). The Ia loci may provide surface markers for particular subpopulations of T lymphocytes, since some of them are

selectively expressed in functionally distinct lymphocyte populations (Murphy, 1978). For example, the Ia-4 determinant coded for by the Ia-4 locus in the I-J subregion was only found on suppressor T lymphocytes (Okumara et al., 1977; Murphy, 1978). The cellular distribution of Ia antigens, coded for by the different I subregions has been given in Table 2.

3.3. Minor histocompatibility antigens in the mouse

The histocompatibility antigens which are coded for by genes outside the H-2 complex are termed minor H-antigens. In the mouse these antigens are known as non H-2 alloantigens. Presumably, the antigens coded for by genes of the T-region do not belong to this group of non H-2 alloantigens (Klein, 1978). In the mouse about 40 of these minor H-antigens have been defined with congenic mouse strains (Klein, 1975). The genes coding for non H-2 alloantigens are spread over the entire genome. Some of the non H-2 alloantigens act more strongly than others in graft rejection, and for most of them only a few allelic forms have been described. Probably, the genes coding for non H-2 alloantigens are by no means as polymorphic as the genes of the H-2 complex. It has been suggested that their function is non-immunological in nature, and they would serve normal, household functions in the cell, which require their expression on the cell membrane (Ohno, 1977; Klein, 1978). It was suggested that many more surface antigens may exist in mice than the 40 presently known minor H-antigens (Klein, 1975). The absence of allelic forms of the other surface antigens in the inbred laboratory mouse strains would prevent their immunological detection. For one minor H-antigen, the male specific H-Y antigen, coded for by a gene on the Y-chromosome, no allelic phenotypes have been described yet. The H-Y antigen can cause the rejection of male-derived skin by syngeneic female mice. It appears that only mouse strains of a particular H-2 haplotype can give a good cellular immune response to H-Y antigen (Gasser and Silvers, 1972). This aspect of immune responsiveness to particular antigens will be discussed later (Chapter 6).

Exceptional minor H-antigens are the surface antigens, coded for by the Mls locus. This locus is situated on chromosome number 1. Mls locus coded products are mainly expressed on B lymphocytes of adult mice, on macrophages and tooth germ, but not on T lymphocytes (Festenstein, 1976). In contrast to other minor H-antigens (Bevan, 1975; Gordon et al., 1975), products coded for by the Mls locus can induce strong proliferation of unprimed H-2 compatible T lymphocytes (Festenstein, 1973). Their relevance for skin graft rejection is dubious, and cytotoxic T cells directed to Mls locus coded products cannot be induced (Festen-

stein, 1976). However, both tooth germ transplant survival and graft-versus-host reactivity was influenced by Mls locus incompatibility (Huber et al., 1973; Bartova and Ivanyi, 1975), and therefore the Mls locus products can be considered as histocompatibility antigens.

Five alleles have been described for the Mls locus, based on stimulatory capacity in different strain combinations (Table 3), though it has not yet been formally established that these various stimulatory tissue antigens are all coded for by the Mls locus on chromosome number 1. The Mls-allelic phenotypes vary considerably in their capacity to stimulate T cell proliferation. In man no analogue for the Mls locus has been found as yet.

TABLE 3

STRAIN DISTRIBUTION OF Mls-ALLELES

Allelic form	Stimulatory capacity	Mouse strain
Mls^a	+++	DBA/2; DBA/1; AKR; NZB
Mls^b	-	CBA/H; CBA/H T6T6; C57BL/6; C57BL10 and all congenic strains on B10 background
Mls^c	++	C3Hf; C3H/He; A; SJL
Mls^d	++	CBA/J
Mls^e	+++	C3H/Tif

4. *T LYMPHOCYTE DIFFERENTIATION*

4.1. Generation of T cells in the thymus

Prethymic precursor cells in the bone marrow, derived from pluripotent haemopoietic stem cells and committed to T cell differentiation, arise in the bone marrow and are able to migrate to the thymus (Kadish and Basch, 1976; Abramson et al., 1977). According to Stutman and Good (1969) the migration into and the differentiation of precursor cells in the thymus are very sensitive to histocompatibility differences between the precursor cells and the thymus. In the thymus the committed precursor cells require contact with the thymic stromal cells for their further differentiation. During this differentiation and maturation the precursor cells acquire some new surface antigens (e.g. Lyt-antigens), and other surface antigens come to full expression (e.g. Thy-1 antigen). Recent studies have suggested that during maturation the thymic epithelial cells are important in determining which H-2 specificities the maturing T cells will be able to recognize as self in the periphery (Zinkernagel et al., 1978a).

According to their localization in the thymus thymocytes can be distinguished as cortical and medullary cells. The cortical thymocytes express a high density of Thy-1 antigen on their surface (high Thy-1) and are immature, immunoincompetent cells. In contrast the medullary thymocytes carry a low density of Thy-1 on their surface (low Thy-1) and they constitute the more mature immunocompetent pool of thymocytes. After treatment of mice with cortisone, the cortical thymocytes disappear. The medullary thymocytes are resistant to this treatment. Two main models of T cell development in the thymus have been proposed (reviewed by Shortman et al., 1975). In the first model, the cortical thymocytes would proliferate and mature, gradually losing part of the Thy-1 antigens. Large numbers of the cortical thymocytes die in situ (Joel et al., 1977), but the remaining cells would migrate to the medulla. At this site as low Thy-1 cells they would further mature and subsequently emigrate to the peripheral lymphoid tissues. The second model presumes two separate lines of T cell differentiation, a cortical and a medullary. The cortical lineage would be eliminated largely as a consequence of the anti-self reactivity of these cells (Von Boehmer and Byrd, 1972; Gorczynski and MacRae, 1979). The medullary lineage would be derived from low Thy-1 medullary precursor cells and after a few divisions these cells would give rise to the immunocompetent medullary thymocytes. Finally, the latter cells would migrate to the peripheral lymphoid organs.

The Lyt-surface antigens constitute a system of surface markers which are selectively expressed on functionally distinct T cell populations (see section 4.3.). The Lyt-system consists of three antigens, Lyt-1, Lyt-2 and Lyt-3, which are expressed on T cells only. More than 90% of the thymocytes bear Lyt-1 as well as Lyt-2 and Lyt-3 antigens (i.e. Lyt-123$^+$). The peripheral T lymphocyte population consists of only approximately 50% Lyt-123$^+$ cells the other cells are either Lyt-1$^+$, or Lyt-23$^+$ (Cantor and Boyse, 1975a, b). The Lyt-1$^+$ cells cannot differentiate into Lyt-23$^+$ cells (Huber et al., 1976a), but in peripheral lymphoid organs Lyt-123$^+$ cells may give rise to Lyt-1$^+$ and Lyt-23$^+$ cells (Cantor and Boyse, 1976; Stutman, 1978). On the basis of these findings it was postulated that all thymocytes are originally Lyt-123$^+$, and that the functionally distinct immunocompetent T cell subsets would derive from them in an antigen-independent step, after they have left the thymus (Fig. 2A; Huber et al., 1976a; Cantor and Boyse, 1977).

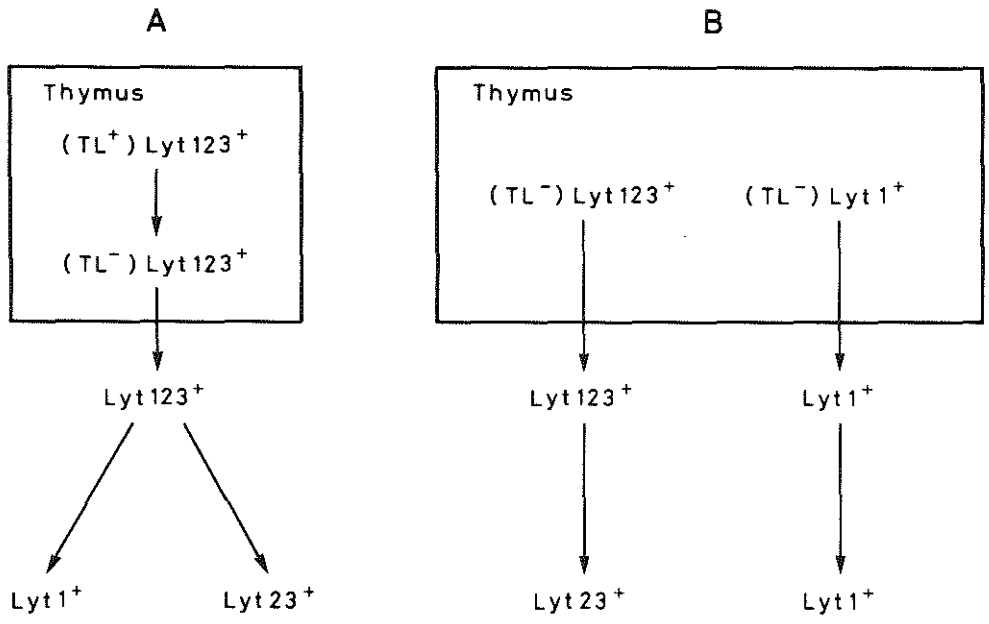

Fig. 2
The development of the functional T cell subsets.
(A) Intrathymic differentiation of a single T cell lineage.
(B) Intrathymic differentiation of two separate T cell lineages.

Recently it was found, however, that the cortisone-resistant T cell pool in the thymus contains a significant proportion of Lyt-1^+ cells, in frequency comparable to that in the peripheral T cell pool (Mathieson et al., 1979). Moreover, it was shown that intrathymically labelled thymocytes, recovered in the spleen 3 hours after the labelling, were partly Lyt-1^+ cells, and partly were Lyt-123^+ cells (Scollay et al., 1978). The latter finding indicates that Lyt-1^+ cells arise intrathymically before migration to the periphery. Altogether these observations suggest, but do not prove, that there exist two separate lines of intrathymic differentiation, one line leading to the Lyt-1^+ cells and the other to the Lyt-123^+ cells (Mathieson et al., 1979). The latter cells might further differentiate into the Lyt-23^+ cells, after their migration to the periphery (Cantor and Boyse, 1976; Scollay et al., 1978). Fig. 2B shows this differentiation pathway.

4.2. Post-thymic T cell differentiation

Peripheral T lymphocytes in the mouse represent a heterogeneous population. Raff and Cantor (1971; 1972a; b) proposed that the peripheral T lymphocytes should be divided into two subpopulations, namely T1 and T2 cells. The population of T1 cells is short-lived as can be shown by their rapid disappearance after adult thymectomy (ATx): they have a half life of 3-4 weeks. Because of its sessile nature the T1 subset is resistant to the in-vivo effects of small doses of anti-thymocyte serum (ATS) (Lance et al., 1973). The T2 cells have a long lifespan after ATx, and since they recirculate, they are sensitive to treatment with ATS. The T1 and T2 cells would correspond to "virgin" and "memory" T cells, respectively. The presence of T2 cells specific for certain antigens in unimmunized animals may be explained by an antigen-independent conversion of cells of the T1 subset into T2 cells, suggesting a predetermined differentiation of T1 cells into T2 cells (Cantor and Boyse, 1975a). The presence of recirculating T lymphocytes in the fetal lamb suggests that in immunologically unstimulated animals T2 cells can arise (Pearson et al., 1976; Cahill et al., 1979). The immunocompetence of the recirculating T cells was not studied, so the relevance of this observation for the maturation of immunocompetent T cells has not been established as yet. The hypothesis that the conversion of T1 into T2 cells is antigen-dependent (Araneo et al., 1977) is very attractive, since this hypothesis fits nicely in the clonal selection theory. This theory states that the specificity of the receptors of lymphocytes depends on a random, antigen-independent process. Due to the short lifespan of the T1 cells, those T1 cells with an irrelevant antigenic specificity disappear, since they are not driven by antigen to differentiate

into long-lived T2 cells. Environmental antigens would continuously select those T1 cells with relevant antigen-receptors.

According to the original hypothesis of Raff and Cantor only T2 cells are responsible for primary immune responsiveness, but other authors suggested that T1 cells may also contribute to primary responses (Kappler et al., 1974; Araneo et al., 1977).

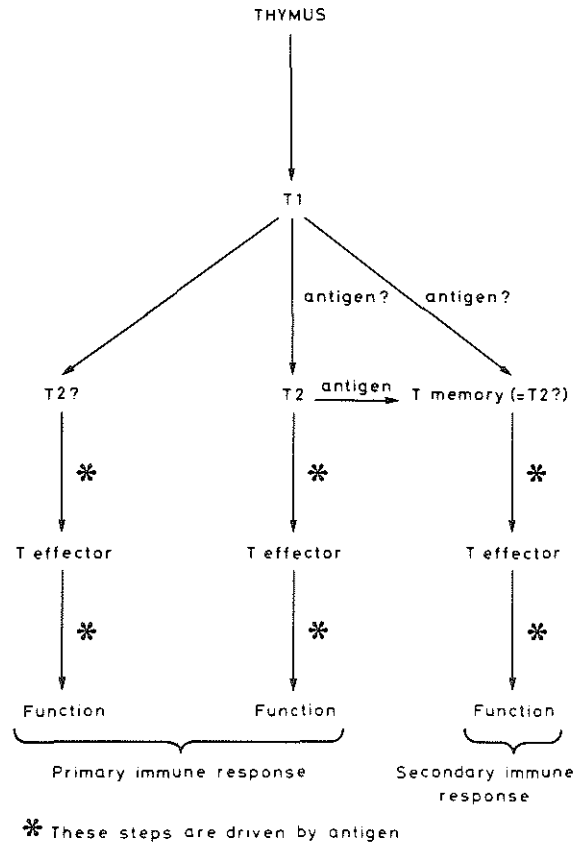

Fig. 3
Scheme of peripheral T cell differentiation.

After deliberate priming with antigen long-lived recirculating T memory cells might also arise, which account for the secondary immune response upon booster injection with the specific antigen. It was suggested that these latter T memory cells can be derived from T1 cells as well as from the already existing T2 cells (Araneo et al., 1977). It remains questionable whether the T memory cells accounting for secondary immune responses

are qualitatively different from the T2 cell population.

Next to T1 and T2 cells, the peripheral T lymphocyte pool constitutes of effector T lymphocytes. The generation of effector T cells is antigen-driven, and these cells carry out the immunological function. The effector T lymphocytes are short-lived cells, and are resistant to treatment with ATS (Araneo et al., 1976).

The differentiation stages of the peripheral T lymphocytes and their relationships as outlined in Fig. 3, were mainly demonstrated in experiments on the T helper system, but it was suggested that this model may also be valid for suppressor activity and the other T cell lines, committed to particular immunological functions, e.g. delayed type hypersensitivity, or cytotoxic activity. On the other hand, it might be that this differentiation model only holds for those T cells which are reactive to conventional antigens.

The clarity of the model depicted above is blurred by the demonstration of synergism of T1 and T2 cells. Such a synergistic effect has been shown in GvH reactivity (Cantor and Asofsky, 1970; 1972; Tigelaar and Asofsky, 1973), T helper functions (Feldmann et al., 1975a; Muirhead and Cudkowicz, 1978), T suppressor function (Feldmann and Kontiainen, 1976), during in-vitro T cell proliferation (Cohen and Howe, 1973) and the generation of cytotoxic T cells (Cantor and Simpson, 1975) in response to allogeneic cells. The synergism may be due to particular experimental conditions, since it can only be seen in models where small numbers of T2 cells are used (Cantor and Simpson, 1975). Therefore, some authors considered T1-T2 synergism as an irrelevant phenomenon (Araneo et al., 1977). Others suggested that T1 cells perform an amplifying function during their differentiation into T2 cells. It is also possible that the T1 population contains amplifier cells which are not subject to further differentiation and thus constitute a separate T cell lineage (Beverley et al., 1977).

4.3. T cell surface markers
4.3.A. General

Functionally different subsets of T lymphocytes can be classified on the basis of their surface antigens. Three distinct surface marker systems will be considered here: those of the Lyt-system, Ia antigens, and the Qa-1 antigen. As discussed in section 4.1. the Lyt-system consists of 3 different antigens, Lyt-1, Lyt-2 and Lyt-3, each of which can occur in two allelic forms. The Lyt-2 and Lyt-3 antigens are always expressed concomittantly on T cells,

and thus three T cell subsets can be distinguished with the Lyt surface markers, namely Lyt-1^+, Lyt-23^+ and Lyt-123^+ T cells.

The Ia antigens are coded for by gene loci in the H-2 complex, and these molecules may perform a regulatory function in the immune system. This became apparent from the finding that immunologically active helper and suppressor factors contain Ia antigens (Munro, 1978). The Qa-1 antigen is a recently detected surface marker, and is coded for by a gene in the T-region (Fig. 1). Qa-1^+ cells occur in the Lyt-1^+ and Lyt-23^+ T cell pool (Stanton et al., 1978).

4.3.B. Surface markers on functional T cell subsets

Based on the Lyt-system the peripheral T cells can be classified into 3 main groups (Table 4):

1. The Lyt-1^+ cells corresponding to the T helper cell pool. These T helper cells include both the T cells, helping B cells in the humoral response, and the T cells which proliferate in a mixed lymphocyte reaction (MLR) in response to LD antigens (Cantor and Boyse, 1975a; b). The MLR-reactive cells are termed T helper cells, since they cooperate with precursor cells of the cytotoxic T cells in the production of cytotoxic effector cells (Schendel et al., 1973). T cells mediating DTH to SRBC or proteins are also Lyt-1^+ cells (Vadas et al., 1976; Huber et al., 1976b). Since they stimulate macrophages they can be considered as a type of T helper cells. Both the precursor and the effector (helper) T cells are Lyt-1^+ cells.
2. The Lyt-23^+ cells represent both the cytotoxic T cells, directed to SD-antigens on allogeneic target cells, and the suppressor T cells, which may inhibit T helper function antigen-specifically or aspecifically (Feldmann et al., 1975b; Cantor et al., 1976; Jandinsky et al., 1976). Both the precursor cytotoxic T cells and the precursor suppressor T cells are Lyt-23^+ (Cantor and Boyse, 1975a; Beverley et al., 1976), and are found in the T2 cell pool (Feldmann et al., 1977). Also the cytotoxic effector cell directed to TNP-modified syngeneic target cells are Lyt-23^+ cells, but in this case the precursors belong to the Lyt-123^+ pool (Cantor and Boyse, 1976; Burakoff et al., 1978).
3. The Lyt-123^+ cells in the peripheral T cell pool were initially thought to be the precursors of the Lyt-1^+ and Lyt-23^+ cells and they were therefore designated as early T cells (T_E). According to some authors the differentiation of Lyt-123 cells into Lyt-23^+ cells is antigen-independent (Cantor and Boyse, 1977), but other authors suggested that this process is driven by antigen (Burakoff et al., 1978). After ATx part

TABLE 4

LYT-PHENOTYPE AND T CELL FUNCTION

Lyt-phenotype 1	Helper precursor in antibody formation
	Helper effector in antibody formation
	Delayed type hypersensitivity to SRBC
	MLR-reactive precursor to LD antigens and H-2K mutant antigens
	Proliferating cells in MLR
Lyt-phenotype 23	Cytotoxic precursor in CML to allo-MHC
	Cytotoxic effector in CML to allo-MHC antigens and a number of modified syngeneic antigens
	Suppressor precursor
	Suppressor effector
Lyt-phenotype 123	Cytotoxic precursor in CML to modified syngeneic antigens
	Cytotoxic effector to tumor associated antigens
	Helper amplifier
	Suppressor amplifier
	Precursor of Lyt-23^+ cells

of the T_E population disappears from the peripheral lymphoid organs, and it was suggested that the T1 and T_E population were identical (Cantor and Boyse, 1977). In analogy with the synergism demonstrated between T1 and T2 cells for T helper function and T suppressor function, it was found that Lyt-123^+ cells could amplify T helper and T suppressor function (Beverley et al., 1977). Additionally, it was shown that the amplifying Lyt-123^+ cells were T1 cells, whereas the Lyt-1^+ and Lyt-23^+ cells were T2 cells (Feldmann et al., 1977). Again these data fit nicely with the hypothesis that T1 and T_E cells are identical.

However, it was found that not all T_E cells are eliminated

within longer periods after ATx. Even normal numbers of T_E cells can still be found in the thoracic duct of ATx mice at a time when the numbers of Lyt-1^+ and Lyt-23^+ cells in peripheral organs are already decreasing (Simpson and Beverley, 1977). Finally, it was shown that precursors of cytotoxic T cells directed to modified syngeneic antigens on target cells (i.e., minor H-antigens and tumour associated antigens) may be found in the Lyt-123^+ cell pool (Cantor and Boyse, 1976; Pang et al., 1976; Simpson and Beverley, 1977; Burakoff et al., 1978). Moreover, the cytotoxic effector cells, directed to syngeneic tumour cells may also be Lyt-123^+ cells (Shiku et al., 1976). These data indicate that the Lyt-123^+ cell population cannot be considered identical to T1 cells, but suggest that they may be represented in the T1, T2 and effector T cell population.

A more refined classification of peripheral T cells can be achieved, when, in addition to the Lyt-system, Ia- and Qa-antigen markers are also related to T cell function. Thus, the cytotoxic Lyt-23^+ cells can be distinguished from the Lyt-23^+ suppressor cells by the selective presence of the Ia antigens on the latter population (Murphy, 1978). Cytotoxic T cells probably do not carry Ia antigens. By means of the Qa-1 antigen the Lyt-1^+ helper T cells can be divided into a Qa-1 positive, and a Qa-1 negative population (Cantor et al., 1978). The Lyt-1^+, Qa-1+ T helper population has the ability both to enhance antibody formation by B cells and to stimulate suppressor T cells, inhibiting the T helper function. Thus a feed-back system in the humoral response has been demonstrated. T helper cells cooperating with B cells in the antibody response to SRBC, and DTH-reactive T cells may also be distinguished from each other, on the basis of the Ia system. Some T helper cells are probably Ia^+ cells, whereas the DTH reactive T cells are Ia^- cells (Huber et al., 1976b; Okumara et al., 1977). This finding may indicate a qualitative difference between some of the T helper cells and the DTH-reactive T cells. Further study on the surface markers of T cells mediating T helper activity and DTH-reactivity should be performed, in order to settle this problem. Such studies should not be restricted to the antigen SRBC.

5. *T CELL FUNCTIONS*

5.1. Helper functions in the humoral immune response

For optimal humoral responses to most conventional antigens the cooperation of helper T cells with antigen-reactive B cells is required. These T cells help in stimulating B cells to produce IgM, IgG and IgA antibodies (Taylor and Wortis, 1972; Benner et al., 1974; Van Muiswinkel et al., 1975). The mechanism of T-B cooperation is not fully understood, although it has become clear that both antigen-specific (Taussig, 1974; Munro and Taussig, 1975) and non-antigen-specific (Schimpl and Wecker, 1972) soluble T cell factors can account for B cell activation. The molecular weight of the antigen-specific factors is in the order of 50,000 Dalton, and these helper factors contain I-A region coded products (Taussig et al., 1975).

Helper T cell function can be assayed by in-vitro culturing of limited numbers of primed helper T cells together with a non-limiting number of non-primed B cells and the specific antigen. The number of antibody forming cells per culture is thought to reflect the helper activity of the added T cells. Using this assay Araneo et al. (1977) studied the kinetics of helper activity in mice immunized with the thymus-dependent antigen SRBC. They showed that helper activity in the spleen appeared by day 2 or 3 after primary i.v. immunization, peaked on day 4 and declined subsequently. The activated helper T cells were short-lived, but priming of mice with SRBC also induced long-lived recirculating memory cells which could promptly generate activated helper cells after booster with the specific antigen. Araneo et al. (1977) proposed that the activated helper cells should be called effector cells, in order to distinguish these short-lived cells from their precursors which are largely long-lived, recirculating T lymphocytes (T2 cells). It was found that "virgin" T precursor cells which are also short-lived, may contribute to the pool of effector cells after primary immunization (Araneo et al., 1976). The generation of effector T cells from the short-lived "virgin" T1 precursor cells appeared to be a more time-consuming process than their generation from the T2 precursor cells. The authors did not exclude the possibility, however, that the T1 cells may pass through a T2-like differentiation stage, during their differentiation into effector T cell (Fig. 3).

5.2. Resistance to intracellular bacteria and viruses

The resistance to facultative intracellular bacteria is mediated by T cells (Campbell, 1976). Immunization of mice with low doses of viable intracellularly growing bacteria can induce a state of immunity, which can protect these animals towards an otherwise lethal dose of these bacteria (Campbell, 1976). Vaccination with killed bacteria without the use of adjuvants generally does not evoke such a state of cell-mediated immunity (Bloch and Segal, 1955; Collins, 1971; 1973). During the immune response to the infecting microorganisms S-phase T lymphoblasts are delivered into the thoracic duct (Lefford et al., 1973a; McGregor et al., 1978; McGregor and Logie, 1973). With these cells a state of resistance can be transferred to normal syngeneic hosts. It has also been shown that the protective T cells are functionally short-lived, non-recirculating cells (North, 1973; Lefford et al., 1973a; b). However, they have the capacity to migrate aspecifically into inflammatory foci (McGregor and Logie, 1974). At these sites they may perform their protective function by the release of cell products, the so called "lymphokines", if they encounter the specific antigen (Mackaness, 1971; Simon and Sheagren, 1971; North and Spitalny, 1974; McGregor and Kostiala, 1976). Lymphokines cause the accumulation of mononuclear cells at the inflammatory site, and can aspecifically increase their bactericidal potency.

At longer intervals after vaccination, protection can be adoptively transferred by recirculating small lymphocytes (Lefford et al., 1973a). Challenge of the recipient with a high dose of bacteria most probably results in transformation of the injected small lymphocytes and the accelerated production of short-lived blast cells which mediate the protective function. This process is associated with proliferation (North, 1975). Thus, the immunizing injection with facultative intracellular bacteria leads to:

1. a short-lived state of active resistance to the bacteria, and
2. a long-lived state of memory, during which period a rapid recall of resistance can be elicited by the second injection of the same bacteria (Collins, 1973; North and Deissler, 1975).

Resistance to viruses can also be mediated by T cells. Cell-mediated immune responses to acute virus infection have especially been studied by infecting mice with ectromelia virus. It has been shown that spleens from these mice contained T lymphocytes capable to lyse ectromelia infected target cells specifically during in-vitro incubation for a couple of hours (Gardner et al., 1974). Such cytotoxic T lymphocytes are probably also responsible for the in-vivo elimination of the virus-infected cells (Blanden and Gardner, 1976). Spleen cells from mice which had been infect-

ed 2 weeks to 16 months before with ectromelia virus could be restimulated during in vitro culture for 2 days with virus infected syngeneic macrophages or spleen cells to produce "secondary" cytotoxic T cells (Gardner and Blanden, 1976). Primary cytotoxic responsiveness to virus infected target cells could not be induced in vitro, which implies a major quantitative or qualitative difference between unprimed T cells and "memory" T cells present in mice after recovery from an acute virus infection.

The above mentioned experiments indicate that functionally different T cell subpopulations mediate the resistance to intracellular bacteria and viruses. Apparently, elimination of intracellular bacteria is aspecifically performed by macrophages activated by sensitized antigen-specific T lymphocytes. Virus-infected cells, on the other hand, are destroyed by cytotoxic T cells themselves. Destruction of bacteria-containing cells by cytotoxic T cells would unfavourably result in the release and dissemination of the bacteria. Lysis of virus-infected cells during the eclipse phase of the virus will eliminate the virus. It should be borne in mind that elimination of viruses is not entirely mediated by cytotoxic T cells, since antibodies also may contribute to the elimination of freely occurring viruses and virus-infected cells (Doherty and Zinkernagel, 1974; Perrin et al., 1977).

In cell-mediated immunity to bacteria as well as to viruses antigen-specific T memory is induced by the primary infection. These T memory cells account for the capacity of accelerated and enhanced generation of effector cells upon restimulation with the infectious agent in both cases. It remains unclear whether the persistence of small amounts of immunogenic material is essential for the maintenance of T memory cells. Persisting viable organisms are probably not necessary for the propagation of T cell-mediated resistance to bacteria (Lefford and McGregor, 1974).

5.3. Reactivity to histocompatibility antigens

Allogeneic organ or cell grafts generally induce an immune response of the host against the graft which ultimately may result in its rejection. This type of immune reaction is termed host-versus-graft (HvG) reaction. The opposite, the graft-versus-host (GvH) reaction can occur when immunocompetent cells are introduced into an allogeneic recipient. A GvH disease will develop if the host is incapable of mounting a sufficient immune response to the transplanted immunocompetent alloantigen-reactive cells. In the latter situation the grafted immunocompetent cells mount an immune response which causes injuries to the tissues of the host and ultimately may cause its death. In-vitro techniques are commonly

used to assay the capacity of responder cells to proliferate (MLR) or to produce cytotoxic T cells (CTL) after stimulation with allogeneic cells. This section will deal with the cellular aspects of the in-vivo and in-vitro immune response to histocompatibility (H-)antigens, presented on allogeneic cells.

5.3.A. Host-versus-graft reaction

The host-versus-graft (HvG) reaction has been mostly studied by means of skin transplantation which is a convenient and very sensitive organ transplant assay for detection of histocompatibility (H-)antigens in the mouse. An acute first set skin graft rejection (i.e. within 3 weeks after transplantation) occurs when donor and recipient either differ in antigens coded for by the MHC or in multiple minor H-antigens. Chronic rejection, occurring beyond the 3rd week after transplantation, occurs when donor and recipient only differ in a limited number of minor H-antigens. Grafting across single regions of the MHC (K, I or D) in congenic recombinant mouse strains, results in equal survival times of the grafts, namely about 3 weeks, but grafts across both the K- and I-region are generally rejected much earlier (Klein, 1975). This might be a cumulative effect of K- and I-region coded antigens, as has been similarly demonstrated for graft rejection caused by multiple minor H-antigens. Minor H-antigen differences may also contribute to the rejection of MHC incompatible grafts, since the rejection time for skin grafts differing by MHC-coded antigens and multiple minor H-antigens is often shorter than in the case of differences in MHC-coded antigens only (Billingham et al., 1954; Graff and Bailey, 1973).

Presensitization of mice with an allograft results in accelerated rejection of the second set graft. This acceleration of graft rejection is more pronounced for minor H-differences than for major H-differences (Hildemann, 1970). The presensitization by means of an H-2 incompatible allograft is not fully specific for the donor strain, since the rejection time for third party grafts carrying a different H-2 haplotype than the donor strain of the first graft, was intermediate between that of a first set and second set graft rejection, even if minor H-differences were eliminated (Klein and Murphy, 1973). These findings suggest cross-reactivity between unrelated H-2 haplotypes, may be of public antigens.

Graft rejections across major as well as across minor H-antigen differences are mainly mediated by T lymphocytes. According to Cohen and Livnat (1976) T cell activation can occur in the skin graft itself, where a subset of circulating immunocompetent T

lymphocytes comes into contact with cells which carry the allogeneic H-antigens. These activated T cells subsequently migrate to the draining lymphatic tissue and recruit another subset of T lymphocytes which proliferates, and differentiates into specific effector cells mediating the graft rejection. The nature of the T cells involved in second set graft rejection has not been yet fully established. Possibly, long-lived effector T cells, induced by the first allograft, may account for the second set graft rejection, although the contribution of a true secondary immune response by restimulation of long-lived T memory cells has not been excluded (Brent et al., 1962). Recent experiments by Hall et al. (1977; 1978a; b) in the rat, indicate that non-recirculating long-lived T memory cells induced by a primary MHC-incompatible skin graft may account for the second set graft rejection of a cardiac allograft. Since no increased immunological activity was seen in the lymphatic tissues during the second set reaction, the authors suggested, that these memory cells are already existing "end" cells, mediating the effector function without the requirement of proliferation or differentiation within a lymphoid organ.

To test the thymic-dependence of precursor T cells and memory T cells involved in the first set, and second set graft rejections, respectively, Hall et al. (1978b) thymectomized rats either before or after grafting. They observed that neither the pool-size of precursors of effector cells mediating first set graft rejection, nor that of the precursors of the "memory" cells, nor that of the "memory" cells themselves was affected by ATx. Lance et al. (1973) showed that depletion of the recirculating T cell pool by means of ATS treatment in vivo markedly prolonged skin graft rejection times. Thus, the precursor T cell pool accounting for first set skin graft rejection consists of recirculating T cells, and these cells may therefore belong to the T2 cell pool. Wood and Monaco (1972) reported that thymectomy several weeks previously potentiated the effectiveness of ATS treatment in the prolongation of the survival time of allogeneic grafts. It follows that the thymus is required for the recovery of the recirculating alloreactive precursor T cell pool after depletion by ATS. On the other hand, the presence of the thymus is not essential for maintenance of the population of alloreactive memory T cells (Hall et al., 1978b). Elimination of T cell memory for major H-antigens by ATS treatment has been difficult to achieve (Lance, 1968; Russell and Monaco, 1967). This observation can be understood on the basis of the data provided by Hall et al. (1978a; b) in the rat, who showed that the memory cells are non-recirculating, and can thereby escape from the effect of ATS treatment. Compared to T cells from unprimed animals, the above described memory cells have a greatly increased potency of alloreactivity,

measured by adoptive transfer of lymphoid cells derived from primed donors. It is surprising that the second set allograft rejection across MHC differences is not as strikingly accelerated despite the presence of these memory cells (Hall et al., 1977; 1978b).

5.3.B. Graft-versus-host reactivity

A graft-versus-host (GvH) reaction occurs when immunocompetent cells are introduced into allogeneic recipients and it may accompany HvG reactions after organ transplant, when the transplant itself contains alloreactive cells. GvH reactions can be assayed in vivo by mortality, splenomegaly and popliteal lymph node assays, which are generally performed in irradiated, neonatal and normal adult hosts, respectively. Differences between the I-region of the MHC of donor and host induce stronger GvH reactions, than differences in the other regions of the MHC (Klein, 1975). Minor H differences alone can also be sufficient for the induction of GvH reactivity (Cantrell and Hildemann, 1972). Presensitization of donors will result in a more vigorous GvH reaction, only if minor H-antigens are involved (Lind and Szendberg, 1961; Ford and Simonsen, 1971).

As might be expected GvH reactions are mainly mediated by T lymphocytes (Sprent and Miller, 1971; Cantor and Asofsky, 1972). Surprisingly large numbers of the peripheral T cell pool (1-10%) are able to respond to a host of a given allogeneic H-2 haplotype, as compared to the very small fraction (0.01%) of T lymphocytes responding to conventional antigens or minor H-antigens (Simonsen, 1967; Ford et al., 1975).

Cantor and Asofsky (1970; 1972) showed synergism between peripheral blood lymphocytes and thymocytes, and between peripheral blood lymphocytes and anti-lymphocyte serum (ALS) treated spleen cells in the GvH reaction. In these experiments peripheral blood was used as a source of T2 cells. Thymocytes and ALS treated spleen cells were considered as a source of T1 cells. These latter cells would actually mediate the GvH reaction, while the T2 cells would merely amplify this effector function. It should be stressed, however, that these results were obtained with the splenomegaly assay; contrasting results might be obtained in other GvH assays.

5.3.C. In vitro assays for alloreactivity

Co-culture of lymphocytes derived from two allogeneic individuals may result in blast transformation and mitotic activity (Bain et al., 1964; Bach and Hirschhorn, 1964). This T cell dependent in-vitro phenomenon is called the mixed lymphocyte reaction (MLR), and is generally considered as the in-vitro analogon of both the

proliferation phase during a GvH reaction (Wilson, 1971), and the induction phase of a HvG reaction (Häyry et al., 1972). Generally an one-way MLR is used, i.e. the proliferation of the lymphoid cells of one individual is inhibited by antimitotic treatment, so that these cells can only act as stimulators. The lymphocytes of the other individual act as responders to the histocompatibility antigens of the stimulator cells. During a MLR, cytotoxic T lymphocytes (CTL) are generated which can specifically lyse lymphoblasts, derived from the original stimulator individual (Cerottini et al., 1970a, b). Peak numbers of CTL are found after the peak of proliferative activity (Cerottini and Brunner, 1974). This lytic reaction, called cell-mediated lympholysis (CML), might be the in-vitro counterpart of the effector phase of the in-vivo allograft reaction (Cerottini and Brunner, 1974). Both blast cells and small lymphocytes can mediate CML (Shortman et al., 1972). In-vivo CTL are generated after injection of allogeneic cells or after allogeneic skin transplantation. It was found that, at the time of skin graft rejection, the number of CTL is maximal (Cerottini and Brunner, 1974), but the presence of CTL is not always so well correlated with the rejection of the allograft (Wilson, 1974). Not only can CTL be found at the rejection site, but also large numbers of non-lymphoid alloreactive cells (Häyry and Roberts, 1977). Thus, it is not clear what contribution is made by the different subpopulations of T lymphocytes and non-lymphoid cells to the process of graft rejection. During acute GvH reactions CTL are also generated, but their role in the symptoms of GvH remains doubtful, since in-vivo and in-vitro generated CTL do not produce GvH reactivity in a splenomegaly assay (Sprent and Miller, 1971; Rouse et al., 1972; Rouse and Wagner, 1972). In contrast, the same CTL did accelerate graft rejection.

After the peak of the blast response in an one way MLR to H-2 incompatible cells, the number of small lymphocytes increases (Häyry and Andersson, 1974). This indicates that a proportion of the blast cells reverts to small lymphocytes. Selective recovery of the blast cells and subsequent culture of these blast cells without antigen on a syngeneic feeder layer, revealed that these small lymphocytes indeed develop from blast cells. These small "secondary" lymphocytes have at least two characteristics, dissimilar from non-primed T cells:

1. They proliferate in a secondary MLR with the original stimulator cells in an accelerated fashion.
2. They could be recovered up to 5 months after i.v. transfer into T cell deprived syngeneic recipients. When tested in vitro in a secondary MLR they give rise to a prompt CML to the original stimulator cells (Häyry and Andersson, 1974).

Thus, these "secondary" lymphocytes apparently have the functional properties of memory T cells. The generation of CTL from their memory precursor cells is not necessarily accompanied by proliferation (MacDonald et al., 1975). This might explain the observation that the memory cells themselves can sometimes perform an effector function (Häyry and Andersson, 1974).

Proliferation in a primary MLR occurs when responder and stimulator cells differ in the I-region of the MHC; K- or D-region differences can induce only weak proliferative activity (Schendel et al., 1973). On the other hand, strong CML can be induced to K- or D-region coded antigens of stimulator cells and only comparatively weak CML to I-region coded antigens (Billings et al., 1977). Similarly as in GvH a high proportion of T cells responds to the stimulator cells of a given haplotype in an one way MLR and CML to allogeneic H-2 incompatible cells (Jones, 1973; Bevan et al., 1976; Lindahl and Wilson, 1977; Teh et al., 1977). The CTL predominantly recognize the private determinants of the SD-antigens, but some public antigens may also induce a rather strong and specicific CML (Lindahl et al., 1975). This CML reactivity to public antigens may account for the considerable cross-reactivity of CTL sometimes found for third-party target cells in primary CML and for third-party stimulator cells in the secondary CML (Corley, 1977).

In addition to K- or D-region coded alloantigens, sometimes the presence of I-region coded alloantigens is required for an optimal CML to the latter SD antigens (Schendel et al., 1973). This observation led to the assumption that a separate T cell population, stimulated by I-region coded determinants, proliferates in MLR and cooperates with precursors of CTL reactive to K- or D-region coded antigens to produce an optimal CML. Shortly thereafter it was shown that the helper T cells and cytotoxic T cells carry different Lyt surface antigens (Cantor and Boyse, 1975a; b). The helper effect was only demonstrated under experimental conditions in which the numbers of precursors of CTL are very small. Recently, it has been shown that a similar T cell cooperation is required for the generation of CML in a MLR across a mutant H-2K locus difference. It appeared that Lyt-1^+ T helper cells were required for the induction of Lyt-23^+ killer cells directed against the target cells from the H-2K mutant strain. It is very likely that these helper cells were not induced by LD antigens, but by antigenic determinants on the H-2K locus coded molecule (Melief et al., 1979). The need for helper cells appeared to be more stringent in the case of CML to the H-2K locus mutant-derived cells than in a CML to K- and I-region coded alloantigens. Probably many fewer clones of CTL precursors are directed against antigens coded for

by a mutant H-2K locus than against H-2K and H-2I coded alloantigens. These experiments suggest that the requirement for Lyt-1^+ helper T cells depends on the number of antigen-reactive precursor CTL during the MLR.

Minor H-antigens can hardly induce an MLR, with the exception of products of the Mls locus on chromosome number 1. Mls locus incompatible, H-2 compatible stimulator cells can induce a MLR comparable in strength to MLR observed in H-2 incompatible combinations (Festenstein, 1973). In-vivo priming of mice with single or multiple minor H-antigens by means of intraperitoneal injection of allogeneic H-2 compatible spleen cells or by skin grafting leads to considerable MLR and CML responsiveness of the recipient's cells against these minor H-antigens. This also occurs in the absence of a stimulatory Mls locus product (Bevan, 1975; Gordon et al., 1975). Obviously, in-vitro MLR and CML to minor H-antigens, except those coded for by the Mls locus, require the presensitization of the mice used as donors for the responder cells. This might indicate that the population of unprimed T cells must undergo a quantitative or qualitative change in order to obtain the capacity of inducing MLR and CML to minor H-antigens. Recent frequency analyses of precursor T cells responsible for MLR and CML before and after in-vivo priming, suggest an increase in the frequency of the specific T cells as the most likely explanation (MacDonald, personal communication).

5.3.D. Conclusions

To summarize, both HvG and GvH reactivity are largely dependent on T lymphocytes. In-vitro assays provided data on: (1) the existence of functionally different T cell subpopulations involved in alloreactivity and (2) the relative importance of major and minor H-antigens in the activation of alloreactive T cells (Table 5). The contribution of alloaggressive CTL in the rejection of H-2 incompatible grafts is beyond doubt (Häyry and Roberts, 1977). In contrast, it is questionable whether CTL play a significant role in GvH (Dennert, 1976), since their GvH activity could not be demonstrated in the splenomegaly assay (Rouse et al., 1972). In other GvH assays CTL might prove to be more effective. Memory can be seen in alloreactivity against both major and minor H-antigens, but the cellular basis of memory to minor H-antigens is not completely clear as yet. Long-lived non-recirculating T lymphocytes induced by a primary H-2 incompatible allograft might function as effector cells in a second set graft rejection. These cells might be identical to the small "secondary" lymphocytes induced in a primary MLR to H-2 incompatible cells. Cooperation of different subsets of T cells has been shown in vitro, and in

the GvH assay. Cooperation of different T cell subsets may also be involved in the in-vivo host-versus-graft reaction (Röllinghof et al., 1977). In addition, it became apparent from the experiments of Cohen and Livnat (1976) that a particular subpopulation of T lymphocytes, the initiator T lymphocytes, can recruit another T cell subset to generate effector cells against a graft.

TABLE 5

ROLE OF HISTOCOMPATIBILITY ANTIGENS IN THE VARIOUS ASSAY SYSTEMS FOR CELLULAR IMMUNITY

Histocompatibility antigen	Function			
	Primary MLR	Primary CML	GvH	HvG
K,D	+	+++	±	++
I	+++	+	++	++
Mls	+++	-	?	?
Minor H, except Mls	-	-	+	+

5.4. Delayed type hypersensitivity

Delayed type hypersensitivity (DTH) is the only form of allergy which is classified as a cell-mediated immune phenomenon, since it can be mediated by antigen-reactive T lymphocytes without the essential involvement of B lymphocytes or their products. Skin reactions due to DTH are distinguished from antibody-mediated skin reactions of immediate type hypersensitivity by the delayed onset of the skin lesions of the former type. The DTH reaction is characterized by a slowly developing induration, erythema and oedema which becomes maximal at about 24-96 hr after its elicitation, depending on the species tested and the antigen used (Crowle, 1975). Furthermore, Chase (1945) showed that DTH could be transferred to non-immune guinea-pigs by injection of living peritoneal exudate cells from sensitized donors, but not by antiserum. A state of DTH will arise after appropriate sensitization, and its expression can be elicited either locally or systemically by local or systemic challenge with the specific antigen, respectively. Skin testing, footpad challenge and ear testing are all examples of local cutaneous elicitation of DTH, while shock, body temperature changes, or haemorrhagic changes in the lungs may occur after intravenous systemic challenge of sensitized animals (Crowle, 1975).

5.4.A. Classification

DTH was originally described as a manifestation of bacterial allergy (Zinsser, 1921; Zinsser and Müller, 1925) because of its common association with bacterial infection. Later on it became apparent that a great number of other antigens, e.g. foreign proteins (Jones and Mote, 1934), simple chemicals (Landsteiner and Chase, 1937), and transplantation antigens (Brent et al., 1958), also could induce DTH. The phenomenology of DTH varied considerably with the different antigens used. Thus, it was realized that not all DTH responses are similar and that the term DTH might stand for a heterogeneity of immunological processes. Classic tuberculin type DTH is produced by the injection of antigen emulsified in Freund's complete adjuvant (FCA) or in other mixtures containing products of Mycobacterium tuberculosis (Freund, 1956). Similar reactivity occurs after mycobacterial, fungal or viral infection. The classic tuberculin type state of DTH is long-lasting and stable. Once a state of tuberculin type DTH is established, desensitization is not easily achieved by second injection of antigen (Rothschild et al., 1934; Benacerraf and McCluskey, 1963). The transient state of DTH seen in man and guinea-pigs after sensitization with antigen-antibody complexes (Uhr et al., 1957) or small amounts of antigen, administered either emulsified in an adjuvant not containing mycobacteria or without adjuvant, was termed Jones-Mote type hypersensitivity (Raffel and Newel, 1958). Dose and route of immunization are decisive for the induction of such a type of DTH. A Jones-Mote type reactivity occurs early after immunization and is evanescent, waning with the appearance of antibody (Lagrange et al., 1974; Parish, 1972). It is sensitive to a second injection of antigen (Coe and Salvin, 1964). Contact sensitivity reactions which occur after painting the skin with simple chemicals like oxazolone or dinitrochlorobenzene, are often considered as Jones-Mote type hypersensitivity reactions although the state of DTH can be maintained during a long period after the sensitization (Crowle, 1961). Antibodies seem to play a role in contact sensitivity (Zembala and Asherson, 1970). In mice both a stable, long-lasting state of classic DTH and an evanescent Jones-Mote DTH can be induced, just as in man and guinea-pigs.

Histologically, skin reactions of delayed onset are characterized by a local infiltration of predominantly mononuclear cells. It was shown that monocytes are essential to enact a DTH reaction elicited in the skin (Lubaroff and Waksman, 1967) or footpad (Volkman and Collins, 1972). Polymorphonuclear cells are often seen in the reaction site also, especially in mice (Crowle, 1975). Jones-Mote type skin reactions in guinea pigs are generally associated with the local accumulation of basophils. As a result

Jones-Mote type allergy in guinea-pigs was termed cutaneous basophil hypersensitivity (Richerson et al., 1970). Tuberculin type DTH to haptens in guinea-pigs may also be associated with a basophil-rich infiltration of the skin test site (Askenase et al., 1976). It has been suggested that antibodies as well as T cell factors play a role in the accumulation of basophil-rich infiltrations in the reaction site (Askenase et al., 1976).

5.4.B. In-vitro assays for delayed type hypersensitivity

In-vitro assays have been developed to test the presence of cells mediating a state of DTH. Most in-vitro tests make use of the capacity of sensitized T lymphocytes to produce humoral substances, called lymphokines, which act on macrophages (Waksman, 1979) The release of lymphokines is an antigen-dependent process. Macrophage migration inhibition factor (MIF) is one of the many lymphokines. The migration inhibition assay is thought to be the in-vitro correlate of tuberculin type DTH only, since during Jones-Mote type hypersensitivity no MIF activity can be demonstrated when sensitized lymphocytes and macrophages are incubated together with the specific antigen (Ohmichi et al., 1976; Himeno et al., 1977). This finding can be taken as an argument to distinguish tuberculin type and Jones-Mote type DTH at the cellular level. On the other hand, the negative MIF tests in Jones-Mote type hypersensitivity can also be explained by the possible interference of antigen-antibody complexes in this assay. Besides MIF production is only one aspect of the in-vivo DTH reaction. In-vitro proliferation and blastogenesis upon antigenic stimulation might reflect the early in-vivo events in the draining lymphatic tissue during the induction of DTH (Davies et al., 1969). The in-vitro blastogenesis seems to be a good correlate for Jones-Mote type DTH.

5.4.C. The cellular basis of delayed type hypersensitivity

Several authors have attempted to characterize the cellular mediator of DTH. Originally this mediator was suggested to be a functionally short-lived lymphocyte (Neveu et al., 1963), as well as a long-lived T lymphocyte, i.e. a typical recirculating memory cell (Chase, 1963). It was later proposed that in tuberculin type DTH both long-lived and short-lived T lymphocytes might mediate DTH reactivity (Lefford and McGregor, 1974).

In studies on contact sensitivity it has been shown that removal of the draining lymph nodes during the proliferation stage inhibits the subsequent development of DTH (Turk and Stone, 1963; Neveu et al., 1963). Once a state of DTH has been established, extirpation of the draining lymph nodes did not affect the DTH reactivity of the animal (Turk and Stone, 1963). It was concluded

that the immunologically stimulated cells leave the regional lymph nodes in the early phase of the sensitization period (Turk, 1975). Asherson and Zembala (1973) showed that after immunization of mice with oxazolone cells of various lymphoid organs successively became capable of transfer of the contact sensitivity to non-immune recipients. These results are compatible with the notion that after the induction stage seeding of long-lived reactive cells to other lymphoid organs occurs (Asherson et al., 1974). An argument for the existence of long-lived DTH-reactive cells is the finding that tuberculin type hypersensitivity persists for a long time after infection of rats with viable BCG. Even elimination of viable mycobacteria did not affect the level of DTH (Lefford and McGregor, 1974). However, it can be argued that immunogenic bacterial remnants persist in such animals. Other evidence for the case of long-lived DTH-related effector cells was the observation that recipients of immune spleen or peritoneal exudate cells remained hypersensitive for periods of more than one month (Chase, 1963; Lagrange and Mackaness, 1975). Treatment of mice, which were donors for sensitized cells, with the antimitotic agent vinblastine did not impair their capacity to transfer DTH (Lagrange and Mackaness, 1975; North and Deissler, 1975). Thus, it was concluded that persistent DTH to purified protein derivative (PPD), L. Monocytogenes, and SRBC is mediated by long-lived cells which do not or hardly proliferate. This vinblastine resistance of DTH-related T cells was only observed at rather long intervals after immunization.

The recent observation that thoracic duct lymphocytes from rats immunized with living BCG were not able to transfer DTH to normal rats at longer intervals after immunization (Kostiala et al., 1978), provides contradictory evidence to the view that the T cell mediating tuberculin type DTH is a long-lived recirculating cell. This opinion was also opposed by earlier experiments which showed that chronic thoracic duct drainage did not impair the level of DTH, as might be expected if sensitivity would be mediated exclusively by recirculating lymphocytes (Coe et al., 1966). Moreover, it has never been shown that small lymphocytes have the capacity to migrate into inflammatory foci, while this is a characteristic of lymphoblasts (Russell et al., 1975).

Another phenomenon which has been considered to support the possible existence of long-lived DTH-reactive effector cells is the capacity of anamnestic DTH in animals primed many months previously. Booster of such animals with the specific antigen resulted in a sudden accelerated increase of DTH reactivity (Crowle, 1975; Tamura and Egashira, 1976). It was believed that the anamnestic response depends on the same long-lived memory

cells which account for the persistent state of DTH (Crowle, 1975).

At present, convincing evidence exists that short-lived T cells can perform an effector function in both Jones-Mote type DTH and the initial stage of tuberculin type DTH (Kettman and Mathews, 1975; Kettman and Turner Lubet, 1976; Askenase et al., 1977; Van der Kwast et al., 1977). Consequently, it was proposed that after immunization for tuberculin type DTH a short-lived blast cell would mediate initial DTH reactivity, while after longer periods a long-lived effector cell would account for the DTH responsiveness (North and Deissler, 1975; Lefford and McGregor, 1978). Persisting antigen or a high antigen dose would be required to drive a particular T cell subclass to become such a long-lived cell (Askenase et al., 1977).

An intriguing problem is the physiological role of the DTH-reactive T lymphocytes. DTH reactions are known to play a role in a number of pathological processes, e.g. granuloma formation, some autoimmune diseases, neurological involvement in leprosy and in contact eczema (Turk, 1975). These DTH-related phenomena can be considered as derangements of the normal immune response to pathogenic agents. Cell-mediated immunity and delayed type hypersensitivity have often been used as synonyms, as was suggested by the development of DTH after allografting, and during resistance to bacterial infection or viruses. Only T helper function for humoral responses did not coincide with an active state of DTH. In fact, an inverse relationship was generally found between T helper activity and DTH reactivity (Lagrange et al., 1974; Parish, 1972). A recent study of Lagrange and Mackaness (1978) indicated that after i.v. priming with high doses of SRBC suspended in saline, DTH-reactive effector cells can indeed be found in the spleen together with T helper cells. It was suggested that antigen-antibody complexes retain these DTH-reactive cells in germinal centres, where they might perform a helper function.

In view of these data it seems worthwhile to consider the hypothesis that somewhere in the differentiation pathway of precursor T cells to effector T cells, either directed against bacteria, mediating cytotoxic functions for virus-infected cells and allogeneic cells, or mediating helper function in the humoral response (Kerckhaert, 1974), a stage exists at which the cells can exhibit DTH reactivity to such an antigen. The discrepancies observed between the occurrence of DTH reactivity and several in-vivo T cell functions can be explained by central recruitment of antigen-reactive cells which would not allow the expression of peripheral DTH reactivity. Also, the appearance of suppressor (T) cells acting upon DTH-reactive T cells (Ramshaw et al., 1976;

Asherson and Zembala, 1975) might obscure the correlation between DTH-reactivity and other T cell functions. Since the cellular basis of DTH reactivity has not been elucidated as yet, the hypothesis that the DTH-reactive cell is a common differentiation step for all "functional" T cell subsets can neither be rejected nor accepted.

6. ANTIGEN RECOGNITION

6.1. T cell activation

About 2 decades ago Mitchison (1954) and Lawrence (1959) suggested that successful cell-mediated immunity to bacterial antigens could only be expected when these antigens were presented on the surface of the individual's cells. The association of self-antigens with the foreign bacterial antigen was thought to lead to cell-mediated immunity to these bacteria. In 1973 Rosenthal and Shevach reported that T cells, derived from guinea pigs immunized with FCA could be restimulated to proliferate in vitro by adding PPD, presented together with syngeneic or semiallogeneic macrophages, but not when the antigen was given together with allogeneic macrophages. Later studies confirmed these results (Paul et al., 1977). Blocking of this T cell proliferation induced by antigen-pulsed macrophages was achieved by addition of alloantisera directed to the Ia antigens specific for the macrophages of the strain which could effectively present the antigen to the immune T cells (Rosenthal and Shevach, 1973; Thomas et al., 1977). However, antisera raised against the native antigen, PPD, could not block the stimulatory capacity of these antigen-pulsed macrophages (Ellner et al., 1977; Thomas et al., 1977). Altogether these observations suggested that the sensitized T cells are triggered to proliferate and to produce lymphokines (Ellner et al., 1977) by recognition of the antigen in association with cell surface antigens coded for by the major histocompatibility complex (MHC), but not by the antigen alone.

Since in-vivo primed guinea pig F1 (P1 x P2) T cells can be restimulated by antigen-pulsed macrophages from either parental strain (here indicated as P1 and P2), these F1 T cells might consist of 2 different antigen-reactive populations: one set primed to respond to the antigen associated with specific surface determinants of P1 macrophages and the other to the antigen associated with specific surface determinants of P2 macrophages. Evidence for this hypothesis was provided by selective killing of proliferating T cells restimulated by antigen-pulsed macrophages from one parental strain. It was shown that the remaining T cells were only capable of responding to the antigen, when associated with macrophages from the other parental strain (Paul et al., 1977). The experimental data mentioned so far were obtained in a model which measured restimulation of previously primed T cells by soluble antigens associated with syngeneic or semiallogeneic macrophages. A number of other reports showed similar results in man and mouse (Bergholtz and Thorsby, 1977; Kappler and Marrack, 1976; Pierce et al., 1976). The latter authors demon-

strated that in antigen-primed F1 (P1 x P2) mice 2 sets of immunocompetent T cells can be distinguished on the basis of the specificity of their antigen receptor: F1-derived T cells recognize an antigen in association with a MHC-coded antigen of the one or the other parental strain. Antigen receptors of sensitized T cells apparently have a dual specificity, namely, for the antigen and for a self-MHC-coded cell surface antigen. This phenomenon of obligatory recognition of antigen in association with MHC-coded antigens has been termed MHC-restriction, or, in the mouse, H-2 restriction.

To test whether this phenomenon of H-2 restriction is predetermined, prior to the encounter of T cells with antigen, Bevan (1977a) and Zinkernagel et al., (1978a) reconstituted irradiated parental strain (P1) recipients with haemopoietic cells from F1 (P1 x P2) mice, and allowed these mice a few months to repopulate the lymphoreticular system. Finally, these chimaeric mice were immunized with minor H-antigens (Bevan, 1977), or vaccinia virus (Zinkernagel, et al., 1978a). In both cases it appeared that the CTL were preferentially activated by the antigen, presented in association with P1-derived MHC-coded antigens, and not when presented in association with P2-derived MHC-coded antigens. Zinkernagel et al. (1978a) showed conclusively that the genotype of the thymic environment imposed this MHC-restriction on the developing F1 (P1 x P2) T cells, prior to their encounter with antigens. It was similarly demonstrated that F1 (P1 x P2) T cells, helping antibody production (Waldmann et al., 1979; Bevan, 1978) or mediating DTH reactivity (Miller et al., 1978) recognize antigens, only when associated with macrophages, which have the same MHC-coded surface antigens as they encountered in the thymic environment in which these T cells were generated. The presence of MHC-coded antigens on the epithelial cells of the thymus (Hoffman-Fezer et al., 1978; Rouse et al., 1979; Wiman et al., 1978) ensures that maturing T cells can indeed learn self-preference in the thymus.

In general, T cells recognize antigens only in association with self-MHC products on the cell surface of antigen-presenting cells. However, arguments against this type of obligatory self-recogniton were obtained from other studies, showing that unprimed peripheral T cells could be sensitized by antigens presented on completely allogeneic macrophages. In most of these experiments alloreactive T lymphocytes were eliminated, in order to avoid a cytotoxic response to these allogeneic macrophages. It appeared that the remaining, unprimed T cells could be activated with antigens, presented on fully MHC incompatible cells (Thomas and Shevach, 1977; Heber-Katz and Wilson, 1976; Wilson

et al., 1977). In other experiments, using fully MHC-incompatible chimaeras, produced by reconstitution of irradiated strain A mice with haemopoietic cells from strain B, Matzinger and Mirkwood (1978) recently showed that cytotoxicity to minor H-antigens could be induced by minor H-antigens presented on cells with the H-2 haplotype of the reconstituting strain B. This result is contradictory to what would be expected from Zinkernagel's hypothesis that T cells only recognize antigens in assocation with the H-2 antigens encountered in the thymus in which they are educated. However, other authors failed to observe stimulation of unprimed T cells by allogeneic antigen-bearing macrophages (Janeway et al., 1978; Bennink and Doherty, 1978; Shearer and Schmitt-Verhulst, 1977). More investigations are required to settle this matter which has important theoretical implications (see section 6.3.).

6.2. T cell recognition in the effector phase

Obviously, antigen recognition is necessary for all classes of sensitized T cells to exert their effector function (Fig. 3). Therefore, a similar MHC restricted recognition of antigens by effector T cells can be expected during the expression of their function as that observed during the activation of their precursors. Not all H-2 coded antigens play an identical role in H-2 restriction. The antigens coded for by the different subregions of the H-2 complex are selectively involved in antigen recognition by the various functional T cell subpopulations (Table 6). As a rule, the same H-2 antigens which restrict the effector function of a particular T cell subpopulation are also responsible for restricting the induction of these T cells.

It has been shown that T helper cells cooperate with B cells for the production of antibodies to thymus-dependent antigens only when they have identical I-A regions of the MHC (Kindred and Shreffler, 1972; Katz and Benacerraf, 1975). Transfer of resistance to Listeria monocytogenes by immune T cells appeared to be similarly restricted by MHC-coded antigenic determinants (Zinkernagel et al., 1977). The host had to be I-A region identical to the effector T cells in order to be protected efficiently by those cells. However, resistance of recipient mice to various virus infections was not restricted by the I-A region, but was only achieved when immune donor T cells had an identical K- or D-region of the MHC as that of the host (Doherty et al., 1976). One year earlier it had been shown that in-vitro recognition and subsequent lysis of virus infected target cells by virus specific cytotoxic T cells could only occur when the K- or D-region of the

TABLE 6

REGIONS OF THE H-2 COMPLEX INVOLVED IN ANTIGEN-RECOGNITION BY THE VARIOUS FUNCTIONAL T CELL SUBPOPULATIONS

Restricting H-2 region	Type of antigen	T cell subpopulation
I-A	conventional	T helper in antibody formation
	conventional	Resistance to Listeria
	conventional	DTH to proteins
K,D	modified syngeneic	CTL to virus-infected cells
	modified syngeneic	CTL to minor H-antigens
	modified syngeneic	CTL to low-density TNP-altered cells
	modified syngeneic	DTH to virus
no	modified syngeneic?	CTL to high-density TNP-altered cells
no	MHC-coded	CTL to K-, I- and D-region coded alloantigens
no	?	CTL to T-region coded alloantigens
K,I-A,D	?	DTH to contact sensitizers

responding T cells was the same as that of the infected target cells (Zinkernagel and Doherty, 1975). Uninfected MHC-compatible target cells were not lysed, and inhibition of lysis of ^{51}Cr-labelled infected target cells was only observed in the case of addition of unlabelled virus infected cells with the same K- or D-region as that of the labelled target cells. T cell mediated cytotoxicity to multiple minor H-antigens is similarly restricted by K- or D-region coded antigens (Bevan, 1975).

Transfer of DTH reactivity also requires matching of certain parts of the MHC between the donors of sensitized T cells and the re-

cipients. However, the region(s) involved in MHC restriction, are determined by the antigen (Table 6). Some antigens require matching of the I-A region only, others of the K- or D-region, and a third group of the K-, I- or D-region. Thus, using proteins as antigens, the MHC region involved in restriction is I-A, whereas in the case of DTH to virus infected cells, an identical K- or D-region in donor and recipient is required for successful transfer of DTH reactivity. In contact sensitivity, the mutual identity of donor and recipient's K-, I-A or D-region is sufficient for successful transfer (Vadas et al., 1977; Miller et al., 1977).

As stated above, helper T cells cooperating with B cells, require identical I-A regions. It is possible that this I-A restriction also holds for helper T cells cooperating with cytotoxic T cells (Zinkernagel et al., 1978b; Von Boehmer et al., 1977). A number of helper factors, produced by helper T cells, or macrophages, often contain Ia antigens coded for by the I-A subregion (Erb and Feldmann, 1975). T suppressor cells can communicate with other cells by the production of antigen-specific or aspecific factors, which contain Ia antigens, coded for by the I-J subregion (Tada, et al., 1976) or I-C subregion (Rich et al., 1979). The observation that a number of these helper and suppressor factors are restricted by the MHC in their interaction with target cells, indicates that at least a proportion of the regulatory T cells are MHC restricted in their action.

In contrast to the T cell responses to most antigens, cell-mediated immunity to cell surface antigens of allogeneic cells coded for by either the K-, I- or D-region is not restricted by MHC-coded antigens (Klein, 1978). Apparently, the immune response to antigens which themselves are involved in MHC restriction is not subject to restriction. Cytotoxic T cell responses to T-region coded antigens on allogeneic cells are also unrestricted by MHC-coded antigens (Klein, 1978). With regard to cytotoxicity to TNP-modified cells, H-2 restriction could not be demonstrated provided that a high density of TNP molecules is present on the cell surface (Pohlit et al., 1979).

6.3. The antigen receptor of T cells

6.3.A. Immune regulatory genes

The important role of MHC-coded surface determinants in antigen-recognition by T cells makes it obvious that the immunogenicity of an antigen is not only determined by the characteristics of the antigen itself, but also by its capacity to associate with MHC-coded molecules. It is known that some genes in the MHC can

indeed determine the level of immune responsiveness to certain antigens. These Ir genes can be found in the I-region (Katz and Benacerraf, 1975) as well as in the K- or D-regions of the MHC (Zinkernagel et al., 1978c; Von Boehmer et al., 1978). Recently, it was clearly demonstrated by Von Boehmer et al. (1978) that these Ir genes do not code for the antigen receptor of the T cell. They demonstrated that, irrespective of the genotype of the T cells, the genotype of the thymus in which these T cells differentiated and matured, determines the responsiveness of the T cells to a particular antigen. Thus, since it was possible to change low-responder T cells into phenotypically high-responder T cells, the Ir genes of the T cell do not themselves code for the antigen receptor. It is very likely that Ir gene products and the antigens responsible for H-2 restriction are the same. The effect of Ir gene coded antigens on the immune responsiveness can be explained in two (not mutually exclusive) ways:

1. The Ir gene coded antigens might be unable to associate with the antigen and would thus fail to provide an immunogenic complex for induction of T effector cells and for the expression of T cell function, or
2. The Ir gene coded antigens might influence the T cell receptor repertoire during the T cell differentiation in the thymus.

The latter possibility can be understood if it is assumed that the combination of an antigen X plus a particular MHC-coded antigen is identical to a normal mouse membrane component. No response to antigen X will then occur because of self-tolerance (Schwartz, 1978). It has also been suggested that MHC-coded antigens present in the thymus, interact with T cell receptors that recognize these self-antigens. Mutation of the genes coding for the receptors recognizing self-antigens results in the formation of a mutant receptor, recognizing a foreign antigen. Because some antigens cannot be recognized by any of the occurring receptor mutants, the set of receptors for foreign antigens does not encompass all foreign antigens. The sets of antigens "recognized" by the receptors of T cells, i.e. the antigen repertoire, will be determined by the MHC of the thymus, if it is assumed that mutants of receptors for self-MHC-coded antigens of mouse strain A are different from those for self-MHC-coded antigens of strain B (Jerne, 1971; Von Boehmer et al., 1978). In this way an explanation is provided for the fact that strain A is a responder to antigen X, while strain B is a non-responder, since the latter is not able to generate mutant receptors for this same antigen (Jerne, 1971; Von Boehmer et al., 1978).

It has been demonstrated that any one particular cytotoxic T cell has receptors with specificity for either K-region coded products or for D-region coded products, but not for both (Zinkernagel and

Doherty, 1975). This genetic exclusion of the presence of receptor with specificity for K- or D-region coded products stands in contrast to the presence of both K- and D-region coded antigenic determinants on the same cell. The consequence of the resulting two distinct subsets of T cells, each showing receptor specificity exclusively for K- or D-region coded antigens, is that the individua animal is not dependent on a single "Ir gene" for a particular pathogenic organism but on two different "Ir genes". The set of T cells with the most suitable receptor for the antigen will be stimulated, at the expense of the set of T cells which have a low responder trait for this antigen (Zinkernagel et al., 1978c).

The occurrence of many alleles of the genes present in the MHC of any one species can also be explained by the proposed influence of MHC-coded antigens on the T cell receptor repertoire. On the basis of this hypothesis, it follows, that the polymorphism of the MHC is advantageous for the maintenance of the species. The existence of many alleles of a certain "Ir gene" minimizes the overall incidence of low responsiveness to life-threatening pathogenic organisms (Zinkernagel et al., 1977).

6.3.B. Models of the T cell receptor

A number of alternative models have been proposed to explain MHC-restricted recognition of antigens by T cells (Dutton et al., 1978): the altered-self model and the dual recognition model (Fig. 4). In the altered-self hypothesis it is assumed that an antigen X associates with the MHC-coded antigen to form a hybrid molecule or a neoantigen. This complex molecule is then recognized by a single receptor for this altered-self MHC-coded antigen. The dual recognition hypothesis states that a T cell has two different receptors, one for antigen X, and another for a particular self-MHC-coded antigen. A variant of the dual recognition hypothesis, the separate site hypothesis, assumes that a single receptor recognizes the antigen X and the self-MHC-coded antigen (Fig. 4). Cross-linking of two of these identical receptors would result in the activation of the T cells.

In the thymus the T cells learn to recognize self-MHC-coded antigens and there they develop their receptor repertoire. These T cell receptors are generated endogeneously; they are not passively adhered to the cell surface (Von Boehmer et al., 1979). According to the altered-self model T cells which have a receptor for antigens only slightly different from self-MHC-coded antigens will be positively selected in the thymus. According to the dual recognition theory, in the differentiation of T cells one receptor is positively selected for its (low) affinity for self-MHC-coded

antigens in the thymus, and the other receptor, of independent origin, is negatively selected on the basis of non-reactivity with self-antigens.

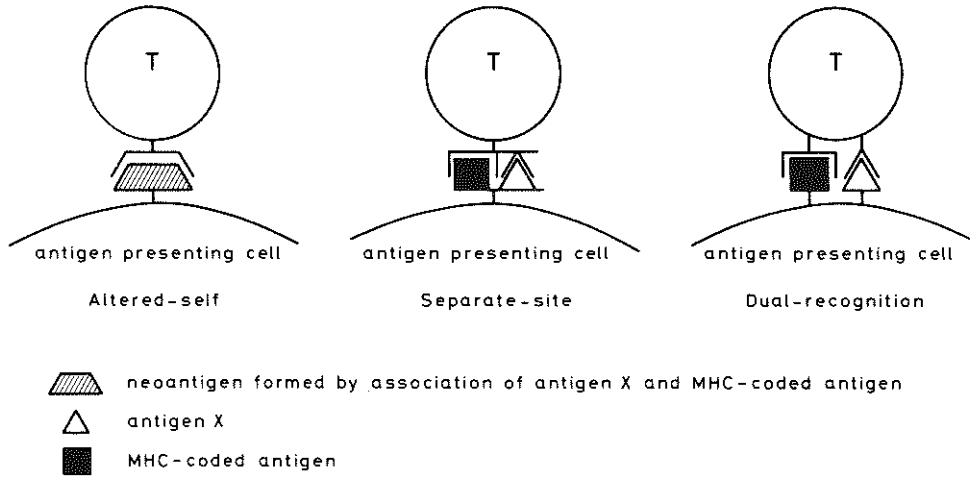

Fig. 4
Models of the antigen receptor of T cells.

According to the altered-self hypothesis, allo-MHC-coded antigens must be considered as identical to altered-self antigens. Since all T cells are reactive to altered-self antigen, and might cross-react with allo-MHC-coded antigens, the high number of alloreactive T cells is not suprising if the altered-self hypothesis is accepted (Matzinger and Bevan, 1977). Support for this hypothesis can also be found in observations that H-2 restricted killer cells specific for antigen X can lyse allogeneic target cells expressing neither the antigen X, nor the restricting self-MHC-coded antigen (Bevan, 1977a; Finberg et al., 1978; Von Boehmer et al., 1979). Reports showing self-preference of immunocompetent peripheral T cells rather than absolute self-restriction in antigen recognition, also argue for the altered self-hypothesis. This is because, in principle, no difference exists between modified self-MHC-coded antigens and modified nonself-MHC coded antigens since both are formed by the interaction of a MHC-coded molecule and a foreign antigen. The altered-self hypothesis predicts the existence of cells in a normal T cell population that are specifically reactive in a H-2 restricted manner to modified H-2 incompatible allogeneic cells. Thus, T cell receptors may exist which recognize

both antigen X in association with a particular allo-MHC-coded antigen, and antigen Z in association with a self-MHC-coded antigen (Doherty and Bennink, 1979). The failure to find this "cross-reactivity" in some studies (Shearer and Schmitt-Verhulst, 1977) would appear to reflect the existence of only a limited number of T cell receptors with such a cross-reactivity.

The dual recognition theory does not explain the high frequency of T cells which are specific for allo-MHC-coded products, since no positive selective force exists in the thymus for T cells with this type of receptor. On the other hand, it has been suggested recently that the number of alloreactive T cells, directed only to MHC-coded antigens, is not as large as has generally been assumed. A preliminary observation reported by Marbrook et al. (1978), showed that the number of precursor CTL for H-2 alloantigens was much less if assayed in the absence of accompanying minor H-alloantigens. The dual recognition theory can explain this finding by assuming that the anti H-2 antigen receptors have a wide range of affinity for these H-2 alloantigens. Cells with a low affinity receptor for an H-2 alloantigen require binding of the anti-X receptor with a minor H-antigen for optimal stimulation.

At present, the altered-self hypothesis is the most accepted one, mainly because of it's attractive simplicity and the demonstration that T cells which are specifically reactive to allo-MHC-coded antigens can indeed recognize foreign antigens in an H-2 restricted manner. The self-preference, rather than absolute restriction, in antigen recognition, demonstrated in several studies (e.g. most recently of Blanden and Andrew, 1979), also points to a T cell receptor commensurate with the altered-self hypothesis.

6.3.C. The variable part of the T cell receptor

The antigen receptor repertoire of T cells and B cells is partly the same. Despite the demonstration of a number of differences in antigen recognition between T and B cells, a common gene pool, coding for parts of the T cell as well as the B cell receptor, cannot be ruled out (Lindahl and Rajewski, 1979). The antigen receptor of B cells consists of a surface immunoglobulin molecule composed of heavy (H-)chains and light (L-)chains. The immunoglobulin molecules partly have a constant amino acid sequence; the part determining the antigen specificity of the receptor has a variable sequence. Variable regions can be found on the H-chains (V_H) as well as on the L-chains (V_L). The antigen specificity of the V_L and V_H chains are thought to result from

a random process of somatic mutation of genes coding for the V_L and V_H chains of the immunoglobulin molecules (V-genes) (Jerne, 1971). The antigen-binding sites of such an immunoglobulin receptor lie within the V-region (Jerne, 1971). Antibodies specific for the antigen-recognition sites in the V-region of the immunoglobulin receptor (anti-idiotypic antibodies) can effectively inhibit antigenic stimulation of antigen-specific B cells. With these anti-idiotypic antibodies it has been demonstrated that T cells and B cells can utilize similar V-gene products when responding to an antigen (Binz and Wigzell, 1975; Cosenza et al., 1977). In addition, it has been suggested that different functional T cell subsets selectively utilize either V_H or V_L gene-coded structures for antigen recognition in contrast to B cells which use both (Lonai et al., 1978). It is uncertain whether genes in the H-2 complex can influence the T cell receptor idiotypes. Thus, while Krammer and Eichman (1977) presented data which clearly showed that the T cell receptor idiotypes, recognizing alloantigens, are controlled by H-2 genes, Bellgrau and Wilson (1978) were unable to demonstrate MHC-polymorphism of T cell receptors specific for alloantigens.

The cellular basis of delayed type hypersensitivity (DTH) directed either to the particular thymus-dependent antigen, SRBC, or to non H-2 coded tissue antigens is described in the papers I, II, III and IV, added to this thesis. Low doses of SRBC, suspended in saline, were used as the immunizing antigen. Consequently, DTH-related T cell functions could be studied under experimental conditions in which the possible influence of persisting antigens was limited. The results obtained with the antigen SRBC, were compared with results from experiments using longer persisting antigens, viz. minor H-antigens, presented on viable H-2 compatible cells (paper IV). The genetic requirements for induction and expression of DTH to minor H-antigens were investigated in two different models, the graft-versus-host (GvH) assay and the host-versus-graft (HvG) assay (paper V and VI).

The first paper (appendix paper I) concerns a study of anamnestic or secondary DTH to SRBC. In this paper the kinetics of secondary DTH to SRBC after optimal i.v. priming and booster was compared with the kinetics of the primary DTH after optimal i.v. immunization. The T cell dependence of the phenomenon of secondary DTH was also investigated.
Appendix paper II further characterizes the T cells accounting for secondary DTH. By means of parabiosis experiments, it was tested whether DTH-related T memory cells are long-lived, potentially circulating cells, which can persist in the absence of antigen. Furthermore, it was studied whether the DTH-related T memory cells accounting for secondary DTH were sensitive to subcutaneous (s.c.) injection with ATS, which implies that they are recirculating cells (Levey and Medawar, 1967). The role of proliferation in the secondary DTH response was investigated by treatment of primed mice with antimitotic agents before or after the booster injection.
Appendix paper III deals with the contribution of short-lived (T1) and long-lived (T2) precursor cells to primary DTH and DTH-related T memory. The methods used in this study were thymectomy of adult mice (ATx) and ATS pretreatment. The results of this study were compared with those of Araneo et al. (1977) on the T helper cells.
Appendix paper IV described the kinetics of primary and secondary DTH to multiple minor H-antigens, or a single minor H-antigen. These minor H-antigens were presented on viable cells, H-2 compatible with the recipient. It was also tested whether secondary DTH-responsiveness can be evoked to multiple minor H-antigen incompatible spleen cells which simultaneously present incompatible H-2 alloantigens. On the basis of the results

reported in this paper it was speculated that persistent DTH as observed in studies on tuberculin type DTH is dependent on the continuous antigen-driven differentiation of long-lived recirculating T memory cells into functionally short-lived DTH effector cells.

In appendix paper V the role of the Mls locus coded H-antigens was studied in DTH assays, based on the HvG model as described in appendix paper IV and the GvH model, as described previously by Wolters et al. (1978). The latter authors described a DTH assay which is fit to measure the development of H-antigen induced DTH effector T cells during a GvH reaction.

In appendix paper VI the influence of H-2 compatibility of injected allogeneic spleen cells on (1) induction of DTH-effector T cells and memory T cells, (2) the booster phase and (3) the elicitation of the DTH reactivity to minor H-antigens was examined with congenic mouse strains. The capacity of T cells, sensitized to multiple minor H-antigens, for mounting a local GvH reaction in different H-2 congenic mouse strains was investigated with the immune lymphocyte transfer assay.

SUMMARY

Peripheral T cells with a particular immunological function (e.g. helper function, suppressor function, resistance to bacteria) can be subdivided according to their differentiation stage (Fig. 3). Short-lived "virgin" T1 cells are cells, which have recently originated in the thymus and they can differentiate into the long-lived T2 cells. These, unprimed, T2 cells are mainly involved in the primary T cell immune response, since after immunization they can rapidly differentiate into the short-lived effector T cells. Priming of mice not only results in a primary immune response, but also in the formation of T memory cells which, upon restimulation with the specific antigen, account for secondary immune responsiveness. These T memory cells are long-lived, potentially recirculating cells, and they do not perform an effector function, unless restimulated. It is not known, whether the unprimed T2 cells are qualitatively different from the memory T cells accounting for secondary immune responsiveness. Memory might also be a quantitative phenomenon, resulting from the increase in the number of antigen-specific T2 cells.

From the data provided by literature it appears that a few exceptions to this general plan of peripheral T cell differentiation may exist:

1. T cells whose function it is to amplify the immunological function of T2 cells have the properties of T1 cells. These T1 cells probably do not differentiate into T2 cells before they have carried out amplifying function.
2. T "memory" cells accounting for second set graft rejection across major H-differences are possibly non-recirculating effector cells with a long lifespan.
3. T cells accounting for persistent tuberculin type DTH were thought to be long-lived T (memory) cells with an effector function.

Our results suggest that the T cells involved in the transient state of DTH to SRBC or in the persistent state of DTH to minor H-antigens can be classified according to the above mentioned scheme of peripheral T cell differentiation, without evoking additional T cell populations with exceptional properties.

H-2 complex coded antigens play a different role under GvH conditions than under HvG conditions. During HvG reactions the K- and D-region coded products constitute the major target antigens of the cytotoxic reaction, whereas in GvH the I-region coded antigens are most important. These I-region coded antigens can strongly stimulate T lymphocyte proliferation in primary MLR. Similarly, Mls locus products can strongly stimulate a primary MLR. In this

respect, the Mls locus products stand in contrast to the other minor H-antigens which are not able to induce a primary MLR. In parallel with the comparatively strong GvH inducing capacity of I-region coded alloantigens, we found a similar capacity of stimulatory Mls-locus antigens to produce DTH effector cells under GvH conditions. Under these conditions, the other minor H-antigens only slightly contributed to the DTH response.

Peripheral T cells generally recognize antigens in association with self-MHC-coded antigens. This also applies to DTH-reactive T cells. Data from the literature suggested that the region(s) of the H-2 complex involved in recognition of antigens by DTH-reactive T cells is (are) determined by the type of immunizing antigen. Our study with congenic mouse strains indicated that DTH reactivity to minor H-antigens was restricted by the K- and D-region of the H-2 complex.

In appendix paper I the kinetics of primary and secondary DTH to SRBC are compared. It was shown that secondary DTH was characterized by an earlier appearance of the state of DTH, an earlier peak reactivity and a somewhat increased intensity of the DTH response, as compared to the primary DTH response. Secondary DTH responsiveness could be elicited at a time when no detectable primary DTH was present, for up to at least one year after optimal i.v. priming. The capacity of secondary DTH could be adoptively transferred into lethally irradiated recipients by means of Thy-1.2$^+$ spleen cells. The cellular basis of this secondary DTH responsiveness was further investigated by the experiments presented in appendix paper II. Parabiosis, for a three week period, of non-immunized mice and mice primed at least 2 months previously, resulted in the transfer of DTH-related T memory cells to the non-immunized partners. The presence of antigen in the non-immunized partner can virtually be excluded, and the capacity of secondary DTH reactivity in these mice, at long intervals after termination of the parabiosis, indicates that DTH-related T memory cells are long-lived, potentially circulating cells, which can persist in the absence of antigen. S.c. treatment of i.v. primed mice with a dose of ATS, which depletes the recirculating T cell pool, did not inhibit the expression of secondary DTH responsiveness in i.v. boosted mice. Secondary DTH reactivity in s.c. boosted mice was inhibited by this ATS treatment. The DTH-related T memory cells probably consist partly of non-recirculating sessile cells and partly of rapidly recirculating T cells. It was further demonstrated that the T memory cells themselves do not perform a detectable effector function, but their progeny, which arises from them in an antigen-driven step accompanied with proliferation.

In appendix paper III the contribution of short-lived T1 and long-lived T2 precursor cells to primary DTH and DTH-related T memory was investigated. It seemed feasible that only one, long-lived, population of recirculating T cells accounts for primary DTH. Furthermore, both T1 and T2 cells contribute to the population of T memory cells accounting for secondary DTH. The presence of the thymus, and probably of T1 cells, seems to be required for the generation of antigen-reactive T2 cells. The generation of T2 cells can take place without deliberate immunization. It was postulated, on the basis of the experiments described in this paper, that T1 cells can compensate for the absence of T2 cells during generation of T memory cells, but T2 cells cannot do so for the loss of T1 cells. Antigen stimulation might at first stimulate the more mature T2 cell pool to carry out their immunological function. If necessary, the T1 cell pool can supplement the contribution of the T2 cell pool to the immune response. The possibility that the short-lived effector T cells can revert to T memory cells in the absence of antigenic stimulation was not investigated.

In appendix paper IV it is shown that a single s.c. immunization of mice with viable allogeneic H-2 compatible spleen cells can induce a persistent state of DTH to multiple minor H-antigens as well as generating DTH-related T memory cells specific for these antigens. Boosting of such primed mice at long intervals after priming resulted in a secondary type DTH reactivity. Secondary DTH to semi-allogeneic H-2 incompatible spleen cells could not be induced. It was suggested in this paper that persistent DTH might be dependent on the continuous antigen-driven differentiation of long-lived recirculating T memory cells into functionally short-lived DTH effector cells.

In appendix paper V it was demonstrated that under GvH conditions created with H-2 compatible, minor H-antigen incompatible mouse strain combinations, DTH-reactive cells were generated which were specific for Mls coded antigens. These cells only occurred if Mls locus antigens were involved which are stimulatory in primary MLR. The contribution of other minor H-antigens was minimal under these GvH conditions. In contrast, under HvG conditions the influence of the Mls locus coded antigens was negligable, while under these HvG conditions the other minor H-antigens played an essential role. It was concluded that the Mls locus resembles the I-region in that both are mainly involved in GvH reactions and primary MLR.

The genetic requirements for (1) induction of DTH effector T cells and DTH-related T memory cells, (2) restimulation during the booster phase in secondary DTH and (3) the elicitation of DTH reactions

to minor H-antigens, were investigated in the experiments described in appendix paper V. Optimal induction of primary DTH and DTH-related memory for minor H-antigens required identical H-2 haplotypes in the immunizing cells and the recipient. Boosting was not strongly dependent on the H-2 haplotype of the cells used for booster injection. The expression of the DTH-reactivity to minor H-antigens was restricted by K- and D-region coded antigens. Elicitation of a DTH reaction to minor H-antigens with fully H-2 incompatible cells led to a comparatively weak, though significant DTH reaction. Our results in addition to those obtained by other authors, provide support for the hypothesis that different T cell subpopulations (e.g. Lyt-1^+ and Lyt-23^+ cells) can mediate DTH reactivity.

SAMENVATTING

Perifere T lymfocyten, die een bepaalde immunologische functie vervullen (b.v. helper functie, suppressor functie, resistentie tegen bacterien) kunnen worden ingedeeld op grond van hun differentiatiestadium (Fig. 3). Kortlevende "virginale" T1 lymfocyten zijn cellen, die onlangs in de thymus zijn ontstaan, en deze T1 cellen kunnen verder differentieren tot de langlevende T2 cellen zonder dat er van opzettelijke immunisatie sprake is. T2 cellen leveren de belangrijkste bijdrage tot de primaire T cellulaire immuunresponse. Na immunisatie kunnen de T2 cellen snel differentieren tot de kortlevende effector T cellen, die de uiteindelijke immuunreaktie teweeg brengen. Immunisatie van muizen leidt niet alleen tot een primaire immuunresponse, maar ook tot de vorming van T memory cellen, die, na restimulatie met het specifieke antigeen, verantwoordelijk zijn voor de secundaire immuunresponse. Deze T memory cellen hebben een lange levensduur, zijn in staat tot recirculatie, en oefenen geen effector functie uit, tenzij wederom door het specifieke antigeen gestimuleerd. Het is onduidelijk, of de T2 cellen in het ongeimmuniseerde dier kwalitatief verschillen van de T memory cellen, die verantwoordelijk zijn voor de secundaire immuunresponse. Memory zou namelijk ook kunnen berusten op de toename, ten gevolge van de immunisatie, van het aantal antigeen-specifieke T2 cellen, welke cellen kwalitatief niet zouden verschillen van de "spontaan" gevormde T2 cellen.

Uit literatuurgegevens blijkt, dat mogelijk enkele uitzonderingen op dit algemene model van perifere T cel differentiatie bestaan:

1. T cellen, die de immunologische functie van T2 cellen versterken, de z.g. amplifier T cellen, hebben de eigenschappen van T1 cellen. Deze T1 cellen hoeven waarschijnlijk niet te differentieren tot T2 cellen, alvorens hun "amplifier" functie uit te oefenen.
2. T "memory" cellen, verantwoordelijk voor de "second set" transplantaatafstoting, waarbij verschillen in het H-2 complex een rol spelen, zijn waarschijnlijk niet-recirculerende effector T cellen, met een lange levensduur.
3. T cellen verantwoordelijk voor persisterende tuberculine type vertraagde overgevoeligheid (DTH) zouden langlevende T memory cellen zijn met een effector functie.

Onze resultaten suggereren echter, dat zowel T cellen betrokken bij de kortlevende staat van DTH (Jones Mote type) als T cellen betrokken bij de persisterende staat van DTH (tuberculine type) geklassificeerd kunnen worden volgens het bovengenoemde schema van perifere T cel differentiatie (Fig. 3), zonder de noodzaak om een T cel populatie met uitzonderlijke eigenschappen te postuleren.

Antigenen gecodeerd door het H-2 complex spelen onder transplantaat-versus-gastheer (GvH) omstandigheden een andere rol dan onder gastheer-versus-transplantaat (HvG) omstandigheden (Tabel 5). Tijdens HvG reakties vormen de door de K- en D-regio gecodeerde weefselantigenen de voornaamste antigenen, waartegen de cytotoxische reaktie gericht is, terwijl daarentegen in de GvH reaktie de door de I-regio gecodeerde antigenen het belangrijkste zijn. Deze in de I-regio gecodeerde weefselantigenen veroorzaken een sterke proliferatie van T lymfocyten in de primaire gemengde lymfocytenkweek (MLR). Eveneens kunnen de weefselantigenen, gecodeerd door de Mls locus een primaire MLR induceren. In dat opzicht verschillen de Mls locus gecodeerde antigenen van de andere "minor weefselantigenen, die niet in staat zijn T cel proliferatie in een primaire MLR teweeg te brengen. Parallel aan de krachtige GvH inducerende werking van in de I-regio gecodeerde weefselantigenen, vonden we, dat Mls locus gecodeerde antigenen onder GvH omstandigheden grote aantallen DTH effector cellen doen ontstaan, terwijl hierbij de overige "minor" weefselantigenen geen belangrijke rol spelen.

Perifere T cellen herkennen in het algemeen antigenen in samenhang met een weefselantigeen gecodeerd door het eigen "major histocompatibility complex". Dit verschijnsel, in de muis genoemd H-2 restrictie, geldt ook voor T cellen betrokken bij de DTH response. Gegevens uit de literatuur suggereren, dat het antigeen bepaalt, welke regio(nen) van het H-2 complex betrokken is (zijn) bij de herkenning van het desbetreffende antigeen door de DTH-reactieve T cellen (Tabel 6). Onze studie met congene muizestammen toonde aan, dat DTH voor "minor" weefselantigenen onderhevig is aan restrictie door K- en D-regio gecodeerde weefselantigenen.

In appendix paper I werd de kinetiek van de primaire en secundaire DTH met elkaar vergeleken. Gevonden werd dat secundaire DTH gekarakteriseerd is door een vervroegd ontstaan van de staat van DTH, een eerdere piek, en een enigszins verhoogde kracht van de DTH response, vergeleken met de primaire DTH. Een staat van secundaire DTH kon worden geinduceerd op een tijdstip waarop geen primaire DTH meer aantoonbaar was, en wel tot minstens 1 jaar na intraveneuze (i.v.) immunisatie met de optimale dosis SRBC. De capaciteit tot secundaire DTH kon adoptief worden overgedragen naar letaal bestraalde recipienten door middel van Thy-1.2^{+} miltcellen. De cellulaire basis van dit verschijnsel van secundaire DTH werd verder onderzocht in de experimenten omschreven in appendix paper II. Parabiose gedurende een periode van 3 weken, van niet-geimmuniseerde muizen en muizen, minstens 2 maanden eerder geimmuniseerd, had tot gevolg dat DTH-reactieve T memory cellen overgedragen werden naar de niet-geimmuniseerde partners. De aan-

wezigheid van antigeen in de niet-geimmuniseerde partner kan in feite uitgesloten geacht worden. De capaciteit tot secundaire DTH in deze niet-geimmuniseerde dieren, lang na de beëindiging van de parabiose, toonde aan, dat de DTH-reactieve T memory cellen langlevende cellen zijn, die in de bloedsomloop kunnen voorkomen, terwijl ze blijven voortbestaan in afwezigheid van antigeen. Subcutane (s.c.) behandeling van i.v. geimmuniseerde muizen met antithymocyten serum (ATS), dat de recirculerende T cel populatie uitschakelt, had nauwelijks invloed op het vermogen tot secundaire DTH na intraveneuze booster. De capaciteit tot een secundaire DTH response in muizen geboosterd via de s.c. route ging wel verloren door ATS behandeling. Waarschijnlijk bestaan de DTH-reactieve T memory cellen deels uit niet recirculerende sessiele cellen, en deels uit snel recirculerende T cellen. Verder werd aannemelijk gemaakt, dat door de T memory cellen zelf geen effector functie wordt uitgeoefend, maar wel door hun nageslacht, dat onder invloed van antigenen uit de memory cellen voorkomt. In het geval van immunisatie met SRBC gaat dit laatste proces gepaard met proliferatie van de memory cellen.

In appendix paper III werd de bijdrage van kortlevende T1 en langlevende T2 voorlopercellen aan primaire DTH en de vorming van DTH-reactieve T memory cellen onderzocht. Het leek zeer waarschijnlijk, dat slechts één enkele, langlevende, populatie recirculerende T cellen een rol speelt in de primaire DTH. Verder zouden zowel T1 als T2 cellen bijdragen tot de vorming van DTH-reaktieve T memory cellen, die de secundaire DTH bewerkstelligen. De aanwezigheid van de thymus, en vermoedelijk van T1 cellen, lijkt vereist voor de vorming van antigeen-reaktieve T2 cellen. De vorming van T2 cellen kan plaatsvinden zonder opzettelijke immunisatie. Op grond van de experimenten, die beschreven zijn in dit artikel, werd gespeculeerd dat T1 cellen kunnen compenseren voor de afwezigheid van T2 cellen tijdens de vorming van T memory cellen, maar dat T2 cellen dat niet kunnen bij een tekort aan T1 cellen. Antigene stimulatie zou allereerst de rijpere T2 cel populatie activeren tot het uitoefenen van hun immunologische functie. Zo nodig kan de T1 cel populatie de bijdrage van de T2 cellen voor de immuunresponse aanvullen. De mogelijkheid, dat kortlevende effector T cellen kunnen "terugdifferentieren" tot T memory cellen, wanneer ze niet door antigeen gestimuleerd worden, werd niet in beschouwing genomen.

In appendix paper IV is aangetoond, dat een enkele s.c. immunisatie van muizen met levende allogene H-2 compatibele miltcellen een persisterende staat van DTH tegen "minor" weefselantigenen kan teweeg brengen, tezamen met de vorming van DTH-reactieve T memory cellen specifiek voor deze antigenen. Booster van der-

gelijk geimmuniseerde dieren enkele maanden later leidde tot het optreden van een secundaire staat van DTH. Secundaire DTH tegen semi-allogene H-2 incompatibele miltcellen kon niet worden geinduceerd. Op grond van de gegevens, beschreven in dit artikel werd gesuggereerd, dat persisterende DTH afhankelijk zou zijn van de voortdurende differentiatie, onder invloed van antigenen, van langlevende recirculerende T memory cellen tot functioneel kortlevende DTH effector cellen.

In appendix paper V werd aangetoond, dat GvH omstandigheden, verkregen door bestraalde ontvangers te reconstitueren met allogene miltcellen uit H-2 compatibele donoren, kunnen leiden tot het ontstaan van DTH-reactieve T cellen. Deze DTH-reactieve T cellen zijn alleen gericht tegen Mls locus gecodeerde antigenen, en dan alleen tegen die, welke in een primaire MLR een stimulatoire rol spelen. De bijdrage van de andere "minor" weefselantigenen was minimaal onder deze GvH omstandigheden. In tegenstelling hiermee was onder HvG omstandigheden de invloed van de Mls locus gecodeerde antigenen verwaarloosbaar. In dit laatste geval speelden de andere "minor" weefselantigenen een essentiële rol. Geconcludeerd werd, dat de Mls locus lijkt op de I-regio van het H-2 complex, omdat beide coderen voor weefselantigenen, die een belangrijke rol spelen in GvH en MLR.

De genetische vereisten voor (1) inductie van DTH-reactieve effector cellen en T memory cellen, (2) restimulatie tijdens de booster phase voor de secundaire DTH en (3) het oproepen van DTH reacties tegen "minor" weefselantigenen werd onderzocht in appendix paper VI. Voor optimale inductie van primaire DTH en memory voor "minor" weefselantigenen was H-2 identiteit van de immuniserende cellen met de recipient vereist. De booster phase is waarschijnlijk in mindere mate afhankelijk van het H-2 haplotype van de cellen, die gebruikt worden voor booster injectie. De expressie van de DTH voor "minor" weefselantigenen was alleen krachtig, wanneer de cellen, waarmee de DTH reactie werd opgeroepen, en de recipient de K- en/of D-regio van het H-2 complex gemeen hadden. Het opwekken van een DTH reactie tegen "minor" weefselantigenen met volkomen H-2 incompatibele cellen leidde tot een vrij zwakke, maar wel significante DTH reactie. Onze resultaten, in samenhang met die van andere auteurs, ondersteunen de hypothese, dat verschillende T cel subpopulaties (b.v. Lyt-1^+ en Lyt-23^+ cellen) een staat van DTH tot stand kunnen brengen.

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CURRICULUM VITAE

Na het behalen van het diploma Gymnasium-β aan het Stedelijk Lyceum te Zutphen in 1970 begon ik de studie Geneeskunde aan de Medische Faculteit (later Erasmus Universiteit) te Rotterdam. In 1973 nam ik deel aan een microscopisch onderzoek van het immuunapparaat, onder leiding van Drs. W. van Ewijk, op de afdeling Celbiologie en Genetica van de Medische Faculteit te Rotterdam. Dit werk verrichtte ik eerst in het kader van het prekandidaatsonderwijs, vervolgens, tot augustus 1974, als studentassistent. In juni 1975 behaalde ik het doctoraal examen, in de Geneeskunde en per 1 juli 1975 trad ik in tijdelijke dienst als wetenschappelijk medewerker bij de afdeling Celbiologie en Genetica van de Erasmus Universiteit te Rotterdam. Binnen de afdeling Celbiologie II, onder leiding van Prof.Dr. O. Vos, werd het in het proefschrift beschreven onderzoek verricht.

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Secondary Delayed-Type Hypersensitivity to Sheep Red Blood Cells in Mice: A Long-Lived Memory Phenomenon

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Immunization of mice with sheep red blood cells (SRBC) can induce the capacity to react with a secondary delayed-type hypersensitivity (DTH) immune response upon a booster injection of the antigen. In this paper the kinetics of secondary DTH after intravenous (iv) immunization with various doses of SRBC was studied by means of the foot swelling test. Dose–response studies showed that maximal secondary DTH responsiveness was obtained by iv administration of a priming dose of 3×10^4 SRBC and a booster dose of 3×10^5 SRBC 2 months later. Secondary DTH in such treated mice was characterized by an earlier appearance of the state of DTH, an earlier peak reactivity, and an increased intensity of the DTH response as compared to the primary DTH response. Up to 1 year after priming, a secondary DTH could be elicited, indicating the long-lived character of this memory phenomenon. With increasing intervals between the priming and booster injection, a gradual shift to a later time, of the peak secondary DTH reactivity was found. The capacity of primed mice to react with an increased intensity upon a booster injection could be adoptively transferred into lethally irradiated recipients by means of spleen cells obtained from primed mice. This phenomenon appeared to be highly dependent on Thy 1.2^+ cells and on the booster dose of SRBC. The DTH reaction, evoked in such recipients, showed a prolonged time course.

INTRODUCTION

Secondary-type responsiveness for delayed-type hypersensitivity (DTH) has been described for a number of antigens including proteins (1), a contact sensitivity agent (2), *Mycobacterium tuberculosis* (3), *Listeria monocytogenes* (4), and sheep red blood cells (SRBC) (5). Most authors did not study secondary DTH systematically but noticed a higher percentage of responder animals after two instead of one injection. Only Crowle (6) carefully reviewed these data and described some general properties of secondary DTH: a more rapid sensitization after the second application of the antigen as compared to the first exposure, an increase of the intensity of an already existing primary hypersensitivity, the finite period of its induction in primed mice, and the ability to transfer the property of accelerated sensitization to syngeneic recipients by means of spleen cells. It was proposed that the interval which allows successful restimulation depends on (i) the route of immunization (6) and (ii) the persistence of the state of primary DTH (4, 6). This persistence of the state of DTH would depend on the presence of nonreplicating, vinblastin-resistant cells (4).

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For the antigen SRBC Lagrange and Mackaness (7), using the iv immunization route, failed to demonstrate a secondary DTH response in primed normal mice. However, in previously splenectomized mice (8) or in mice that had been pretreated with cyclophosphamide and *Bacille Calmette Guérin*, an increase of the intensity of DTH was observed after iv injection of a booster dose of SRBC (7). Recently Tamura and Egashira (5) found that subcutaneous (sc) priming and a subsequent booster injection with SRBC resulted in an accelerated DTH responsiveness. They did not study the other above-mentioned properties of secondary DTH. These authors concluded that priming of mice with SRBC induced DTH-related memory cells.

In cell-mediated immunity both short-lived and long-lived memory exist (9). An example of a short-lived memory phenomenon in cell-mediated immunity is the capacity of secondary-type mixed lymphocyte reaction (MLR). A second-set skin graft rejection can be considered as a long-lived memory phenomenon (9).

In this paper we investigated the kinetics of secondary DTH after iv immunization with SRBC. Furthermore, the influences of antigen dose and interval between priming and booster on the reaction pattern were studied.

MATERIALS AND METHODS

Animals. (C57BL/Rij × CBA/Rij) F1 female mice, 20 to 30 weeks old, were used. In one transfer experiment male (C57BL/Rij × CBA/Rij) F1 mice were used, as indicated in the text. The mice were purchased from the Medical Biological Laboratory, Rijswijk, The Netherlands, and the Laboratory Animals Center of the Erasmus University, Rotterdam, The Netherlands.

Antigen and immunization. Sheep red blood cells were commercially obtained, stored, and washed as described previously (10). Primary and secondary immunization was performed by iv injection of the SRBC in 0.5 ml of saline. The challenge dose was administered sc in the instep of the right hind foot.

Preparation of cell suspensions for adoptive transfer. Single cell suspensions were prepared in a balanced salt solution as described in a previous paper from our laboratory (11). Bone marrow was collected from femurs and tibias. Lethally irradiated recipients were injected iv with the appropriate cell suspension and 3×10^5, 3×10^6, or 3×10^7 SRBC in a total volume of 0.5 ml.

Antisera. The production of anti-Thy 1.2 antisera and their use for the selective elimination of T cells in the spleen have been described previously (11).

Cell counts. Viable cells were counted in a hemocytometer using 0.2% trypan blue in phosphate-buffered saline (PBS) as diluent.

Irradiation. The recipient mice received 925 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 of Cu; correction of irradiation for field inhomogeneity; focus-object distance, 53 cm; dose rate, 30–35 rad/min. During irradiation the dose was measured with a Baldwin Ionex dosimeter. The recipient mice were injected iv with the appropriate cell suspension 24 hr after irradiation.

Assay for delayed-type hypersensitivity. DTH reactions were determined by measuring the difference in thickness of the hind feet 24 hr after sc injection of 5×10^7 SRBC in the instep of the right hind leg. In transfer experiments the in-

crease in foot thickness was also measured 48 and 72 hr after challenge. The challenge dose was administered sc in a volume of 0.02 ml by means of a 29-gauge needle. The thickness of the left and right hind foot was measured with a footpad meter as described by Bonta and Vos (12), with some minor modifications. During measurement the mice were anesthetized with ether.

A control group, consisting of nonimmune mice challenged with SRBC in a manner similar to the mice to be tested, was always included. 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 (10). The swelling in control mice varied between 15 and 20%.

RESULTS

Variation of the priming dose. (C57BL × CBA) F1 female mice were primed with either 3×10^4, 3×10^5, or 3×10^6 SRBC iv and boosted with 3×10^5 SRBC iv 6 months later. Different groups of mice were challenged with 5×10^7 SRBC sc in the foot 2, 3, or 4 days after injection of the booster dose. At 24 hr after challenge, the DTH reaction was measured and expressed as the percentage increase of foot thickness. The mice primed iv with 3×10^4 or 3×10^5 SRBC showed an almost equally high response on the second day after the booster injection, whereas mice primed with 3×10^6 SRBC showed a somewhat lower secondary DTH response (Fig. 1). The DTH response, evoked on the third and fourth day after booster injection, was inversely related to the priming dose. Apparently, priming with 3×10^5 SRBC 6 months previously results in a diminished capacity for secondary DTH response in these mice, whereas this dose is optimal for induction of primary DTH (13, and our unpublished results).

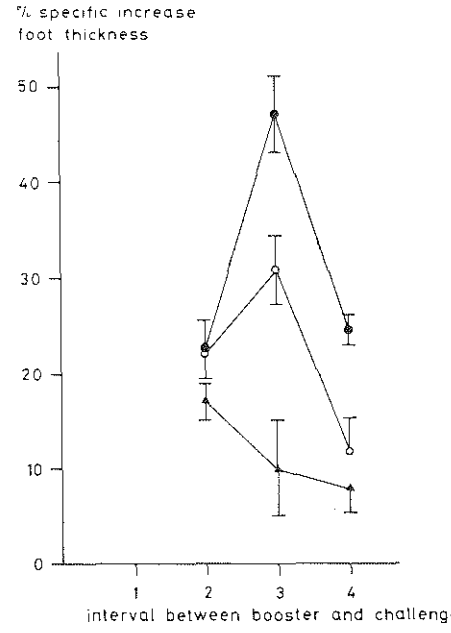

FIG. 1. Effect of variation of the priming dose on the secondary DTH response to SRBC. Mice were primed with either 3×10^4 (●——●), 3×10^5 (○——○), or 3×10^6 SRBC (▲——▲) and boosted with 3×10^5 SRBC 6 months later. Another 2, 3, and 4 days later different groups of mice were challenged. Vertical bars represent 1 SEM ($n = 5$).

TABLE 1

Variation of Interval and Priming Dose on Secondary DTH to SRBC

Priming dose	Interval between priming and booster		
	2 months	3 months	12 months
PBS[a]	1.2 ± 2.9[b]	0.2 ± 2.1	n.d.[c]
3×10^3 SRBC	9.5 ± 3.9	n.d.	n.d.
3×10^4 SRBC	31.2 ± 1.4	31.4 ± 2.5	19.4 ± 2.5
3×10^5 SRBC	16.7 ± 4.4	20.0 ± 2.4	3.0 ± 3.0

[a] PBS = phosphate-buffered saline. These mice were only immunized with 3×10^5 SRBC 2 days before challenge.

[b] Figures represent the mean ± 1 SEM of five mice injected with 3×10^5 SRBC 2 days previously.

[c] n.d. = not determined.

Since the optimal dose for priming might depend on the interval between priming and booster, groups of mice primed with varying doses of SRBC were boosted with 3×10^5 SRBC iv at various times after priming. Table 1 shows the results obtained with mice boosted 2, 3, or 12 months after priming with either 3×10^3, 3×10^4, or 3×10^5 SRBC iv. All mice were challenged on the second day after booster injection. Since in normal mice no primary DTH response can be initiated 2 days after immunization with 3×10^5 SRBC iv, the DTH reaction evoked on the second day after booster is a parameter for the level of secondary DTH. The priming dose of 3×10^4 SRBC appeared to be optimal at all intervals (Table 1).

Kinetics of the secondary DTH. Groups of mice were primed with 3×10^4 SRBC iv and boosted with different doses of SRBC iv 3 months later. Figure 2

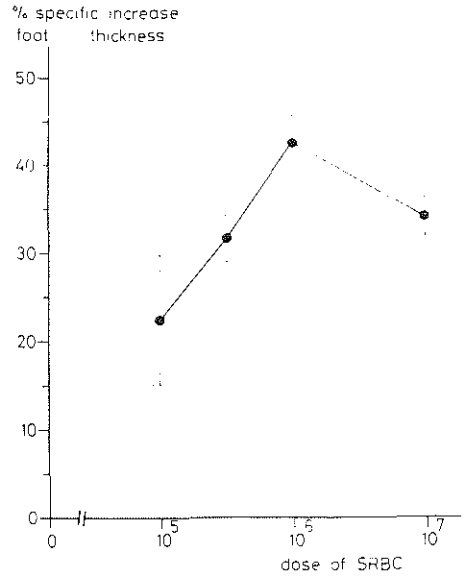

FIG. 2. Dose-response curve for secondary DTH, obtained in mice primed with 3×10^4 SRBC iv 3 months previously. The mice were boosted with varying doses of SRBC and challenged 2 days later. Vertical bars represent 1 SEM ($n = 5$).

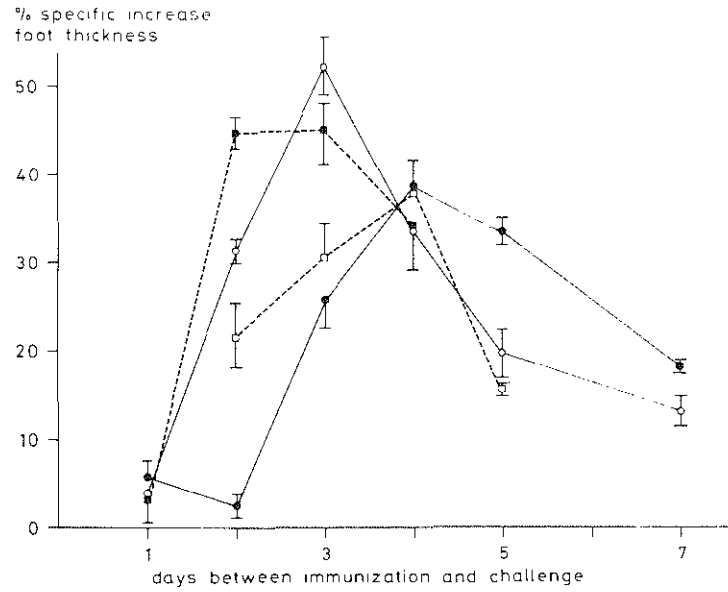

FIG. 3. Kinetics of primary and secondary DTH to SRBC. Mice were primed for secondary DTH with 3×10^4 SRBC and boosted with either 1×10^4 (□ - - - □), 3×10^5 (○——○), or 1×10^6 SRBC (■ - - - ■) 2 months later. A group of nonprimed mice of the same age was also immunized with 3×10^5 SRBC (●——●) in order to induce an optimal primary DTH. DTH responses were determined at different days after the last immunization. Vertical bars represent 1 SEM. Each point is the mean of at least five mice.

shows the dose–response curve when the mice were challenged 2 days after booster injection. It was found that the booster dose of 1×10^6 SRBC evoked the strongest reaction when elicited on the second day after booster.

To compare the course of the primary and the secondary DTH nonprimed mice and mice primed 2 months previously with 3×10^4 SRBC were immunized with 3×10^5 SRBC. The DTH responses of these mice were determined at different times after immunization (Fig. 3). Primary DTH could not be detected before the third day after immunization, reached a peak reactivity on the fourth day, and subsequently declined. Contrarily, secondary DTH was already detectable 48 hr after booster injection, peaked at about 72 hr, and subsequently declined. Generally the peak of the secondary DTH response reached a higher level than peak primary DTH responses to the same dose of antigen. Mice primed 2 months previously with 3×10^4 SRBC and boosted with either 1×10^4 SRBC or 1×10^6 SRBC showed a different reaction pattern (Fig. 3). A booster dose of 1×10^4 SRBC resulted into a shift of the peak response to Day 4, whereas after a booster dose of 1×10^6 SRBC the peak response occurred by Day 2. Nevertheless, the strongest DTH response was obtained in mice boosted with 3×10^5 SRBC iv.

Variation of the interval between priming and booster. In order to study the kinetics of secondary DTH at different intervals after priming, mice were immunized iv with 3×10^4 SRBC and boosted with 3×10^5 SRBC at either 1, 3, 9, 30, 60, or 90 days after priming. The response of primed mice which had not been boosted was also measured. Boosting on the third day after priming could already enhance the existing primary DTH response (Fig. 4). At longer intervals between priming and booster the secondary DTH response further increased. A clear peak

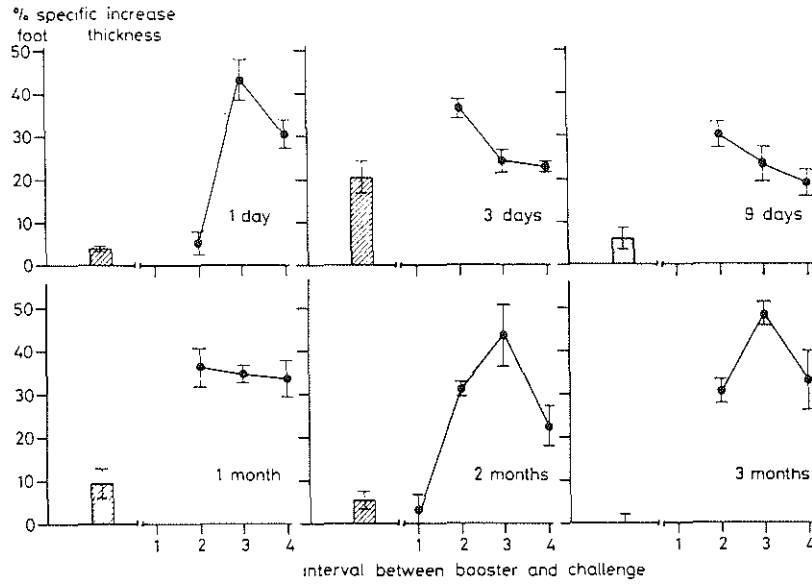

FIG. 4. Effect of variation of the interval between priming and booster upon secondary DTH responsiveness. Mice were primed iv with 3×10^4 SRBC and boosted with 3×10^5 SRBC iv at different intervals. DTH responses were evoked on different days after booster injection (graphs). Columns show the level of still-persisting primary DTH of similarly primed mice 2 days after the indicated interval. These mice had only received the priming dose of 3×10^4 SRBC. Vertical bars represent 1 SEM ($n = 5$).

response on the third day after booster was observed, when a period of 60 days between priming and booster had passed. Thus, the kinetics of secondary DTH was strongly influenced by the interval between priming and booster. Also 3 months after priming, when primary DTH reactivity due to priming could no longer be detected, a typical secondary DTH response could be induced (Fig. 4).

Transfer of the capacity of secondary DTH. Male (C57BL × CBA) F1 mice were primed iv with either 3×10^3 or 3×10^4 SRBC. Six months later, 5×10^7 spleen cells from these mice were transferred together with 3×10^6 normal bone marrow cells and either 3×10^5, 3×10^6, or 3×10^7 SRBC into lethally irradiated recipients. As a control, normal spleen cells were transferred together with 3×10^6 bone marrow cells and the same doses of antigen. Four days after transfer, all recipients were challenged. The DTH reactivity was determined 24, 48, and 72 hr after challenge. Also in these transfer experiments priming with 3×10^4 SRBC accounted for maximal DTH responsiveness upon subsequent transfer and booster with 3×10^5 SRBC (Fig. 5A). Furthermore, it appeared that the DTH response in recipients of immune spleen cells was susceptible to the suppressive action of higher booster doses of antigen, whereas the DTH response in recipients of normal spleen cells was enhanced after boosting with high antigen doses (Figs. 5B and C). In contrast to normal mice, in which the DTH response at 48 hr after challenge is generally diminished to about 50% of the response measured at 24 hr (data not shown), irradiated recipients of normal or immune spleen cells showed a prolonged time course of the DTH response after challenge (Fig. 5).

A subsequent experiment studied whether the observed capacity to transfer secondary DTH to irradiated recipients was dependent on the presence of Thy 1.2$^+$

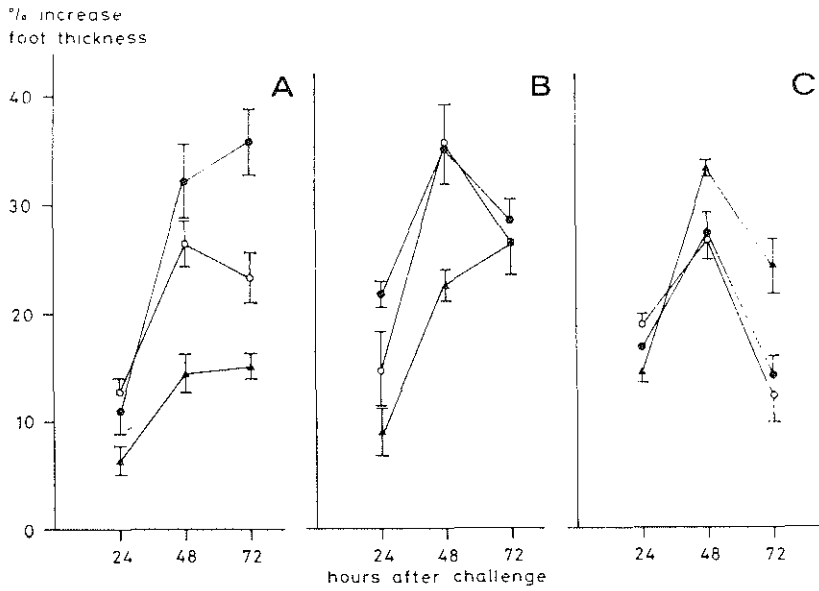

FIG. 5. Transfer of the capacity of secondary DTH into irradiated recipient mice. Irradiated mice were injected iv with either 5×10^7 normal spleen cells (▲——▲) or 5×10^7 spleen cells from mice primed with 3×10^3 (○——○) or 3×10^4 SRBC (●——●) 6 months previously. Together with the spleen cells, 3×10^6 bone marrow cells and either 3×10^5 (A), 3×10^6, (B), or 3×10^7 SRBC (C) were administered. Challenge of the recipients was performed 4 days after transfer, and DTH reactivity was determined at 24, 48, and 72 hr after challenge. Vertical bars represent 1 SEM ($n = 5$). Control mice which had only received 3×10^6 bone marrow cells and 3×10^6 SRBC did not show a significant foot swelling at 24, 48, and 72 hr after challenge.

cells in the inoculum. Spleen cells from female mice, primed with 3×10^4 SRBC iv 6 weeks previously, were treated either with normal mouse serum (NMS) and complement or with anti-Thy 1.2 antiserum and complement. A total of 2×10^7 of these cells were either supplemented or not with 2×10^7 normal spleen cells and injected iv together with 3×10^6 normal bone marrow cells and 3×10^5 SRBC

TABLE 2

Dependence of Secondary DTH Responsiveness on T Memory Cells

Immune spleen cells[a]	Treatment	Nonimmune spleen cells	SRBC	Response (%)
2×10^7	NMS + C[b]	—	3×10^5	56.7 ± 3.7[c]
2×10^7	anti-Thy 1.2 + C	—	3×10^5	16.6 ± 2.4
2×10^7	NMS + C	2×10^7	3×10^5	56.1 ± 6.6
2×10^7	anti-Thy 1.2 + C	2×10^7	3×10^5	26.0 ± 2.8
—	—	2×10^7	3×10^5	21.2 ± 1.7
—	—	2×10^7	—	9.4 ± 1.7

[a] Lethally irradiated recipients were injected with 3×10^6 normal bone marrow cells together with the indicated number of immune and nonimmune spleen cells and SRBC. Immune spleen cells were obtained from isogeneic mice primed with 3×10^4 SRBC iv 6 weeks previously.

[b] NMS = normal mouse serum; C = guinea pig complement.

[c] Figures represent the mean response ± 1 SEM.

into lethally irradiated mice. The mice were challenged on the fifth day after transfer and DTH reactivity was measured 24, 48, 72, and 96 hr after challenge. Maximal responses have been listed in Table 2. It was found that typical secondary DTH responses were elicited only after transfer of NMS-treated immune spleen cells. Addition of normal spleen cells to anti-Thy 1.2-treated immune spleen cells could not restore the capacity of a typical secondary DTH. These results suggest that T cells, and not macrophages or B cells in the immune inoculum, are mainly responsible for secondary DTH responsiveness.

DISCUSSION

This study on DTH to SRBC reports the induction of a state of immunological memory for DTH reactivity in normal mice after priming by the iv immunization route. The failure of Lagrange and Mackaness (7) to demonstrate a secondary DTH to SRBC using the same immunization route can be explained by the strong dependence of this phenomenon on the antigen dose used from priming (Fig. 1 and Table 1) and the interval between booster and challenge (Fig. 3). In the case of iv antigen injection, secondary DTH can be most easily demonstrated after low priming doses (e.g., 3×10^4 SRBC) and a short interval between booster and challenge (e.g., 2 days). This strong dependence of secondary DTH on the priming dose of SRBC was not found by Tamura and Egashira (5). These authors immunized mice with SRBC via the sc route. The variance between their and our results might indicate that suppression of secondary DTH by high priming antigen doses occurs especially after iv immunization. This is consistent with reports in literature attributing suppression of primary DTH mainly to the spleen (8, 14, 15). The dose–response curve for the booster dose in secondary DTH on Day 2 (Fig. 2) is very much like the dose–response curve of primary DTH on Day 3 (data not shown), which suggests that a similar mechanism suppresses the expression of primary and secondary DTH.

The DTH reaction elicited in irradiated recipients of both normal and immune spleen cells showed a remarkably prolonged time course (Fig. 5) as compared to the DTH reaction to SRBC evoked in normal mice, in which the foot swelling rapidly diminishes after 24 hr. This altered time course of DTH reactivity was also reported to occur in guinea pigs pretreated with cyclophosphamide before immunization with 1-fluoro-2,4-dinitrobenzene (15). Evidence was presented that cyclophosphamide treatment causes a depletion of the suppressor cell population acting at the effector cell level (16). In a previous study on DTH to SRBC we have shown that the DTH effector cells have a short-lived functional lifespan (10), and we suggested that continuous migration of DTH effector cells to the site of challenge was required for the establishment of a foot swelling reaction. Thus, the prolonged course of the DTH reaction observed in irradiated recipients might reflect the kinetics of the production of DTH-effector cells derived from the injected inoculum. The adoptive transfer experiments also show that immune spleen cells are more sensitive to suppression by high antigen doses than normal spleen cells (Fig. 5), which can be attributed to the coexistence of both DTH-related memory cells and DTH-related suppressor cells. This latter population might have expanded or increased in reactivity due to the primary immunization.

Variation of the interval between priming and booster showed that the kinetics of secondary DTH changed with time. A clear peak reactivity on the third day

after a booster injection with 3×10^5 SRBC was found only 2 months and later after priming (Fig. 4). This shift of the peak response from Day 2 to Day 3 can be ascribed to a gradual increase of the number of DTH-related memory cells.

Although the secondary DTH as a phenomenon is qualitatively different from primary DTH (Fig. 3), it remains questionable whether the T cells for primary DTH differ qualitatively from the DTH-related T memory cells. Once started, the courses of primary and secondary DTH are very much alike. The earlier appearance of secondary DTH and higher peak reactivity might therefore be attributed to a larger number of antigen-specific DTH-related T cells. The earlier peak reactivity during secondary DTH might then be due to the earlier occurrence of suppression of the state of DTH in such mice. Although in this way the differences between primary and secondary DTH can be explained in quantitative terms, there is some evidence for qualitative differences as well. This can be deduced from comparison of the course of primary DTH to 3×10^5 SRBC with the course of secondary DTH to a booster dose of 1×10^4 SRBC. Both responses have an equally high peak reactivity on the same day (Fig. 3). However, the first appearance of the secondary DTH reactivity after booster with 1×10^4 SRBC is 1 day earlier, which might suggest that precursors for primary DTH are qualitatively different from the DTH-related memory cells.

In cell-mediated immunity, as in humoral immunity (17, 18), both short-lived and long-lived memory has been described (9). Long-lived memory phenomena thus far reported are: the second-set skin graft rejection (19), its *in vitro* correlate, the secondary cell-mediated lympholysis (20); and the recall of resistance to *M. tuberculosis* (21), to *L. monocytogenes* (22), and to *Salmonella enteritidis* (23). The secondary mixed lymphocyte culture (MLC) was considered as a short-lived memory phenomenon only demonstrable for 3–5 weeks after induction of primary MLC according to some authors (24), but for about 5 months according to others (25). Crowle postulated, in his review on DTH in mice (6), that memory for secondary DTH would have a finite life time, depending on the persistence of the original state of hypersensitivity, just as once had been suggested for the second-set skin graft rejection: The accelerated rejection would be due to the persistence of effector cells (19). Since the secondary DTH to SRBC can be demonstrated in absence of a persistent primary DTH and even 1 year after priming with 3×10^4 SRBC, we suggest that secondary DTH represents a true long-lived memory phenomenon, waning only after a long time. In a following paper, evidence will be presented for the long-lived nature of the DTH-related T memory cells and their persistence in the absence of antigen (27).

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Secondary Delayed Type Hypersensitivity to Sheep Red Blood Cells in Mice: Dependence on Long-Lived Memory Cells

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Secondary delayed type hypersensitivity (DTH) to sheep red blood cells (SRBC) in mice is a long-lived memory phenomenon which is characterized by the accelerated reappearance of the state of DTH after a booster injection of the antigen. In this paper the nature of the DTH-related T memory cells accounting for secondary DTH was investigated. Parabiosis of primed and nonprimed mice for a period of 4 weeks resulted in an equally large secondary DTH responsiveness in both partners. This ability was maintained in both members for at least 6 months after termination of the parabiosis. These results indicate that (a) DTH-related T memory cells are potentially circulating cells, and (b) the persistence of these memory cells is not dependent on the presence of the antigen which induced their generation. Subcutaneous (sc) injection of intravenously (iv) primed mice with a small dose of anti-thymocyte serum before boosting did prevent the development of secondary DTH responsiveness in sc boosted mice, but not in iv boosted mice. Treatment of primed mice with vinblastine or azathioprine did not decrease the capacity of adoptive transfer of secondary DTH by means of spleen cells, but passive transfer of secondary DTH was completely abolished by this treatment. These results suggest that (a) SRBC-induced DTH-related T memory cells are nonproliferating, partially sessile, partially recirculating cells, and (b) these memory cells proliferate before they become DTH-related effector cells.

INTRODUCTION

In a previous paper it was shown that secondary delayed type hypersensitivity (DTH) to sheep red blood cells (SRBC) is a long-lived memory phenomenon, which can be demonstrated up to at least 1 year after priming (1). Secondary DTH has been described for a number of antigens but the cellular base of this phenomenon has not yet been investigated (2).

A stable and long-lived state of DTH has been observed after immunization with mycobacteria alone (3, 4), after immunization with antigen emulsified in an adjuvant containing mycobacteria (5, 6), and after pretreatment of mice with Bacillus Calmette-Guérin (BCG) before immunization (7). Such a persistent state of DTH can be considered as an expression of immunological memory and it was proposed that this classical DTH would be mediated by long-lived effector cells which would not require proliferation in order to establish DTH reactivity after passive transfer of these cells into a syngeneic recipient (8, 9). The cells that are responsible for the persistent state of DTH would be different from the DTH effector cells generated early after immunization with, e.g., BCG. This has been proposed by Lefford

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and McGregor (4), who showed that during the early stage after immunization of rats with BCG the cells conferring protective immunity to tuberculosis were vinblastine-sensitive cells, whereas at a later moment these cells were vinblastine resistant and recirculating (10). The same results were obtained when mice were intravenously (iv) immunized with SRBC in saline 2 days after treatment with cyclophosphamide. Initially, the DTH to SRBC was mainly dependent on a vinblastine-sensitive pool of lymphocytes, but later on the DTH response was no longer influenced by vinblastine treatment (11). The persistence of the vinblastine-resistant population might be independent of the antigen used for immunization, although this has not been shown conclusively (11).

The aim of the experiments reported here is to investigate whether long-lived memory cells occur after iv priming with SRBC in saline and to further characterize these DTH-related T memory cells.

MATERIALS AND METHODS

Mice. (C57BL/Rij × CBA/Rij) F1 female mice, 12–16 weeks old, were used. They were purchased from the Laboratory Animals Centre of the Erasmus University, Rotterdam, and the Medical Biological Laboratory, Rijswijk, The Netherlands.

Antigen and immunization. Sheep red blood cells were commercially obtained, stored, and washed as described previously (12). Primary and secondary immunization was done by iv injection of the SRBC in 0.5 ml of saline. The challenge dose of 5×10^7 SRBC was administered subcutaneously (sc) in the instep of the right hind foot.

Parabiotic mice. Parabionts were joined unilaterally under Nembutal (Abbott S.A., Saint-Rémy-sur-Avre, France) anesthesia (70 mg/kg body wt). The mice were united by suturing the peritoneum and abdominal wall muscles without leaving a communication between the abdominal cavities. The connective tissue along the thorax was also sutured. In addition, the scapulae were joined. The skin was closed with linen thread from behind the ears to the femora. Parabiotic mice were separated from each other under Nembutal anesthesia 4 weeks after joining. Again the skin was closed with linen thread.

Anti-thymocyte serum. Anti-mouse thymocyte serum (ATS) was prepared in rabbits as described previously (13). For elimination of recirculating T cells a total volume of 0.2 ml ATS or normal rabbit serum (NRS) was administered sc on the back, in the armpits, and the groins. ATS and NRS were adsorbed once with mouse blood cells (1:1) before use.

Vinblastine and azathioprine. Vinblastine sulfate (Eli Lilli and Co., Indianapolis, Ind.) was iv injected in a dose of 100 μg/recipient, dissolved in a volume of 0.2 ml sterile saline. Azathioprine sodium (Imuran; Burroughs Wellcome and Co., London, England) was iv injected in a dose of 100 mg/kg body wt, dissolved in a volume of 0.25 ml distilled water.

Preparation of cell suspensions. Single-cell suspensions were prepared in a balanced salt solution as described in a previous paper from our laboratory (14). Bone marrow was collected from femora and tibiae. Cells were counted in a Coulter Counter Model B.

Irradiation. For adoptive transfer, the recipient mice received 900 rad whole-body irradiation, generated in a Philips–Müller MG 300 X-ray machine. Details

of the apparatus and the irradiation procedure have been described previously (1). The recipient mice were iv injected with the appropriate cell suspension in a total volume of 0.5 ml 24 hr after irradiation.

RESULTS

Secondary DTH Responsiveness of Nonprimed Mice after Parabiosis with Primed Mice

To investigate the ability of DTH-related T memory cells to circulate in the blood and to study their persistence in the absence of antigen, the technique of parabiosis was used. Within 6 days after joining the mice for parabiosis, blood cells can freely circulate from one partner to the other and within 4 weeks of parabiosis an equilibrium of the freely circulating cells between both partners can be expected (17). In the present study, mice, primed iv with 3×10^4 SRBC 2–3 months previously, were joined for parabiosis with nonimmune mice. As controls similarly primed and nonimmune mice were joined separately. Four weeks after the establishment of the parabiosis all parabionts were separated. Either 3 weeks, 3 months, or 6 months after separation all mice were iv immunized with 3×10^5 SRBC. The challenge injection was given 2 days later, as the response measured on the second day after a booster injection has been shown to be a reliable parameter for the secondary DTH responsiveness (1). The parabiosis of nonimmune mice with primed mice resulted in an equal capacity of secondary DTH of both partners, irrespective of the interval between separation of the parabionts and the booster injection (Table 1). The secondary DTH responsiveness of these mice was as high as in immune mice that had been joined to other immune mice. The level of secondary DTH responsiveness did not decline with increasing interval between separation of the parabiotic mice and the moment of booster injection. Nonimmune mice which had been joined together did not show a significant DTH response on Day 2 after immunization.

TABLE 1

Transfer of DTH-Related Memory by Means of Parabiosis

Parabiotic members tested for DTH[a]	Parabiotic members that had been joined to them	Interval between termination of parabiosis and iv booster injection		
		3 Weeks	3 Months	6 Months
Normal	Normal	−4[b]	n.d.[c]	−2
Immune[d]	Immune	37	n.d.	31
Normal	Immune	31	34	37
Immune	Normal	27	30	31

[a] Mice were iv injected with either 3×10^4 SRBC in 0.5 ml PBS or with 0.5 ml PBS alone, rested for 2–3 months, and joined for parabiosis with either normal or similarly primed mice. Four weeks later all pairs were separated, and at the indicated intervals the mice were injected with 3×10^5 SRBC iv in order to elicit a state of DTH. Two days later all mice were challenged and the subsequent DTH responses measured.

[b] Figures represent the mean response of the group of mice indicated by the left column. Each group consisted of five to six mice.

[c] Not determined.

[d] Immune mice were iv primed with 3×10^4 SRBC at least 2 months before joining for parabiosis.

Sensitivity of DTH-Related T Memory Cells to Treatment with Anti-Thymocyte Serum

Mice, primed with 3×10^4 SRBC iv 6–8 weeks previously, were injected sc with either 0.2 ml ATS or 0.2 ml NRS. These mice were boosted with 3×10^5 SRBC iv either 2 days or 3 weeks later and another 2 days later they were challenged. The ATS treatment could not prevent the development of secondary DTH responsiveness (Table 2). In a further experiment, mice, primed with 3×10^4 SRBC 8 weeks previously, were similarly treated with ATS or NRS. Ten days or 4 weeks later they were boosted sc instead of iv using an antigen dose of 1×10^7 SRBC, which is optimal for the sc route of immunization (data not shown). Another 2 days later the mice were challenged. The subsequent DTH reactions were significantly depressed in ATS-treated mice as compared to NRS-treated mice and resembled a primary DTH response to a sc immunization with 1×10^7 SRBC (Fig. 1A). Similarly primed but nonboosted mice were not capable of a significant DTH response 12 weeks after priming (Fig. 1B).

Effect of Antimitotic Agents on the Passive and Adoptive Transfer of Secondary DTH Reactivity

Mice primed with 3×10^4 SRBC iv and boosted 2 months later with 3×10^5 SRBC iv were iv injected with 100 μg vinblastine in 0.2 ml phosphate-buffered saline (PBS) either 1, 2, or 3 days after the booster injection. At 15 hr after injection of vinblastine, suspensions of the whole individual spleens were iv transferred into normal syngeneic recipients. These recipients were challenged with SRBC immediately after this passive transfer of T cells. The recipients of the spleen cells from vinblastine-treated mice did not show any DTH reactivity (Fig. 2). An iv injection of 100 mg/kg azathioprine at 48 and 24 hr before the passive transfer of the spleen cells also abolished DTH reactivity (Fig. 2).

To test whether the T cells accounting for secondary DTH are sensitive to antimitotic agents, mice, primed with 3×10^4 SRBC iv 8 weeks previously, were treated either by four daily iv injections of PBS or azathioprine (100 mg/kg), or by a single iv injection of vinblastine (100 μg/recipient), 15 hr before adoptive transfer. Spleen cells from these various groups of donor mice were iv injected into 900 R

TABLE 2

Sensitivity of DTH-Related T Memory Cells to Treatment with ATS

Treatment	Interval between treatment and iv booster injection[a]	
	2 Days	3 Weeks
NRS	41 ± 3[b]	35 ± 6
ATS	27 ± 2	36 ± 6

[a] Mice were iv primed with 3×10^4 SRBC 6–8 weeks before NRS or ATS treatment. Two days or 3 weeks later they were boosted iv with 3×10^5 SRBC. Challenge was performed 2 days after the booster injection.

[b] Figures represent mean 24 hr DTH responses ± 1 SEM ($n = 5$). In both groups DTH responses occurred at 24 hr after challenge.

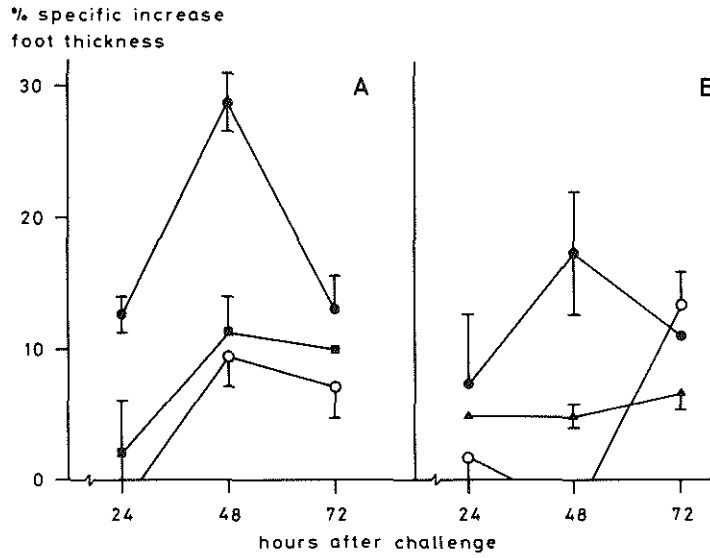

FIG. 1. The susceptibility of DTH-related T memory cells to *in vivo* treatment with ATS. Immune mice were sc treated with 0.2 ml ATS (■), or NRS (●). Ten days (A) or 4 weeks (B) later these mice were sc boosted with 1×10^7 SRBC. A group of nonimmune mice was immunized sc with 1×10^7 SRBC (○). Two days after the booster or immunization all mice were challenged. One group of immune mice was not boosted, but only challenged (▲). At 24, 48, and 72 hr after challenge the foot swelling was measured. Vertical bars represent 1 SEM ($n = 5$).

irradiated mice together with 3×10^6 bone marrow cells and 1×10^5 SRBC as controls were used irradiated mice iv injected with 3×10^6 bone marrow cells and 1×10^5 SRBC. At 4 days after transfer all recipients were challenged and the DTH reactivity was determined. It appeared that neither vinblastine nor azathioprine treatment did affect the scondary DTH responsiveness (Table 3).

DISCUSSION

Both iv and sc immunization of mice with SRBC in saline can induce a transient state of primary DTH to this antigen (7, 16–18). This DTH reactivity can be considered as a Jones–Mote-type of DTH (12, 17), although recently it was shown that histological and other features of the DTH reactivity induced by iv immunization with SRBC in saline are more similar to the tuberculin-type hypersensitivity (19). Convincing evidence exists that this transient state of DTH to SRBC is entirely mediated by functionally short-lived T effector cells (12, 18, 20). The lifespan of T effector cells in DTH reactivity induced by BCG or *Listeria monocytogenes* injection would depend on the interval between immunization and challenge (21–23). Shortly after priming with these antigens the DTH reactivity is dependent on short-lived, nonrecirculating T effector cells (21, 22), while it is assumed that a long-lived DTH-reactive effector cell population mediates the DTH reactivity to these antigens at longer intervals after immunization (21, 23). Persisting antigen or a high antigen dose would be required to drive a particular T cell subclass through a differentiation step in order to become such a long-lived cell (18).

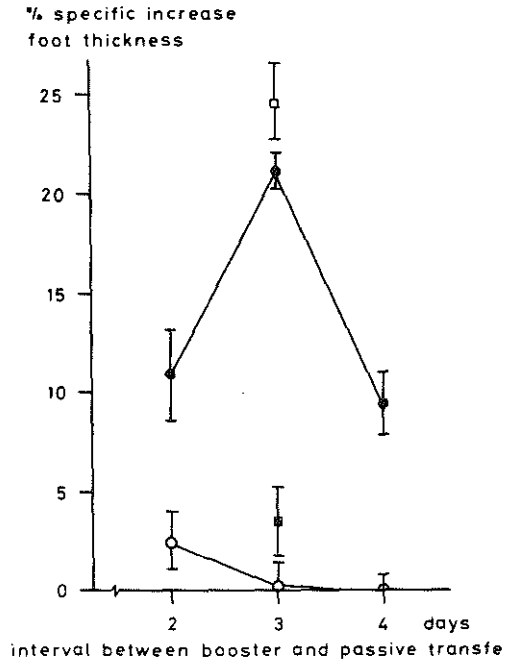

FIG. 2. The immunosuppressive action of vinblastine and azathioprine on the passive transfer of secondary DTH. Groups of donor mice were treated either 1, 2, or 3 days after their booster with a single injection of 100 μg of vinblastine (○) or PBS (●). At 15 hr after this treatment the individual spleens were transferred into normal syngeneic recipients. Two other groups of mice received two iv injections of either 100 mg/kg body wt azathioprine (■) or 0.25 ml PBS (□) on Days 1 and 2 after booster. At 24 hr after the last injection the individual spleens were transferred into normal syngeneic recipients. All recipients were challenged immediately after transfer and the 24-hr DTH response was determined. Vertical bars represent 1 SEM (n = 5).

In a previous paper we have shown that priming of mice with a low dose of SRBC in saline can induce T-cell memory for DTH to this antigen (1). Parabiosis of such SRBC primed and nonprimed mice results in an equal secondary DTH responsiveness in both groups of animals for at least 6 months after the termination of the parabiosis (Table 1). It is unlikely that dissemination of the antigen from the one partner, primed at least 8 weeks before parabiosis, to the other nonprimed partner accounts for the presence and propagation of such a high level of secondary DTH responsiveness in the nonprimed mice, since the mice were primed with a very low dose of SRBC, and SRBC are rapidly degraded in mice, thereby losing their immunogenicity (24, 25). This indicates that the T cells responsible for secondary DTH are long-lived, potentially circulating cells. Their resistance to treatment with vinblastine or azathioprine (Table 3) suggests that they do not or hardly proliferate in the absence of the specific antigen, just as has been proposed for DTH effector cells specific for purified protein derivative (21), *L. monocytogenes* (23), and DTH effector cells induced by extremely high doses of SRBC (11). Based upon the results presented in this paper we suggest an alternative explanation for the resistance of DTH reactivity to vinblastine in these latter situations. Under influence of small amounts of persisting antigen long-lived memory cells might develop into DTH effector cells without additional proliferation, or, alternatively, a proportion of the

TABLE 3

Susceptibility of DTH-Related T Memory Cells to Antimitotic Agents Tested in an Adoptive Transfer System[a]

Treatment of donor mice[b]	Peak DTH response (%)
PBS	51 ± 3[c]
Azathioprine	48 ± 4
PBS	56 ± 4
Vinblastine	51 ± 3

[a] Lethally irradiated recipients were injected iv with 3×10^6 normal bone marrow cells together with 5×10^7 immune spleen cells and 1×10^5 SRBC. Four days after transfer the recipients were challenged and the foot swelling was read on the subsequent day in order to determine the peak DTH response.

[b] Azathioprine was injected iv in a dose of 100 mg/kg body wt (on 4 consecutive days) into a group of three donor mice. Controls received PBS. The last injection was given at 24 hr before the adoptive transfer of the pooled spleen cells. A single iv injection of 100 μg vinblastine was given to three donor mice at 15 hr before the adoptive transfer of the pooled spleen cells. Controls received a single injection of PBS.

[c] Figures represent the mean peak response ± 1 SEM. Lethally irradiated recipients of 5×10^7 normal spleen cells, in addition to 3×10^6 normal bone marrow cells and 1×10^5 SRBC, showed a peak response of 26%.

long-lived memory cells might be in Gl-phase for a rather long time, before they give rise to DTH effector cells. After passive transfer these activated memory cells, which can not be affected by vinblastine treatment (26), may then further mature into DTH effector cells. Proliferation of DTH-reactive precursor cells is probably only required for optimal DTH responsiveness, as has been suggested by experiments on DTH reactivity to major histocompatibility antigens (27). This requirement for proliferative activity of precursors for DTH to SRBC might be due to the number of potentially reactive precursor cells, which is probably larger in case of histocompatibility antigens (28) and after infection with persistent antigens (29). In the case of secondary DTH to SRBC proliferation of memory cells seems to be essential for its expression (Fig. 2).

Labeling studies (30, 31) and functional studies (15) have shown that during parabiosis for a period of 4–5 weeks a nearly complete equilibrium of long-lived T and B cells can be established between both members. Whether an equilibrium of the DTH-related T memory cells indeed arises within this period, remains obscure, as it seems conceivable that at a certain level of DTH reactivity a further increase will be suppressed (32). This is in harmony with the finding that the secondary DTH response of parabionts consisting of one primed and one nonimmune member was as high as in parabionts consisting of two immune mice (Table 1).

It is generally thought that T memory cells performing helper functions in antibody formation, and those reactive to alloantigens belong to the recirculating pool of small lymphocytes (33, 34). Levey and Medawar (35) have shown that sc injected ATS acts on the recirculating lymphocytes, but not on the sessile cells in the lymphoid organs. Attempts to erase memory in order to restore the "virgin" state in primed mice have given conflicting results. Helper T memory cells are sensitive to ip injection of small doses of ATS (36), but abrogation of second set skin graft

rejection by ATS treatment has been difficult to achieve (37, 38). The finding that a single sc injection of a small dose of ATS did not prevent secondary DTH reactivity in mice iv boosted with SRBC (Table 2), suggests that a part of the DTH-related T memory population consists of sessile cells residing in the spleen. This parallels the observation that a part of the B memory cells is sessile or slowly recirculating and residing in the spleen (39). Subcutaneous boosting with SRBC of mice treated with ATS 4 weeks previously did not elicit a typical secondary DTH response, but only a primary type DTH reactivity (Fig. 1). Apparently sc treatment with ATS results in a long-lasting depletion of a subset of rapidly recirculating DTH-related memory cells.

The experiments reported here suggest that nonreplicating T memory cells give rise to DTH effector cells, *only* when restimulated by antigen. The susceptibility of the passive transfer of secondary DTH to treatment with antimitotic agents (Fig. 2) suggests that the development of secondary DTH to SRBC requires proliferation. Thus, the establishment of secondary DTH reactivity to SRBC is not fundamentally different from the situation in humoral immunity (40). In both cases the memory cells need to be restimulated by the antigen which initiated their generation, in order to become functionally active.

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T_1 and T_2 Lymphocytes in Primary and Secondary Delayed Type Hypersensitivity of Mice

I. Contribution in the Response to Sheep Red Blood Cells and to Allogeneic Spleen Cells

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Peripheral T lymphocytes can be subdivided into two populations (T_1 and T_2 cells) based upon the short life span of T_1 cells after adult thymectomy (ATx) and sensitivity of T_2 cells to treatment with anti-thymocyte serum (ATS) *in vivo*. The contribution of the T_1 and T_2 cells to primary and secondary delayed type hypersensitivity (DTH) to sheep red blood cells (SRBC) and to primary DTH to allogeneic cells was studied in mice. T_2 cells were found to account for the development of the state of primary DTH responsiveness after intravenous immunization with SRBC and after subcutaneous immunization with allogeneic cells. No clear cut evidence was found that in the presence of T_2 cells DTH related T effector cells were generated from T_1 cells. In mice selectively depleted for T_1 cells by ATx, the remaining T_2 cells were capable to generate SRBC-specific T memory cells, but not in numbers as large as in non-thymectomized mice. On the other hand, T_1 cells in mice depleted for T_2 cells by ATS treatment, could give rise to normal numbers of SRBC-specific T memory cells. Apparently T_1 cells can compensate for the absence of T_2 cells during generation of T memory cells, but T_2 cells cannot do so for the loss of T_1 cells. From the time curve showing the ATx-induced decline of the population of SRBC-specific T_2 cells, involved in primary DTH responsiveness, the half life was calculated to be 6 to 7 months.

INTRODUCTION

Both in cell mediated and humoral immunity T lymphocytes play an important role (1). Raff and Cantor proposed to subdivide T lymphocytes into two populations, namely T_1 and T_2 cells (2). This subdivision was indicated by studies of Cantor and Asofsky (3, 4), who showed synergism between peripheral blood lymphocytes and thymocytes, and between peripheral blood lymphocytes and anti-lymphocyte serum (ALS) treated spleen cells in the Graft-vs-Host (GvH) reaction. In these experiments the peripheral blood was used as a source of T_2 cells, and thymocytes and ALS treated spleen cells as a source of T_1 cells. Pretreatment of mice with ALS or anti-thymocyte serum (ATS) was found to enrich the spleen for T_1 cells by selective depletion of the pool of recirculating T_2 cells (5). Raff and Cantor suggested that T_1 cells are immature short-lived cells, sensitive to adult thymectomy (ATx), and that T_2 cells are mature long-lived cells, resistant to ATx.

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According to this proposal T_2 cells would be responsible for the primary type immune responses. These T_2 cells would have matured from the T_1 population under influence of environmental antigens (2) or independent of antigenic stimulation (6). Thus, primary immune responsiveness would be relatively ATx resistant. This thesis could be supported by previous studies on the effects of ATx on antibody formation (7–9) and on GvH reactions (7, 8). Later studies on T helper function in antibody formation (10–13) and skin graft rejection (14, 15) generally approved this model. On the other hand, some authors suggested that both T_1 cells and T_2 cells contributed to the primary T helper function (11, 12), and in one study, using cell mediated lympholysis (CML) as a test system, it appeared that the capacity for primary CML fell shortly after ATx (10). The effects of ATx on the capacity to respond in a mixed lymphocyte reaction (MLR) are rather conflicting (10, 16–19).

Concerning secondary type immunity, Raff and Cantor suggested that T memory formation would depend on the presence of T_1 cells. Priming for a secondary type immune response would imply an antigen-driven differentiation of T_1 cells into T_2 cells. Again evidence for this theory was mostly obtained from studies on T helper function in antibody formation (10, 11, 13). This proposal was recently modified by Araneo *et al.* (12), who demonstrated an involvement of both T_1 and T_2 cells in the formation of T memory cells for humoral immunity.

In a previous paper it was shown that priming of mice with SRBC can result in the formation of T effector cells with a short functional life span (20), as well as long-lived T memory cells. These latter cells can give rise to a state of secondary DTH upon a booster immunization.[1] This system was used to evaluate whether Raff and Cantor's original proposal also holds for primary and secondary type cell mediated immunity to SRBC and allogeneic cells.

MATERIALS AND METHODS

Mice. (C57BL/Rij × CBA/Rij) F1 and Balb/c female mice were used. The F1 mice were 6 or 12 weeks old, the Balb/c mice 12 to 15 weeks. They were purchased from the Medical Biological Laboratory, Rijswijk, The Netherlands, and the Laboratory Animals Centre of the Erasmus University, Rotterdam, The Netherlands.

Antigen and immunization. Sheep red blood cells (SRBC) were commercially obtained, stored and washed as described previously (20). Primary and secondary immunization with SRBC was performed by intravenous (iv) injection of the SRBC in 0.5 ml saline. To induce DTH to histocompatibility antigens, mice were sc immunized with 1×10^7 or 5×10^7 Balb/c spleen cells in 0.1 ml saline, equally distributed over both inguinal areas. The challenge dose of either 5×10^7 SRBC or 1×10^7 Balb/c spleen cells was administered subcutaneously (sc) in the instep of the right hind foot.

Adult thymectomy. Adult thymectomy (ATx) and sham-thymectomy (ShTx) was always performed, when the mice were 6 weeks old. The surgery was done as described by Miller (21). The mice were anesthetized by 70 mg/kg body weight Nembutal (S. A. Abbott, Saint-Rémy-sur-Avre, France).

Thymus transplantation. The restorative effects of thymus transplantation on

[1] Kwast, Th.H. van der, H. de Ruiter, and R. Benner. Secondary delayed type hypersensitivity to sheep red blood cells in mice. Its dependence on long-lived memory cells. Submitted for publication.

DTH in ATx mice was investigated by transplantation of a single thymus lobe under the kidney capsule of mice, thymectomized 6 months previously. Thymus lobes were obtained from 300 rad irradiated syngeneic neonatal mice, within 24 hr after birth. The recipient mice were anesthetized by Nembutal. Irradiation of the neonatal mice was done as described previously (22).

Treatment with anti-thymocyte serum. ATS was prepared according to the method of Jooste *et al.* (23) by two injections of New Zealand White rabbits with 5×10^8 Balb/c thymocytes, with an interval of 2 weeks. One week after the second injection the rabbits were bled. For elimination of T_2 cells a total volume of 0.2 ml unabsorbed antiserum was administered sc on the back, in the arm-pits, and the groins.

Assay for Delayed Type Hypersensitivity. DTH reactions were determined by measuring the differences in thickness of the hind feet 24 hr after sc injection of either 5×10^7 SRBC or 1×10^7 Balb/c spleen cells into the instep of the right hind foot. Details of this assay have been given in a previous paper (20). A control group consisting of non-immune mice challenged in the same way as the mice to be tested, was always included. The specific increase in foot thickness was calculated as the relative increase in foot thickness of the immune mice, minus the relative in-

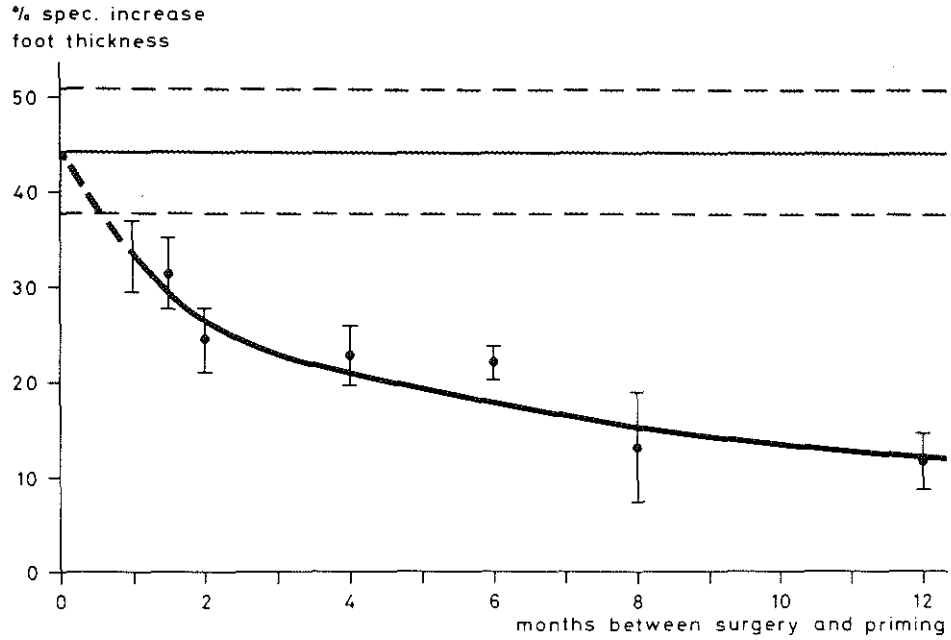

FIG. 1. Effect of ATx upon primary DTH responsiveness to SRBC. Six week old mice were thymectomized or sham-thymectomized and immunized with 3×10^6 SRBC iv at various times after surgery. Another 3, 4, and 5 days later different groups of mice were challenged with 5×10^7 SRBC sc into the instep of the right hind leg, and the 24 hr DTH responses were measured. Only the peak responses of both groups of mice are given. Generally these peaks were elicited on the 4th day after immunization. Hatched area represents the mean responses of the sham-thymectomized mice $\pm$ 1 SD. Closed circles represent the responses of the ATx mice. Each figure is the arithmetic mean of the response of 5 to 15 mice. Vertical bars represent 1 SEM. The decline of primary DTH responsiveness can be described by the reverse exponential function $y = 33.4 - 8.5 \ln x$. $r^2 = 0.91$.

crease in foot thickness of the control mice. The swelling in control mice varied between 12 and 20% for SRBC, and between 25 and 28% for Balb/c spleen cells.

RESULTS

Primary DTH Responsiveness to SRBC at Different Intervals After Adult Thymectomy

To investigate the lifespan of the T cells which give rise to primary type DTH responsiveness, mice were thymectomized or sham-thymectomized at 6 weeks of age. At different intervals after surgery groups of mice were immunized with 3×10^5 SRBC iv, which is an optimal dose for induction of primary DTH reactivity. These mice were challenged 2, 3, 4, or 5 days after priming in order to determine the peak response as the parameter for the height of primary type DTH responsiveness. Independent of the interval between thymectomy and immunization the first DTH reactivity could be demonstrated on Day 3, while the peak response was generally found to occur on Day 4. Up to 1 year after surgery the primary DTH responsiveness remained at the same level in ShTx mice. In ATx mice a gradual decline of primary DTH responsiveness was observed (Fig. 1). Two functions can describe this decline, namely a reverse exponential function ($y = 33.4 - 8.5 \ln x$; $r^2 = 0.91$), and a linear function ($y = 32.1 - 1.9x$; $r^2 = 0.87$). The first function suggests an at random inactivation of a single long-lived population, whereas the second function indicates the participation of two populations of T cells in primary DTH responsiveness, one being short-lived and one being long-lived. In this latter case the contribution of the short-lived T_1 cells could be calculated to be about 25%.

Since ATx might affect the dose-response relationship, groups of mice were primed

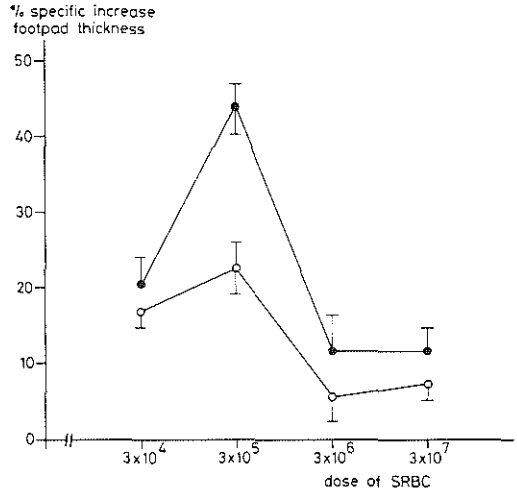

FIG. 2. Effect of ATx upon the primary DTH responsiveness to various doses of SRBC. Six week old mice were thymectomized or sham-thymectomized, and immunized with various doses of SRBC iv 6 months later. Another 3, 4, and 5 days later different groups of mice were challenged and the 24 hr DTH responses were measured. Only the peak responses of both groups of mice are given. These peaks were elicited on the 4th day after immunization. (●) Sham-thymectomized mice, and (○) thymectomized mice. Each figure is the arithmetic mean of the response of 5 mice. Vertical bars represent 1 SEM.

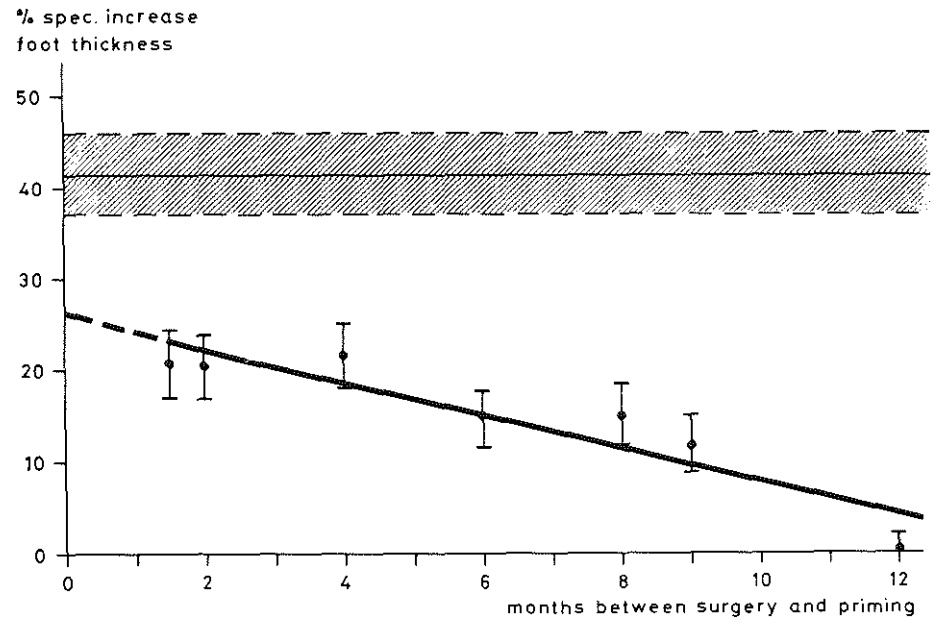

FIG. 3. Effect of ATx upon the generation of memory cells required for secondary DTH responsiveness to SRBC. Six week old mice were thymectomized or sham-thymectomized, and primed with 3×10^4 SRBC iv at various times after surgery. At 6 to 8 weeks after this optimal dose the mice were boosted with 3×10^6 SRBC iv. Another 2 days later these mice were challenged, and the 24 hr DTH responses were measured. Hatched area represents the mean secondary DTH responses of the sham-thymectomized mice ± 1 SD. Closed circles represent the responses of ATx mice. Each figure is the arithmetic mean of 5 to 10 mice. Vertical bars represent 1 SEM. From 6 weeks after ATx onwards, the decline of the capacity to generate DTH-related T memory cells might be described by the function $y = 25.9 - 1.8x$. $r^2 = 0.84$.

with either 3×10^4, 3×10^5, 3×10^6, or 3×10^7 SRBC 6 months after ATx and ShTx. Four days after immunization the peak primary DTH reactivity was determined. For both groups of mice the dose of 3×10^5 SRBC appeared to be optimal (Fig. 2). ATx apparently did not affect the suppression which normally occurs after iv immunization with higher doses of SRBC.

Decline of the Capacity to Generate DTH Related T Memory Cells After Adult Thymectomy

The lifespan of the precursors of the T memory cells which account for secondary type DTH responsiveness, was investigated in mice iv primed with 3×10^4 SRBC at varying intervals after ATx or ShTx. At 6 to 8 weeks after injection of this optimal dose (22) the mice were boosted with 3×10^5 SRBC iv. The challenge dose was administered 2 days later, since the response measured on the second day after the booster injection has been shown to be a good parameter for the secondary DTH responsiveness (22). No significant decline of the capacity to generate T memory cells for secondary DTH responsiveness was observed up to 1 year after ShTx, whereas in ATx mice, already 6 weeks after surgery, a greatly diminished capacity of secondary DTH responsiveness was found (Fig. 3). The subseqent decline of the capacity to generate T memory cells for secondary DTH reactivity was rather gradual.

TABLE 1

Recovery of the Primary and Secondary DTH Responsiveness in Adult Thymectomized Mice After Thymus Transplantation

Surgery[a]	Primary response	Secondary response
ShTx + Thym	49.3 ± 6.6[b]	37.7 ± 4.5
ATx + Thym	37.0 ± 11.2	33.1 ± 4.0
ATx + Sh-Thym	14.3 ± 4.6	12.1 ± 3.0

[a] Thymus transplantation (Thym) or sham-transplantation (Sh-Thym) was performed at 6 months after thymectomy of adult mice. Three months later these mice were primed either for primary DTH (3×10^5 SRBC) or for induction of T memory cells for secondary DTH (3×10^4 SRBC). At 3, 4, or 5 days after immunization for primary DTH these mice were challenged. Only the peak responses of these mice are given. These peaks were elicited on the 4th day after immunization. At 2 months after priming for secondary DTH the mice were boosted with 3×10^5 SRBC iv, and challenged 2 days later. The 24 hr DTH responses are given.

[b] Figures represent the mean response ±1 SEM of 4 or 5 mice.

The Restorative Capacity of Thymus Transplantation on DTH in Adult Thymectomized Mice

It was studied whether the decline of the capacity of primary and secondary DTH responsiveness after ATx is a reversible phenomenon. Therefore, 300 rad irradiated syngeneic neonatal thymus lobes were transplanted under the kidney capsule of ATx and ShTx mice, 6 months after surgery. At 14 days and 3 months after transplantation or sham-transplantation the different groups of mice were immunized with 3×10^5 SRBC iv. Three, 4 and 5 days later the DTH responsiveness was determined. At 14 days after thymus transplantation no recovery of the primary DTH responsiveness was found (data not shown), whereas 3 months after thymus transplantation the mice were capable of an almost normal DTH responsiveness (Table 1). These results suggest that the decrease of primary DTH responsiveness after ATx is due to the loss of T cells and not to a lack of thymic hormones. Thymus transplantation also almost completely restored the capacity of generation of T memory cells in ATx mice, when assayed 3 months after transplantation.

TABLE 2

Primary DTH Responsiveness After Depletion of T_2 Cells by ATS Treatment

Protocol	% specific increase foot thickness
NRS $\xrightarrow{2\text{ days}}$ 3×10^5 SRBC iv $\xrightarrow{4\text{ days}}$ challenge	37.7 ± 3.5[a]
ATS $\xrightarrow{2\text{ days}}$ 3×10^5 SRBC iv $\xrightarrow{4\text{ days}}$ challenge	7.8 ± 0.5
ATS $\xrightarrow{3\text{ days}}$ 3×10^5 SRBC iv $\xrightarrow{4\text{ days}}$ challenge	25.7 ± 4.9

[a] Figures represent the mean ± 1 SEM of 5 mice.

TABLE 3

Thymic Dependence of the Recovery of Primary DTH Responsiveness After Depletion of T_2 Cells by ATS Treatment

Protocol	% specific increase foot thickness
NRS $\xrightarrow{3\text{ days}}$ NRS $\xrightarrow{3\text{ weeks}}$ 3×10^5 SRBC iv $\xrightarrow{4\text{ days}}$ challenge	51.6 ± 2.9[a]
ATS $\xrightarrow{3\text{ days}}$ ATS $\xrightarrow{3\text{ weeks}}$ 3×10^5 SRBC iv $\xrightarrow{4\text{ days}}$ challenge	41.9 ± 4.7
ATx $\xrightarrow{6\text{ weeks}}$ ATS $\xrightarrow{3\text{ days}}$ ATS $\xrightarrow{3\text{ days}}$ 3×10^5 SRBC iv $\xrightarrow{4\text{ days}}$ challenge	−1.6 ± 2.2
ATx $\xrightarrow{6\text{ weeks}}$ ATS $\xrightarrow{3\text{ days}}$ ATS $\xrightarrow{3\text{ weeks}}$ 3×10^5 SRBC iv $\xrightarrow{4\text{ days}}$ challenge	5.8 ± 3.1

[a] Figures represent the mean ± 1 SEM of 5 mice.

Effect of Selective depletion of T_2 cells by ATS-treatment on primary and secondary DTH responsiveness

The role of T_2 cells in primary and secondary DTH responsiveness was investigated by sc injection of mice with 0.2 ml ATS. Treatment of normal mice with a single dose of 0.2 ml ATS sc, 2 days before iv immunization with SRBC, largely abolished their capacity of primary DTH responsiveness. However, 3 days after ATS-treatment the capacity of primary DTH responsiveness had been largely recovered (Table 2). Mice, thymectomized 6 weeks previously, and treated by two sc injections of 0.2 ml ATS were found to be incapable of recovery of primary DTH responsiveness, even when tested 3 weeks after treatment with ATS (Table 3). Thus, the presence of the thymus is required for recovery of primary DTH responsiveness in ATS treated mice.

In another experiment the effect of selective elimination of T_2 cells was investigated for the capacity to generate DTH related T memory cells. Therefore, mice were primed with 3×10^4 SRBC iv 2 days after treatment with ATS. Three weeks later the mice were boosted with 3×10^5 SRBC iv, and challenged another 2 days later. It was found that the secondary DTH responsiveness in the ATS treated mice was equally high as in non-treated control mice (Table 4).

Effect of Adult Thymectomy on Primary DTH Responsiveness to Allogeneic Spleen Cells

Groups of ATx and ShTx mice were sc immunized with either 1×10^7 or 5×10^7 Balb/c spleen cells, 1 month or 7 months after surgery. Challenge was performed

TABLE 4

Capacity of Memory Induction in Mice Depleted of T_2 Cells by ATS Treatment

Protocol	% specific increase foot thickness
3×10^4 SRBC iv $\xrightarrow{3\text{ weeks}}$ 3×10^5 SRBC iv $\xrightarrow{2\text{ days}}$ challenge	39.6 ± 2.1[a]
ATS[b] $\xrightarrow{2\text{ days}}$ 3×10^4 SRBC iv $\xrightarrow{3\text{ weeks}}$ 3×10^5 SRBC iv $\xrightarrow{2\text{ days}}$ challenge	44.3 ± 3.5

[a] Figures represent the mean ±1 SEM of 5 mice.

[b] The same dose of ATS administered 2 days before immunization with 3×10^5 SRBC iv reduced the 4th day primary DTH response from 37.7 to 7.8% (Table 1).

TABLE 5

Effect of Adult Thymectomy on Primary DTH Responsiveness to Allogeneic Spleen Cells

Interval[a]	Immunization dose	Surgery	
		ShTx	ATx
1 month	1×10^7	22.2 ± 3.2[b]	16.8 ± 3.6
7 months	1×10^7	23.6 ± 4.5	23.7 ± 2.9
7 months	5×10^7	31.8 ± 1.5	25.8 ± 2.5

[a] The interval between surgery and immunization has been given.
[b] Figures represent the mean ± 1 SEM of 5 mice.

on these mice 6 days after immunization, since at this interval the DTH reactivity appeared to be maximal (data not shown). It was found that ATx hardly affected the primary DTH responsiveness to allogeneic spleen cells, both after priming with a suboptimal (1×10^7) and an optimal (5×10^7) dose of Balb/c spleen cells (Table 5).

DISCUSSION

In studies on the influence of ATx on the capacity of GvH reactivity (7, 8), T helper function in antibody formation (7–13), and skin graft rejection (14, 15), it was generally found that primary immune responsiveness gradually declined after ATx. For DTH Kappler *et al.* (11) reported a rapid initial fall of the capacity of primary DTH responsiveness to SRBC after ATx, but other authors found an increase in DTH responsiveness to methylated human serum albumin after ATx (24). The explanation for these contradictory results probably is, that the suppression of DTH responsiveness to the latter antigen, in contrast to SRBC (Fig. 2), is regulated mainly by short-lived T cells. The experiments reported here show that primary DTH responsiveness to SRBC declines according to a reverse exponential function, namely, $y = 33.4 - 8.5 \ln x$ (Fig. 1). In such a function only one long-lived population of T lymphocytes needs to be hypothetized to explain the decline of the capacity of primary DTH after ATx. This is in agreement with the original proposal of Raff and Cantor (2), who suggested that the primary immune response is completely dependent on long-lived T_2 cells. The reverse exponential decline indicates an at random inactivation of T_2 cells. From this function ($y = 33.4 - 8.5 \ln x$) the maximal lifespan of the population of T_2 cells specific for SRBC can be extrapolated to be approximately 50 months. The half life of these cells can be calculated to be about 6 to 7 months. From the decline of the capacity to induce DTH related T cell memory in ATx mice, it can be concluded that the long-lived T cells can also contribute to T memory formation.

The observed gradual decline of primary DTH responsiveness to SRBC after ATx is at variance with the data published by Kappler *et al.* (11), who found a rapid decrease of the primary DTH responsiveness. This difference is probably due to the rather young age of 4 to 5 weeks that these authors thymectomized their mice. Our data were obtained with mice which were thymectomized at an age of 6 weeks when the capacity of DTH-reactivity to SRBC had been fully developed (25).

The rapid initial fall of the capacity of secondary DTH responsiveness after ATx

(Fig. 3) suggests that T_1 cells are required for the production of long-lived T memory cells in numbers comparable to normal mice. These latter cells were suggested to belong to the T_2 cell pool [1] (26). The slow further decrease of the capacity to generate DTH related T memory cells after ATx, subsequent to the rapid initial fall (Fig. 3), suggests that T_2 cells can also give rise to T memory cells. Presumably, there is an additive contribution of short-lived and long-lived precursors in the formation of DTH related T memory cells for SRBC, although it cannot be excluded theoretically that T_1 cells have an amplifying effect on the T_2 cells. These data lead us to modify Raff and Cantor's original concept on T memory formation (2), and to suggest that both T_1 and T_2 cells can give rise to T memory cells for DTH. Recently, it has been shown that this also holds for humoral immunity (12). We extend this concept by suggesting that the proportion in which T_1 cells and T_2 cells contribute to T memory development is *only* determined by the number of antigen-specific T_2 cells already present in the animal. This number of T_2 cells may be dependent on the type of antigen chosen for immunization, the environmental antigens of the individual, and on the level of "spontaneous" conversion of T_1 cells into T_2 cells. This "spontaneous" conversion may be either fully antigen-independent, or due to self recognition (6). In addition we postulate that T_2 cells can give rise to new T_2 cells without deliberate immunization. From Fig. 1 it appears that for the antigen SRBC, this occurs at a rate too small to compensate for the natural decline of the pool of T_2 cells. The calculated half life of the SRBC specific T_2 cell population of about 7 months, would then be an overestimation. This can explain the discrepancy between this data and the half life of 4 to 6 months calculated from experiments in which long lived T cells are labeled by means of [^{3}H]-thymidine (27).

From the effect of ATx (Fig. 2) and ATS (Table 2) upon primary DTH responsiveness to SRBC, it can be concluded that in normal mice the conversion of T_1 cells into T_2 cells occurs at a large scale without intentional immunization. This mechanism fully compensates for the natural decline of the T_2 cell population (Fig. 1) up to at least 1 year of life, and for the depletion of the T_2 cell pool by ATS-treatment (Table 2). However, the capacity of primary DTH responsiveness to histocompatibility antigens is not or only slightly influenced by ATx (Table 5), and can thus be maintained independent of T_1 cells. A spontaneous clonal expansion of the population of T_2 cells might account for this observation but it is also conceivable that the DTH reactivity to histocompatibility antigens is not *per se* linearly related to the number of reactive T_2 cells. Possibly for these antigens the number of T_2 cells is larger than required for maximal DTH responsiveness, so that a decline of the number of T_2 cells will not be directly reflected in the height of the DTH response.

In conclusion, these *in vivo* experiments on DTH suggest that (a) DTH related T effector cells (generated after primary immunization with SRBC and histocompatibility antigens) are derived from T_2 cells, (b) T_2 cells can give rise to DTH related T memory cells, similarly as has been shown recently for T helper memory (12), and (c) T_1 cells can supplement this contribution of T_2 cells.

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PRIMARY AND SECONDARY DELAYED TYPE HYPERSENSITIVITY TO MINOR HISTOCOMPATIBILITY ANTIGENS IN THE MOUSE

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SUMMARY

Immunization of mice with viable allogeneic H-2 compatible spleen cells can induce a persistent state of delayed type hypersensitivity (DTH) to these alloantigens, as measured with the footpad swelling test. Boosting of such mice, 2-4 months after priming, induced a typical secondary type DTH reactivity. The capacity of secondary DTH to non H-2 alloantigens could be adoptively transferred from primed mice into irradiated syngeneic hosts by means of nylon-wool non-adherent, Thy-1.2$^+$ spleen cells. Vinblastine treatment of the donor mice did not affect the adoptive DTH responsiveness. These results suggest that a population of long-lived T memory cells contributes to secondary type DTH responsiveness to non H-2 alloantigens. The phenomenon of persistent DTH is discussed in the light of these results. The hypothesis is put forward that persistent DTH is dependent on the continuous antigen-driven differentiation of long-lived, recirculating T memory cells into non-recirculating, functionally short-lived DTH effector cells.

INTRODUCTION

Allogeneic skin grafts or tumor grafts can induce a state of delayed type hypersensitivity (DTH) to histocompatibility (H) antigens in many species, including mice (1). In hamsters (2) and mice (3) subcutaneous (s.c.) injection of viable allogeneic lymphoid cells can also evoke DTH reactivity to H-antigens. Histologically the reaction which is elicited in immune mice by the s.c. injection of intact lymphoid cells, crude membrane extracts or soluble antigens into the footpad appears to be of a typical DTH type (4,5). Kon and Klein (5) showed that such a DTH assay can detect both H-2 and non H-2 alloantigens in mice immunized by a H-2 or non H-2 incompatible skin graft, respectively. Earlier studies of Brent et al. (4) indicated that transplantation of allogeneic skin in guinea-pigs induced a state of DTH, which could also be demonstrated at long intervals after the rejection of the skin. These authors ascribed this phenomenon to a long-lived effector cell population. These cells were suggested to be responsible for the accelerated rejection of a second allogeneic graft as well. However, the contribution of long-lived memory cells to both the

persistent state of DTH and the second set reaction was not excluded.

Painting of mice with contact chemicals (6), injection of mycobacteria (7) or of antigens emulsified in Freund complete adjuvant (FCA) (6,8), can also induce a stable and persistent state of DTH to the specific antigen. Boosting of such primed mice generally results in the occurrence of an anamnestic or secondary DTH (6,7,9). This latter phenomenon is characterized by an initial disappearance of DTH reactivity followed by a rapid rise till above the level of DTH reactivity previous to the booster injection (7,9). Secondary DTH could also be demonstrated in mice, primed intravenously (i.v.) with a low dose of sheep red blood cells (SRBC) suspended in saline (10). The secondary DTH reactivity to SRBC was shown to be dependent on the presence of non-proliferating, long-lived T memory cells which can persist in the absence of antigen (11).

In this paper we describe the inducibility and kinetics of primary and secondary type DTH reactivity to minor H-antigens in the mouse. Furthermore, this paper deals with the nature and specificity of the DTH related memory cells induced by priming with multiple minor H-antigens.

MATERIALS AND METHODS

Mice

Female BALB/c (H-2^d), DBA/2 (H-2^d), C57BL/LiA (H-2^{be}) and (C57BL/LiA x C3Hf) F_1 (H-$2^{be/k}$) mice were purchased from the Radiobiological Institute TNO, Rijswijk, The Netherlands. Female C3Hf (H-2^k) and (C57BL/LiA x CBA/Rij) F_1 (H-$2^{be/q}$) mice were obtained from the Laboratory Animals Centre of the Erasmus University, Rotterdam, The Netherlands. Female AKR/FuRdA (H-2^k), (AKR/FuRdA x C3Hf) F_1 (H-$2^{k/k}$) and (BALB/c x C3Hf) F_1 (H-$2^{d/k}$) mice were bred at our department. The age of all mice ranged between 12 and 20 weeks.

Preparation of cell suspensions

Spleens were removed, brought into a balanced salt solution (BSS) and squeezed through a nylon gauze filter to give a single cell suspension. Bone marrow was collected from femora and tibiae, and a single cell suspension of bone marrow cells in BSS was prepared as described previously (12). Nucleated cells were counted with a Coulter Counter Model B. Viable nucleated cells were counted in a haemocytometer using 0.2% trypan blue in BSS as a diluent.

Antigen and immunization

Primary and secondary immunization was performed with the appropriate allogeneic spleen cells, suspended in a volume of 0.1 ml. The priming and booster dose was 1 x 10^7 spleen cells, unless stated otherwise. A total volume of 50 µl of this spleen cell suspension was s.c. injected by means of a 28 gauge needle in each of both inguinal areas. Ultrasonic disruption of spleen cells suspended in BSS was performed in an ice bath with a MSE 100 W ultrasonic desintegrator, 20 kc/sec, for 3 bursts of 30 sec with an interval of 30 sec. Skin grafting was performed according to a modification of the method of Billingham and Silvers (13). The skin was removed aseptically from the donor and freed of the panniculus carnosus by cautious scraping with a scalpel. The recipient was anaesthetized with Hypnorm (Philips Duphar BV, Amsterdam, The Netherlands) and the panniculus carnosus of the graft bed was kept undamaged while removing the back skin.

Selective depletion of T lymphocytes or B lymphocytes

The method described by Julius et al. (14) for the depletion of B lymphocytes was used. After incubation for 45 min at 37°C on prewashed nylon wool (Fenwal Laboratories, Morton, Grove, Illinois) approximately 90% of the recovered lymphoid cells were T lymphocytes as shown by indirect membrane fluorescence staining using a specific rabbit anti-mouse thymocyte serum and a FITC-conjugated goat anti-rabbit immunoglobulin antiserum (15). Anti-Thy 1.2 treatment to eliminate T cells was performed as described in a previous paper of our laboratory (12).

Irradiation

The recipient mice received 800 rad of whole body irradiation as described in a previous paper (10). The recipient mice were i.v. injected with the appropriate number of spleen cells and 3 x 10^6 bone marrow cells suspended in 0.5 ml BSS within 4 hr after irradiation.

Vinblastine treatment

At 24 hr and 12 hr before adoptive transfer Vinblastine sulphate (Eli Lilli & Co., Indianapolis, Ind.) was i.v. injected in a dose of 100 µg/recipient, dissolved in a volume of 0.1 ml sterile saline. This dose is sufficient to abolish passive transfer of DTH reactivity (11).

Assay for delayed type hypersensitivity

DTH reactions were determined by measuring the difference in thickness of the hind feet 24 hr after s.c. injection of 8 x 10^6 spleen

cells in the instep of the right hind leg. In adoptive transfer experiments the increase in foot thickness was also measured 48, 72, 96 and 120 hr after challenge. The challenge dose was administered s.c. in a volume of 20 µl by means of a 28 gauge needle. The thickness of the left and right hind foot was measured with a footpad meter as described by Bonta and De Vos (16), with some minor modifications. During measurement the mice were anaesthetized with ether.
A control group, consisting of non-immune mice challenged with the same spleen cells as the mice to be tested, was always included. 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 control mice ranged between 15 and 28%.

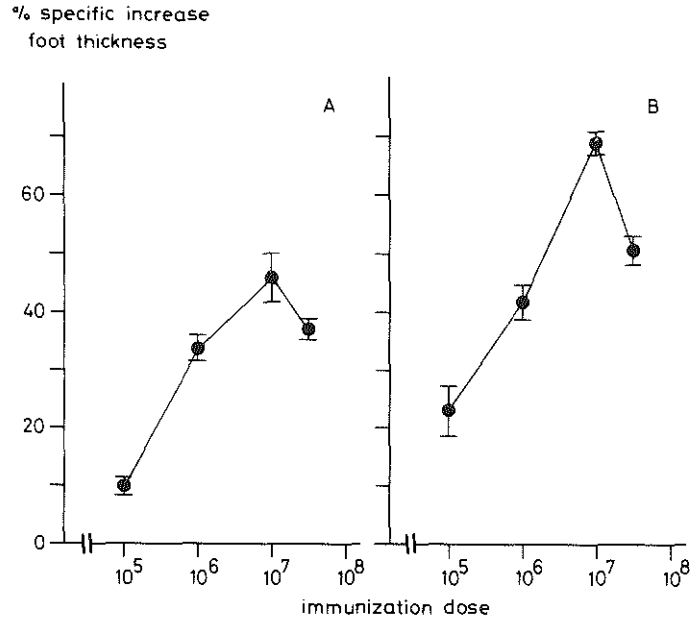

Fig. 1
Dose-response curve for primary DTH to multiple non H-2 alloantigens. C3Hf mice were injected s.c. with varying doses of AKR spleen cells and challenged on Day 5 after immunization (A). DBA/2 mice were similarly immunized with varying doses of BALB/c spleen cells and challenged on Day 5 (B). Vertical bars represent 1 SEM (n = 5).

RESULTS

Dose-dependence of the primary DTH response to multiple non H-2 alloantigens

C3Hf mice were immunized s.c. with either 10^5, 10^6, 10^7 or 3 x 10^7 viable allogeneic H-2 compatible AKR spleen cells and challenged with AKR spleen cells by s.c. injection into the right hind leg 5 days later. DBA/2 mice were similarly immunized with different doses of viable H-2 compatible BALB/c spleen cells, and challenged with BALB/c spleen cells 5 days later. In both mouse strain combinations the maximal 24 hr DTH response was found after immunization with 1 x 10^7 allogeneic H-2 compatible spleen cells (Fig. 1).

Induction of primary and secondary DTH responsiveness to multiple non H-2 alloantigens by injection of viable spleen cells

To investigate the kinetics of primary and secondary DTH reactivity to multiple non H-2 alloantigens, groups of BALB/c, DBA/2 and C3Hf mice were s.c. immunized for primary DTH, or primed and boosted for secondary DTH by injection of DBA/2, BALB/c and AKR spleen cells, respectively. All immunizations were done by s.c. injection of 1 x 10^7 viable nucleated spleen cells. The interval between priming and boosting for secondary DTH was at least 3 months. At varying intervals after primary immunization or booster injection groups of mice were challenged with the specific antigen. In all three strain combinations it appeared that the course of the secondary DTH response differed considerably from the primary DTH response (Fig. 2A, B and C). Primary DTH reactivity to non H-2 alloantigens became detectable from Day 3 onwards, with a clear peak reactivity on Day 5 after immunization. Thereafter the level of primary DTH reactivity declined to reach a plateau level which persisted for at least 3 months (points not shown in figures). The secondary DTH responsiveness had similar characteristics for all three mouse strain combinations. At 24 hr after booster injection the DTH reactivity had disappeared, but by Day 3 after booster the DTH response of the boosted mice reached its peak and surpassed the level of pre-existing DTH in primed mice.
To test whether a similar difference exists in primary and secondary DTH to H-2 incompatible spleen cells, C3Hf mice were immunized for primary and secondary DTH with the dose of 1 x 10^7 (BALB/c x C3Hf) F_1 spleen cells. At varying intervals after primary and secondary immunization different groups of mice were challenged with (BALB/c x C3Hf) F_1 spleen cells and the DTH reaction was determined. The kinetics of the primary and secondary DTH response to these H-2 incompatible cells did not differ to the same extent as observed for allogeneic H-2 compatible spleen cells. Furthermore, the kinetics of the response to H-2 incompatible spleen cells was

different from that to allogeneic H-2 compatible spleen cells. The most striking difference was the slow development of DTH reactivity and the lower peak reactivity attained in case of immunization with H-2 incompatible spleen cells (Fig. 2D).

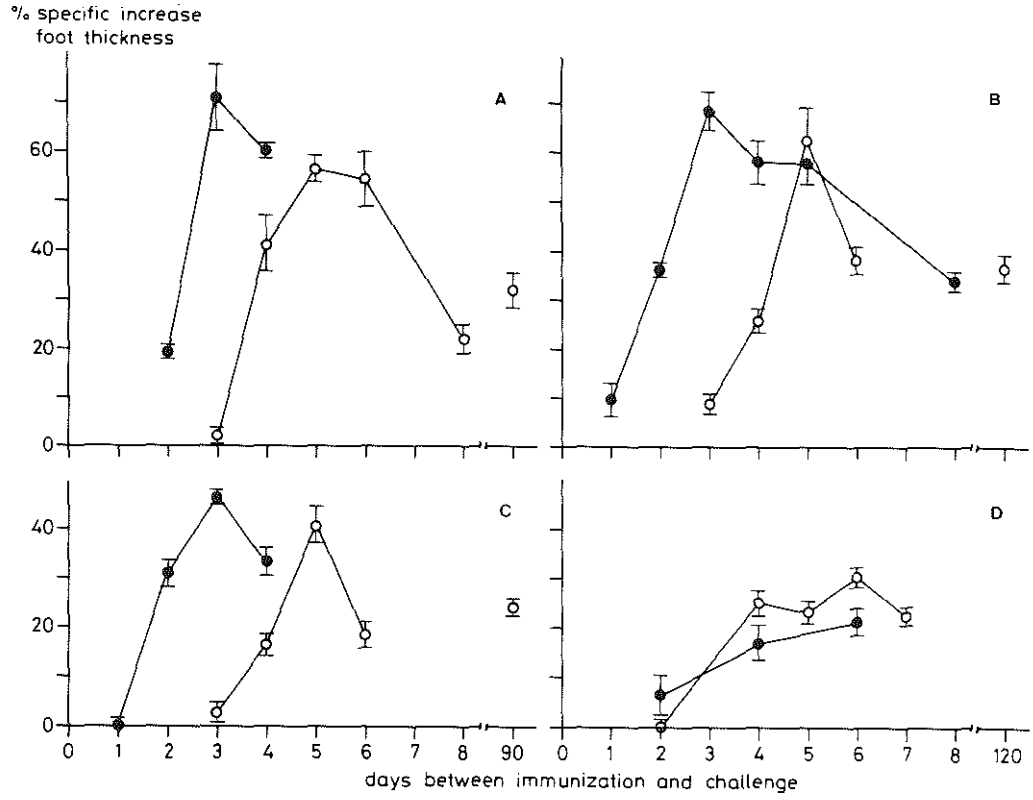

Fig. 2
Primary and secondary DTH responsiveness to allogeneic H-2 compatible or H-2 incompatible spleen cells. The time course of the primary DTH (○) and secondary DTH (●) response of DBA/2 mice to H-2 compatible BALB/c spleen cells (A), of BALB/c mice to H-2 compatible DBA/2 spleen cells (B), of C3Hf mice to either H-2 compatible AKR spleen cells (C) or to H-2 incompatible (BALB/c x C3Hf)F_1 spleen cells (D) was followed. In all strain combinations the priming and booster dose was 1 x 10^7 viable spleen cells. The persistent primary DTH reactivity at the moment of booster, at least 3 months after priming, was also determined. Vertical bars represent 1 SEM (n = 5).

Priming for secondary DTH to multiple non H-2 alloantigens by injection of crude membranes or skin grafting

To exclude a role of persisting viable spleen cells in the ability of primed mice to respond with a secondary type DTH response, C3Hf mice were either grafted with dermis obtained from AKR mice or injected s.c. with ultrasonically disrupted AKR spleen cells. Primary DTH responsiveness to viable and ultrasonically disrupted AKR spleen cells was determined at varying intervals after the immunization. The ultrasonically disrupted spleen cells gave rise to very weak DTH reactivity (Table 1). Booster injection with 1×10^7 viable AKR spleen cells caused a clear secondary DTH response in both cases, as measured on Day 3 after booster (Table 1).

TABLE 1

INDUCTION OF DTH-RELATED MEMORY IN C3Hf MICE BY AKR SKIN GRAFTING OR INJECTION WITH ULTRASONICALLY DISRUPTED AKR SPLEEN CELLS

Priming	Interval	Booster[a]	day of challenge	specific DTH response
Viable cells[a]	-	-	3	6.5 ± 2.0
Skin graft	90 days	viable cells	3	31.1 ± 4.0
U.D. cells[b]	-	-	5[c]	9.8 ± 1.0
U.D. cells	-	-	24	6.5 ± 2.7
Viable cells	-	-	24	33.7 ± 2.0
U.D. cells	21 days	viable cells	3	30.7 ± 1.8
U.D. cells	90 days	viable cells	3	45.7 ± 2.9

a. The priming dose and booster dose of viable cells was always 1×10^7 spleen cells.

b. The dose of ultrasonically disrupted (U.D.) spleen cells was equivalent to 1×10^7 viable cells.

c. The DTH reactivity after immunization with U.D. cells is shown on the 5th day when maximal responsiveness was found.

Primary and secondary DTH responsiveness to the male specific H-Y antigen

To study the capacity of primary and secondary DTH to a single non H-2 antigen the H-Y antigen was chosen. Therefore (C57BL x CBA) F_1 female mice were s.c. immunized for primary and secondary DTH by 1×10^7 syngeneic male spleen cells. At varying intervals after primary and secondary immunization various groups of mice were challenged with syngeneic male spleen cells, and the 24 hr DTH reaction was measured. It appeared that a single immunization with the male specific H-Y antigen could induce DTH effector cells (Fig. 3). The difference in time course of the primary and secondary DTH reactivity to H-Y antigen revealed that s.c. priming with male spleen cells in addition induced DTH related memory for the H-Y antigen.

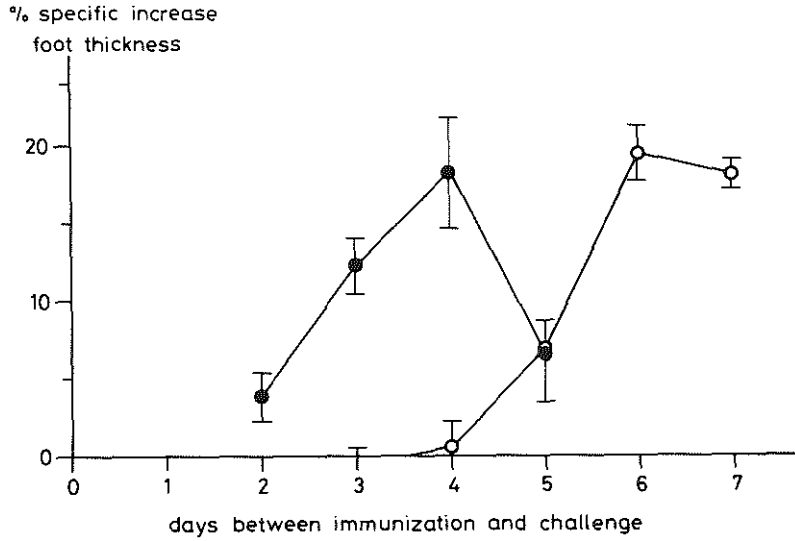

Fig. 3
Primary (O) and secondary (●) DTH responsiveness to H-Y antigen. Female (C57BL x CBA) F_1 mice were immunized for primary DTH or primed and boosted for secondary DTH by s.c. injection of 1×10^7 syngeneic male derived spleen cells. DTH reactivity was determined at different days after the last immunization. Vertical bars represent 1 SEM (n = 5).

Adoptive transfer of secondary DTH to non H-2 alloantigens with T cells

The cellular basis of secondary DTH responsiveness to non H-2 alloantigens was investigated by means of adoptive transfer experiments.

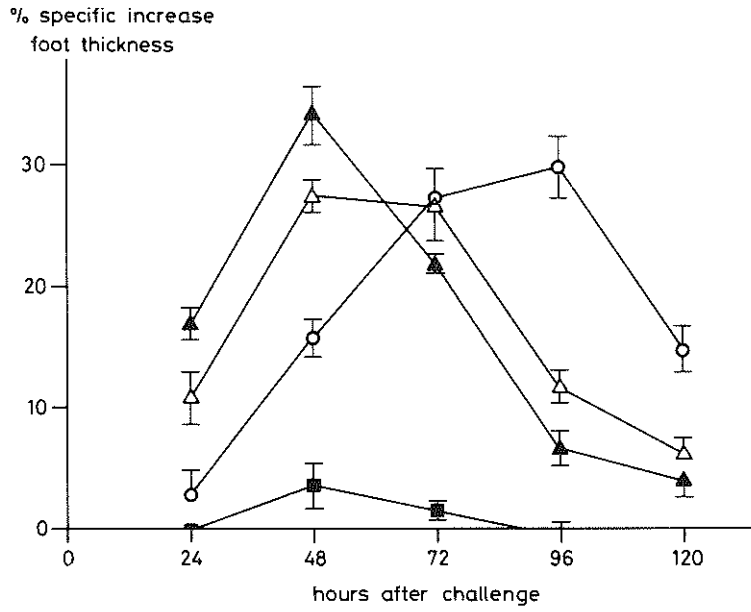

Fig. 4
Dependence of secondary DTH responsiveness to multiple non H-2 alloantigens on T memory cells. Lethally irradiated C3Hf mice were injected with 3 x 10^6 syngeneic bone marrow cells together with 2 x 10^7 viable immune or non immune (O) spleen cells. Immune spleen cells were obtained from C3Hf mice primed s.c. with 1 x 10^7 AKR spleen cells 2 months previously, and were enriched for T cells by nylon wool incubation (▲), depleted from T cells by anti-Thy 1.2 serum treatment (■), or were treated with normal mouse serum (△). All recipients were s.c. immunized with 3 x 10^5 AKR spleen cells immediately after adoptive transfer, and challenged 4 days later. At different intervals after challenge the specific increase of foot thickness was determined. Vertical bars represent 1 SEM (n = 5).

C3Hf mice, primed s.c. with 1 x 10^7 AKR spleen cells 2 months previously, were used as donor mice of spleen cells. A number of 2 x 10^7 viable spleen cells was adoptively transferred into 800 R irradiated C3Hf mice after selective depletion of Thy-1.2$^+$ cells by in vitro anti-Thy-1.2 serum treatment, or after T cell enrichment by incubation on nylon wool. Furthermore, one group of recipients received normal mouse serum treated spleen cells from primed C3Hf mice, and another group was injected with 2 x 10^7 spleen cells derived from non-primed C3Hf mice. Control mice did receive spleen cells. All groups of recipients were reconstituted with 3 x 10^6 C3Hf derived

bone marrow cells and immunized s.c. with 3 x 10^5 AKR spleen cells. On Day 4 after transfer and immunization all mice were challenged and during the following days the foot swelling was measured. The results indicate that the secondary DTH reactivity was strongly affected by anti-Thy-1.2 antiserum treatment of the spleen cells, whereas the ability of secondary DTH response was unaffected when nylon wool purified T cells were transferred (Fig. 4).
In a similar set up the proliferative activity of DTH-related memory was investigated. This was done by studying the vinblastine sensitivity of the pool of T cells responsible for the adoptive transfer of secondary DTH. BALB/c mice, primed s.c. with 1 x 10^7 viable DBA/2 spleen cells 4 months previously, received at 24 and 12 hr before adoptive transfer either 100 µg vinblastine or 0.1 ml BSS i.v. Thereafter 5 x 10^7 spleen cells from these groups of donor mice were i.v. injected into 750 R irradiated BALB/c mice together with 3 x 10^6 BALB/c derived bone marrow cells. Furthermore, one group of recipients received spleen cells from unprimed donors and another group was injected i.v. with bone marrow cells only. All groups were immunized s.c. with 3 x 10^5 DBA/2 spleen cells immediately after cell transfer, and challenged 5 days later. It appeared that vinblastine treatment did not diminish the capacity of secondary DTH responsiveness to DBA/2 spleen cells (Table 2).

Specificity of DTH-related memory for the multiple non H-2 alloantigens used for its induction

In order to investigate the specificity of the DTH-related memory for multiple non H-2 alloantigens, female (C57BL x C3Hf) F_1 mice were primed s.c. with 1 x 10^7 viable female AKR spleen cells. Two months later these mice were boosted with either 1 x 10^7 male AKR spleen cells or 1 x 10^7 male (C57BL x C3Hf) F_1 spleen cells. Challenge of these mice with the antigen used for booster injection was performed on Day 3. One group of primed (C57BL x C3Hf) F_1 mice was challenged with AKR spleen cells without a previous booster injection with this antigen. Furthermore, primary DTH reactivity to female AKR and male (C57BL x C3Hf) F_1 spleen cells was induced, and assayed on Day 3 or 6 after immunization. The data presented in Table 3 show that (C57BL x C3Hf) F_1 mice are indeed capable of DTH reactivity to H-Y antigen, but priming of these mice with female AKR spleen cells could not induce the capacity of secondary DTH responsiveness to H-Y antigen upon booster injection with syngeneic male spleen cells.

TABLE 2

SUSCEPTIBILITY OF DTH-RELATED T MEMORY CELLS TO TREATMENT WITH VINBLASTINE

Treatment of donor mice[a]	Transferred cells[b]	DTH reactivity[c] at 48 hr	72 hr
----	5×10^7 nonimmune spleen cells	-1.0±1.1	14.0±3.8
PBS	5×10^7 immune spleen cells	18.8±1.3	33.4±1.8
Vinblastine	5×10^7 immune spleen cells	21.4±1.7	37.0±2.1

a. BALB/c mice primed 4 months previously with 1×10^7 DBA/2 spleen cells, were injected twice with either 100 µg Vinblastine or 0.1 ml PBS. These injections were given at 24 hr and 12 hr before adoptive transfer.

b. Together with the spleen cells 3×10^6 syngeneic bone marrow cells were injected i.v. into the 750 R irradiated BALB/c hosts. Immediately after transfer all recipients were immunized s.c. with 3×10^5 DBA/2 spleen cells.

c. Five days after adoptive transfer and immunization all mice were challenged. At 24, 48, 82 and 96 hr after challenge the specific increase of foot thickness was determined. Figures represent the mean responses ± 1 SEM (n = 4) at 48 and 72 hr. The peak response of the groups of mice receiving immune spleen cells occurred at 72 hr after challenge.

DISCUSSION

In this paper a stable and persistent state of DTH to non H-2 alloantigens has been described, which was induced by a single s.c. injection of mice with viable allogeneic H-2 compatible spleen cells, suspended in saline. The time course of this DTH resembled the state of DTH to purified protein derivative of tuberculin (PPD) after immunization with viable mycobacteria (7). S.c. injection of viable allogeneic H-2 compatible spleen cells might lead to a condition in which a part of the injected cells remains intact in the host for a rather long time period. The experiments of Jacobsson et al. (17) indeed suggested that semi-allogeneic H-2 compatible cells can survive in a normal host for at least 2 weeks, in contrast to H-2 incompatible cells. The existence of long-lived DTH related T memory cells requiring

TABLE 3

SPECIFICITY OF DTH-RELATED MEMORY INDUCED BY MULTIPLE NON H-2 ALLOANTIGENS

Priming[a]	Booster	Challenge	Day of challenge	Specific DTH response[b]
AKR ♀	-	AKR ♀	3	17.2 ± 1.6
AKR ♀	-	AKR ♀	55	18.5 ± 2.7
AKR ♀	AKR ♀	AKR ♀	3	46.8 ± 4.6
(C57BL x C3H)F_1 ♂	-	(C57BL x C3H)F_1 ♂	3	-0.2 ± 1.5
(C57BL x C3H)F_1 ♂	-	(C57BL x C3H)F_1 ♂	6	16.1 ± 1.7
(C57BL x C3H)F_1 ♂	(C57BL x C3H)F_1 ♂	(C57BL x C3H)F_1 ♂	3	13.2 ± 1.4
AKR ♀	(C57BL x C3H)F_1 ♂	(C57BL x C3H)F_1 ♂	3	2.0 ± 1.5

a. Female (Black x C3H)F_1 mice were immunized for primary and secondary DTH reactivity by s.c. injection of 1 x 10^7 viable spleen cells as indicated in the table. The interval between priming and boosting for secondary DTH was 2 months.

b. Figures represent the mean specific increase of foot thickness ± 1 SEM (n = 5).

antigenic restimulation in order to become able to perform an effector function has been demonstrated in our previous paper on secondary DTH to SRBC (11). The adoptive transfer experiments presented in this paper suggest that such a population of hardly proliferating, long-lived T memory cells is also present in mice which show a persistent level of DTH, and have the capacity of secondary DTH responsiveness to multiple non H-2 alloantigens (Table 2). Similarly, after immunization with viable mycobacteria, not only non-recirculating DTH-related T effector cells are generated (18), but also a population of recirculating cells which can differentiate into such DTH effector cells (19). A stable and persistent state of DTH has never been observed after short lasting immunization with non-viable agents alone (20), although such immunization procedures can induce a long-lasting capacity to react upon reimmunization with secondary DTH reactivity (10). These data together lead us to hypothesize that a persistent state of DTH is dependent on the continuous antigen-driven differentiation of long-lived, recirculating T memory cells into non-recirculating, functionally short-lived DTH effector cells. According to this hypothesis the persistence of immunogenic material is required for the persistence of DTH reactivity.

It cannot be excluded, however, that the increase in DTH reactivity during anamnestic DTH can also be mediated by the progeny of effector cells undergoing an extra proliferation cycle upon booster injection with minor H-antigens. The sudden disappearance of DTH reactivity 24 hr after the booster injection might reflect the recruitment of these effector cells either to the injection site or to the regional lymph nodes, where this further proliferation might take place. DTH-related memory for non H-2 alloantigens could also be found in primed mice at a moment when they hardly show DTH reactivity to these antigens (Table 1). Obviously the absence of a detectable number of DTH effector cells doesn't preclude DTH-related memory.

Combined *in vivo* and *in vitro* experiments provided evidence that alloreactive T cells, stimulated *in vitro* in a mixed lymphocyte reaction (MLR) with H-2 incompatible spleen cells can revert into long-lived small lymphocytes after the injection into syngeneic recipients (21). Upon *in vitro* restimulation with the specific H-2 alloantigens these small lymphocytes gave rise to cytotoxic T lymphocytes in an accelerated fashion as compared to the generation of cytotoxic T lymphocytes in a primary MLR (21). On the other hand, MLR and *in vitro* induced cytotoxicity to single or multiple non H-2 alloantigens could generally be induced only after *in vivo* priming of mice with the specific non H-2 alloantigens (22-24). Therefore, MLR and *in vitro* induced cytotoxicity to non H-2 alloantigens might require either a quantitative or a qualitative change in the population of precursor cells reactive to these antigens (25). The discrepancy in time course

between primary DTH and the appearance of cytotoxicity to minor H-antigens in vivo (23,26) suggests that T cells mediating DTH to non H-2 alloantigens and T cells performing cytotoxic functions to these antigens either belong to a different subpopulation or represent a different stage of differentiation of the same cell line.

While T cells recognizing non-self H-2 antigens in association with multiple minor H-differences are more numerous than T cells that recognize multiple minor H-antigens (27,28), immunization with H-2 alloantigens always gives rise to a slowly developing and relatively weak DTH reactivity as compared with non H-2 alloantigens (Fig. 2D; other data not shown). It was shown by Hall et al. (29,30) that a primary allograft incompatibility for major H-antigens greatly increased the potency of alloreactive cells upon adoptive transfer to reject an allogeneic skin graft. However, neither the second set rejection of H-2 incompatible grafts (31) nor secondary DTH responsiveness to H-2 incompatible cells (Fig. 2D) are markedly different from the primary responses to major H-antigens, in contrast to responses to non H-2 antigens (31; Fig. 2A-C). Experiments are in progress to reveal the underlying cause of this discrepancy.

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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 an 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) F_1, the Mls^b locus antigens were 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) F_1 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

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

Injection of lymphocytes into Mls locus incompatible neonatal F_1 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) F_1 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 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) F_1 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 have 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. Using this assay we have now studied the capacity of Mls locus antigens to induce such DTH T effector cells after (semi-)allogeneic spleen cell transplantation into 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-2^d, Mls^b) female mice and DBA/2 (H-2^d, Mls^a) male mice were purchased from the Medical Biological Laboratory TNO, Rijswijk (ZH), The Netherlands. (BALB/c x DBA/2) F_1 (H-$2^{d/d}$, Mls$^{b/a}$) female mice were obtained from the Radiobiological Institute TNO, Rijswijk

(ZH), The Netherlands. B10.D2 (H-2^d, 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-2^k, Mls^c) male mice and (C57BL/Rij x CBA/Rij) F_1 (H-$2^{b/q}$) female mice were bred at the Laboratory Animals Centre of the Erasmus University, Rotterdam, The Netherlands. (BALB/c X C3H/f) F_1 (H-$2^{d/k}$, $Mls^{b/c}$) female and AKR/FuRdA (H-2^k, Mls^a) male mice were bred in our own department. The age of the mice ranged between 15 and 30 weeks. When mice from either sex were used in anyone experiment, the combinations were always chosen in such a way that anti-H-Y responses were impossible.

Preparation of cell suspensions

Mice were killed with carbon dioxide. Immediately after killing, the spleens were removed and placed in 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 (i.v.) injection of 5 x 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 1 x 10^7 spleen cells in a volume of 0.1 ml of BSS. The cells were injected subcutaneously (s.c.), and equally distributed over both inguineal 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 carried out 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.). 5 x 10^5 (100 µl) responder cells (R) and 5 x 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 1 x 10^6 responder cells or 1 x 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 3H-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:

$$\text{spec.cpm} = \text{cpm}(5\text{x}10^5\ \text{R} + 5\text{x}10^5\ \text{S}) - \frac{\text{cpm}\ (1\text{x}10^6\ \text{R}) + \text{cpm}\ (1\text{x}10^6\ \text{S})}{2}$$

The activity of 1 x 10^6 R cells ranged from 500 to 2500 cpm, and the activity of 1 x 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 s.c. immunization with 1 x 10^7 allogeneic or syngeneic spleen cells (treated with 25 µg mitomycin C per ml), the mice were injected i.v. 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 allogeneically reconstituted recipient mouse was transferred i.v. 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. 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 ranged between 12 and 22%.

RESULTS

Kinetics of MLC responses to Mls locus antigens

The capacity of the Mls locus antigens of our mice to induce proliferation was tested in an 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) F_1, 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, but 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) F_1 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) F_1 spleen cells. Both BALB/c and DBA/2 spleen cells did proliferate upon stimulation with H-2 incompatible (C57BL/Rij x CBA/Rij) F_1 spleen cells, with a maximum response on day 3. These results confirm 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. 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, after continued cultivation Mls^a locus antigen can induce a higher level of proliferation than Mls^c.

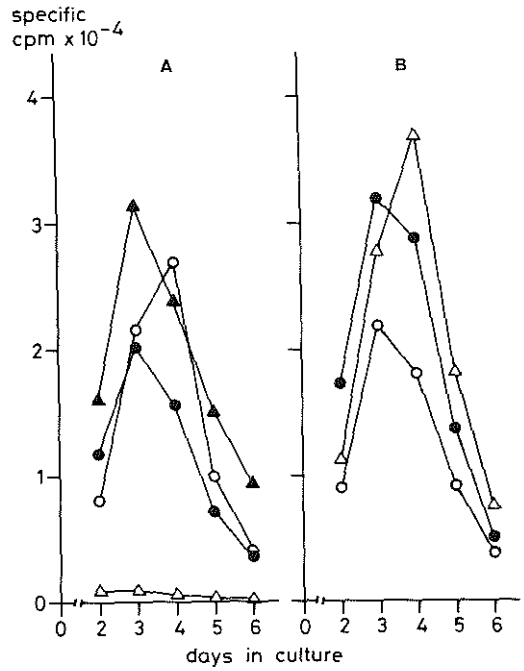

Fig. 1
Kinetics of ^{3}H-thymidine incorporation in one way MLC after stimulation with Mls antigens. Responder (R) and stimulator (S) cells were always spleen cells. S cells were treated with 25 µg mitomycin C per ml.
(A) BALB/c (R) → (BALB/c x DBA/2) F_1 (S) (○); DBA/2 (R) → (BALB/c x DBA/2) F_1 (S) (△). 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) F_1 (S) (●); DBA/2 (R) → (C57BL/Rij x CBA/Rij F_1 (S) (▲). (B) AKR/FuRdA (R) → C3H/f (S) (○); C3H/f (R) → AKR/FuRdA (S) (△). For comparison the response of AKR/FuRdA cells in an H-2 incompatible combination is shown: AKR/FuRdA (R) → (BALB/c x C3H/f) F_1 (S) (●). Each experimental point represents the arithmetic mean of three separate experiments. Values are corrected for background (35 cpm).

Cellular changes in the spleen after Mls locus differing spleen cell transplantation

The cellular changes in the spleen of lethally irradiated (BALB/c x DBA/2) F_1 mice inoculated with either 5 x 10^7 BALB/c, DBA/2 or (BALB/c x DBA/2) F_1 spleen cells were studied. After reconstitution with BALB/c and especially with DBA/2 spleen cells, an earlier in-

crease of cellularity was found as compared to 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.

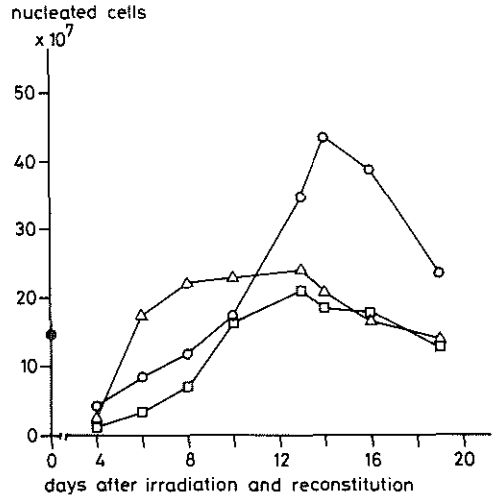

Fig. 2
Recovery of the number of nucleated cells in the spleen of lethally irradiated (BALB/c x DBA/2) F_1 mice, reconstituted with either 5×10^7 BALB/c (○), DBA/2 (△) or (BALB/c x DBA/2) F_1 (□) spleen cells. The figure representing the value 0 days after irradiation was obtained from non-irradiated (BALB/c x DBA/2) F_1 control mice. Each experimental point represents the arithmetic mean of 4 mice.

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) F_1 mice and reconstitution with 5×10^7 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 2×10^7 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) F_1 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) F_1 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 against 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 anti-host DTH response in the Mls locus differing (BALB/c x DBA/2) F_1 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.

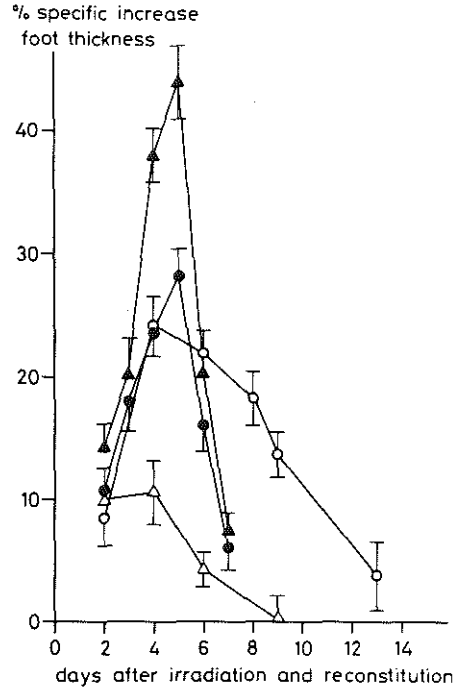

Fig. 3
Development of anti-host DTH reactivity in the spleen of lethally irradiated (BALB/c x DBA/2) F_1 mice, inoculated with 5×10^7 BALB/c (○) or DBA/2 (▲) spleen cells and in the spleen of lethally irradiated (C57BL/Rij x CBA/Rij) F_1 mice, inoculated with 5×10^7 BALB/c (●) or DBA/2 (▲) spleen cells. Each experimental point represents the arithmetic mean ± 1 SEM of two different experiments (n=10).

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
GvH[a]	BALB/c	(BALB/c x DBA/2) F_1	DBA/2	25.5±0.7[c]
	BALB/c	(BALB/c x DBA/2) F_1	B10.D2	0.5±1.2
system	immunizing cells	responder	challenge	response
HvG[b]	(BALB/c x DBA/2) F_1	BALB/c	DBA/2	39.2±2.2
	(BALB/c x DBA/2) F_1	BALB/c	B10.D2	28.4±3.0

a. GvH reactions were elicited by i.v. injection of 5×10^7 BALB/c spleen cells in lethally irradiated (BALB/c x DBA/2) F_1 mice. After 5 days the recipient spleen cells were transferred into BALB/c mice and these mice were challenged with either DBA/2 or B10.D2 spleen cells.

b. HvG reactions were elicited by s.c. injection of 1×10^7 (BALB/c x DBA/2) F_1 spleen cells in BALB/c mice. Five days later these mice were challenged with either DBA/2 or B10.D2 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.

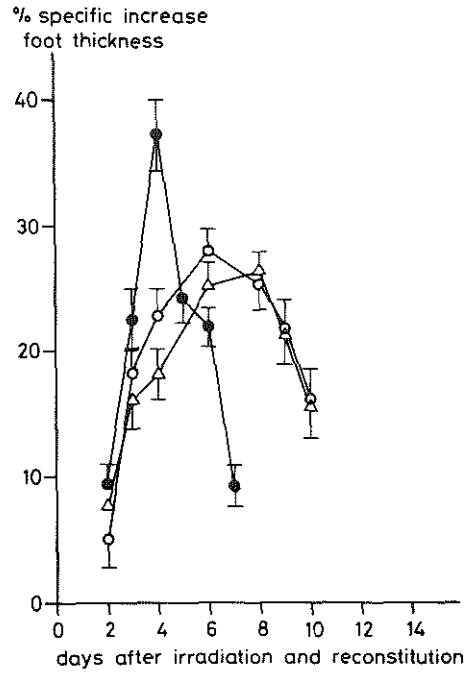

Fig. 4
Development of anti-host DTH reactivity in the spleen of lethally irradiated C3H/f mice inoculated with 5 x 10^7 AKR/FuRdA spleen cells (O), in the spleen of lethally irradiated AKR/FuRdA mice inoculated with 5 x 10^7 C3H/f spleen cells (Δ), and in the spleen of lethally irradiated (BALB/c x C3H/f) F_1 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).

In order to study a second Mls locus differing, H-2 compatible combination, AKR/FuRdA and C3H/f mice were tested. The transferable antihost 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 around days 6-8 after reconstitution. Thereafter, the reactivity gradually declined. The anti-host DTH reactivity of AKR spleen cells in H-2 incompatible, Mls locus incompatible (BALB/c x C3H/f) F_1 mice was also 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 maintain the anti-host DTH reactivity on a high level, as is the case in H-2 compatible combinations.

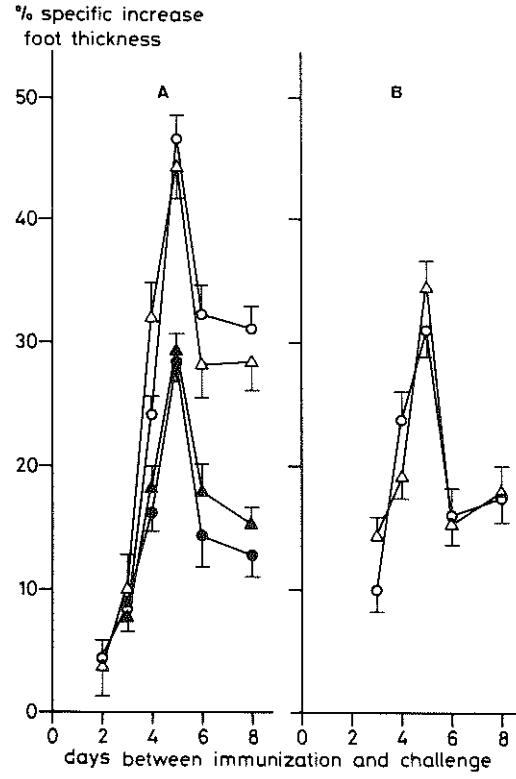

Fig. 5
(A) Primary DTH responsiveness of BALB/c (O) and DBA/2 (▲) mice s.c. immunized with 1 x 10^7 spleen cells from (BALB/c x DBA/2) F_1 mice; or BALB/c (●) and DBA/2 (▲) mice s.c. immunized with 1 x 10^7 spleen cells from (BALB/c x DBA/2) F_1 mice which were lethally irradiated (850 rad) previously.
(B) Primary DTH responsiveness of C3H/f (O) mice s.c. immunized with 1 x 10^7 AKR/FuRdA spleen cells, and DTH responsiveness of AKR/FuRdA (▲) mice s.c. immunized with 1 x 10^7 C3H/f spleen cells. Each experimental point represents the arithmetic mean of $\pm$ 1 SEM of a group of at least 5 mice.

TABLE II

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) F_1	BALB/c	3202±212	5.8±0.4
(BALB/c x DBA/2) F_1	DBA/2	999± 54	3.8±0.4
BALB/c	BALB/c	552± 44	-
DBA/2	DBA/2	266± 20	-

a. Immunization was performed s.c. with 1 x 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.

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 immunized s.c. with 1 x 10^7 (BALB/c x DBA/2) F_1 spleen cells and AKR and C3H/f mice were immunized s.c. with 1 x 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 against non H-2 histocompatibility antigens other than Mls locus products. This was clear from the relatively high DTH response of BALB/c mice, immunized with DBA/2 (Mls^a) spleen cells, challenged with B10.D2 (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) F_1 spleen cells were irradiated (850 rad) in vivo before harvesting. As can be seen from Fig. 5A, in this case also 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.

Proliferative activity in the development of anti-graft immune reactivity

The proliferative activity in the development of anti-graft DTH reactivity after s.c. immunization of BALB/c and DBA/2 mice with 1×10^7 (BALB/c x DBA/2) F_1 spleen cells was determined in vivo in the inguinal lymph nodes. Both in BALB/c and in DBA/2 mice an increased proliferative activity was observed at 4 days after immunization (Table II). 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 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 correlated fully with the capacity to induce an one-way MLC response. Thus, in the BALB/c versus (BALB/c x DBA/2) F_1 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 (Fig. 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) F_1, the Mls^b locus antigen was not able to initiate in vitro proliferation (Fig. 1) or 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 histo-incompatible combination (9). In the earlier study 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 component of reacting lymphocytes with the ^3H-thymidine suicide technique, showed that the maximum proliferative activity preceded the maximal anti-host DTH reactivity (9). It is also possible that in Mls locus

differing 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 not cytotoxic T cells (13,14). However, until this moment the results have been 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 histo-incompatibilities 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. Röllinghof 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 Bach et al. in which LD-antigen 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 antigens. Our experiments with cross-reacting (12) non H-2 alloantigens revealed that the anti-host T effector cells in DTH are directed mainly against the Mls locus antigen and not against other non H-2 alloantigens (Table 1). In studies with congenic mice (manuscript in preparation), 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 against I-region antigens, and not against K- and D-region antigens. Just like Mls products, I-region antigens stimulate T cell proliferation in MLC (18). The inability of K and D products to efficiently stimulate T cell proliferation in vitro (18) again coincided with the inability to evoke DTH T effector cells during a GvH reaction. Klein et al. (19) were able to demonstrate cytotoxic effector cells against I-region antigens; thus, 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 I). Other non H-2 differences are probably responsible for most of the DTH reactivity after immunization with H-2 compatible spleen

cells (Table 1). Although T effector cells specific for Mls locus products could not be detected, a distinct proliferation in the inguineal lymph nodes 4 days after s.c. immunization with H-2 identical, non H-2 differing spleen cells was found (Table II). Thus, other non H-2 alloantigens 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.

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H-2 RESTRICTED RECOGNITION OF MINOR HISTOCOMPATIBILITY ANTIGENS IN DELAYED TYPE HYPERSENSITIVITY

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SUMMARY

Subcutaneous (s.c.) immunization of mice with viable allogeneic H-2 compatible spleen cells can induce a persistent state of delayed type hypersensitivity (DTH) to minor histocompatibility (H)antigens which can be evaluated with a footpad swelling assay. The importance of H-2 compatibility of the injected spleen cells with the recipient for (1) the elicitation of the DTH-reaction, (2) the induction of DTH-related effector and memory T cells, and (3) the activation of T memory cells was examined with congenic mouse strains. Spleen cells sharing either the K- or D-region of the H-2 complex with the recipient could elicit strong DTH-reactions to minor H-antigens, though somewhat less than did fully H-2 compatible allogeneic spleen cells. H-2 incompatible cells, or cells sharing I-region coded antigens only elicited relatively weak, though significant, DTH-reactivity to the minor H-antigens. Similar H-2 requirements for recipient mice were demonstrated in the immune lymphocyte transfer assay. Optimal induction of primary DTH and DTH-related T memory cells for minor H-antigens also required H-2 identity of the immunizing cells and the recipient. To some extent, H-2 incompatible cells were able to induce primary DTH-reactivity and memory for minor H-antigens. The secondary DTH-reactivity was not or slightly dependent on the H-2 haplotype of the cells used for booster injection.
It is concluded that the DTH-related effector cells are restricted in their recognition of minor H-antigens by K- or D-region coded antigens. Presumably, macrophage processing of the allogeneic spleen cells after primary and secondary immunization accounts for the capacity of the minor H-antigens to activate unprimed T cells and memory T cells when these antigens are presented on H-2 incompatible cells.

INTRODUCTION

Allogeneic skin grafting of mice can induce a state of delayed type hypersensitivity (DTH) to minor histocompatibility (H-)antigens (Kon & Klein, 1976). In a previous paper we have shown that subcutaneous (s.c.) injection of allogeneic H-2 compatible spleen

cells also leads to a strong and stable state of DTH to non H-2 alloantigens (Van der Kwast et al., in press). Mls locus products did not influence the intensity of the primary DTH to minor H-antigens (Van der Kwast et al., in press; Wolters et al., submitted for publication). Grafting of allogeneic skin may also result in the appearance of sensitized cells in the draining lymph nodes whose antigraft reactivity can be assayed by intracutaneous transfer into recipients identical to the donor strain of the graft (Brent et al., 1962; Streilein & Billingham, 1970). This immune lymphocyte transfer (ILT) causes a local Graft-versus-Host (GvH) reaction, characterized by oedema and infiltration, which is maximal at about 24 hr. ILT-reactions to multiple minor H-antigens or to a single minor H-antigen can be demonstrated in mice (Streilein & Billingham, 1970). S.c. priming of mice with allogeneic H-2 compatible spleen cells not only induces primary DTH-reactivity, but also DTH-related T memory cells (Van der Kwast et al., in press). These T memory cells account for the occurrence of secondary DTH after booster injection with the specific antigen. Secondary DTH to minor H-antigens is characterized by the rapid increase of DTH-reactivity till above the level of pre-existing DTH. The peak reactivity can be observed about 2 days earlier than in primary DTH. Transfer experiments revealed that DTH-reactivity to proteins could only be transferred into recipients with an identical I-A-region of the MHC, while contact sensitivity to dinitrofluorobenzene was transferable to recipients which have either the same K-, I-, or D-region as the sensitized donor strain (Vadas et al., 1977; Miller, et al., 1977). Transfer of DTH to viral antigens required K- or D-region compatibility between donor and recipient (Zinkernagel, 1976). H-2 restricted cell-mediated immunity to multiple minor H-antigens has been demonstrated in vitro by means of the cell-mediated lympholysis (CML) assay. Since in-vivo priming of mice with a skin graft or by injection of cells is required for the in-vitro induction of CML, only secondary responses to minor H-antigens could be assayed in CML (Gordon et al., 1975; Bevan, 1975). It was then found that efficient lysis only occurred when responder cells and target cells have identical K- or D-regions of the H-2 complex. In this paper the in-vivo relevance of the phenomenon of H-2 restriction was investigated for the effector phase of primary DTH to minor H-antigens. Furthermore, the optimal conditions for primary and secondary immunization for DTH reactivity to minor H-antigens were studied.

MATERIALS AND METHODS

Mice

Female BALB/c ($H\text{-}2^d$) and DBA/2 ($H\text{-}2^d$) mice were purchased from the

Radiobiological Institute TNO, Rijswijk, The Netherlands. Female C3Hf ($H\text{-}2^k$), B10.A ($H\text{-}2^a$), A.SW ($H\text{-}2^s$), and B10.AQR ($H\text{-}2^{y1}$) mice were obtained from the Laboratory Animals Centre of the Erasmus University, Rotterdam, The Netherlands. Female B10.Br ($H\text{-}2^k$) and B10.D2 ($H\text{-}2^d$) mice were kindly provided by the University of Nijmegen, The Netherlands and the Central Bloodtransfusion Service in Amsterdam, The Netherlands. Female BALB.B ($H\text{-}2^b$), CBA/J ($H\text{-}2^k$) and CBA.M523 ($H\text{-}2^{ka}$) mice were kindly provided by the Basel Institute for Immunology, Basel, Switzerland. Female A ($H\text{-}2^a$), A.TL ($H\text{-}2^{t1}$), B10.T (6R)($H\text{-}2^{y2}$) and A.TH ($H\text{-}2^{t2}$) mice were commercially obtained from OLAC Ltd., Bicester, U.K. Female Swiss ($H\text{-}2^s$) and B10.ScSn ($H\text{-}2^b$) mice were commercially obtained from TNO, Zeist, the Netherlands. Female AKR/FuRdA ($H\text{-}2^k$) mice were bred in our department. The age of the responder mice ranged between 12 and 20 weeks.

Preparation of cell suspensions

Spleens or lymph nodes were removed, placed in a balanced salt solution (BSS) and squeezed through a nylon gauze filter to provide a single cell suspension. Nucleated cells were counted with a Coulter Counter Model B. Viable nucleated cells were counted in a haemocytometer using 0.2% trypan blue in BSS as a diluent.

Antigen and immunization

Primary and secondary immunization was performed with the appropriate allogeneic spleen cells, suspended in a volume of 0.1 ml. The priming and booster dose was always 1×10^7 spleen cells. A total volume of 50 µl of this spleen cell suspension was injected s.c. by means of a 28-gauge needle in each of both inguineal areas.

Assay for delayed type hypersensitivity

DTH-reactions were determined by measuring the difference in thickness of the hind feet 24 hr after s.c. injection of 8×10^6 spleen cells into the instep of the right hind leg. The challenge dose was administered s.c. in a volume of 20 µl by means of a 28-gauge needle. The thickness of the left and right hind feet was measured with a footpad meter as described by Bonta and De Vos (1965), with some minor modifications. During measurement the mice were anaesthetized with ether.
A control group, consisting of non-immune mice challenged with the same spleen cells as the mice to be tested, was always included. 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 control mice ranged between 15 and 25%.

Assay for immune lymphocyte transfer reactivity

The ILT-reactivity in lymph node cells from immunized donors, directed against H-antigens of a particular host was determined by s.c. injection of 5 x 10^6 of these lymph node cells into the right hind foot of the host to be tested. The hosts received a s.c. injection into the left hind foot of 5 x 10^6 lymph node cells from non-immune mice, syngeneic with the immunized donor mice. This latter injection may result in a normal lymphocyte transfer (NLT) reaction. For ILT and NLT the cells were injected in a volume of 40 µl with a 28 gauge needle. Preliminary results indicated that injection of 5 x 10^6 lymph node cells resulted in a maximal ILT-reactivity to minor H-antigens at 24 hr after transfer. A control group consisting of recipient mice syngeneic to the immunizing donor mice, was always included. These mice were similarly injected with immune and nonimmune lymph node cells, into the right and left hind feet, respectively.
The thickness of both injected feet was measured as in the DTH assay. The specific ILT-reactivity was calculated as: $(ILT\text{-}NLT)_{test} - (ILT\text{-}NLT)_{control}$, and was expressed in 10^{-2} cm.

RESULTS

H-2 restricted recognition of minor H-antigens during the expression of primary DTH-reactivity

To investigate the genetic requirements for expression of DTH to minor H-antigens, groups of mice were immunized s.c. with 1 x 10^7 allogeneic H-2 compatible spleen cells and challenged five days later. This challenge was performed with spleen cells derived from H-2 congenic mouse strains, which have a genetic background either fully (Table 1, Exp. I-III) or partly (Table 1, Exp. IV and V) identical to that of the immunizing spleen cells. Subsequently, the 24 hr DTH-reactions were measured. It appeared that rather strong DTH-reactions could be evoked by spleen cells exhibiting either the same K-region (Table 1, Exp. Ib) or the same D-region (Table 1, Exp. IIb) coded antigens, though fully H-2 compatible spleen cells elicited still higher responses (Table 1, Exp. I and IIa). On the other hand, comparatively weak, though significant, DTH-reactions were evoked by fully H-2 incompatible spleen cells (Table 1, Exp. I-IVc). I-region compatibility with the recipient alone was not sufficient for elicitation of stronger DTH-reactions than were evoked by fully H-2 incompatible spleen cells (Table 1, Exp. III-Vb,Vd). The existence of I-region restriction was not excluded by these experiments, because of the comparatively low

TABLE 1

H-2 RESTRICTION OF PRIMARY DTH REACTIVITY TO NON H-2 ALLOANTIGENS

Exp.		Recipient	Donor[1]	Challenge	Response[2]	Shared region of the H-2 complex
	a	Swiss	A.SW	A.SW	51 ± 4	K, I, S, D
I	b	Swiss	A.SW	A.TL	42 ± 3	K
	c	Swiss	A.SW	A	21 ± 2	-
	a	DBA/2	B10.D2	B10.D2	63 ± 3	K I, S, D
II	b	DBA/2	B10.D2	B10.T(6R)	32 ± 1	D
	c	DBA/2	B10.D2	B10.ScSn	10 ± 1	-
	a	C3Hf	B10.Br	B10.A	22 ± 2	K, I_{A-E}
III	b	C3Hf	B10.Br	B10.AQR	10 ± 2	I_{A-E}
	c	C3Hf	B10.Br	B10.ScSn	9 ± 2	-
	a	C3Hf	AKR	A	23 ± 1	K, I_{A-E}
IV[3]	b	C3Hf	AKR	A.TL	11 ± 2	I, S
	c	C3Hf	AKR	A.TH	7 ± 3	-
	a	A	B10.A	CBA/J	18 ± 2	K, I_{A-E}
V[3]	b	A	B10.A	CBA.M523	4 ± 2	I_{A-E}
	c	B10.A	A	CBA/J	40 ± 3	K, I_{A-E}
	d	B10.A	A	CBA.M523	26 ± 3	I_{A-E}

1. Recipient mice were immunized s.c. with 1 x 10^7 viable spleen cells derived from H-2 compatible donor mice.

2. Five days after s.c. immunization the mice were challenged. At 24 hr after challenge the specific increase of foot thickness was determined. Figures represent the mean response ± 1 SEM (n=5).

3. In these experiments the challenge was performed with spleen cells from congenic mice with a genetic background different from that of the immunizing spleen cells.

DTH reactions in the essential mouse strain combinations. It is concluded that the expression of the DTH-reactivity to multiple minor H-antigens is strongly restricted by the K- and D-region of the H-2 complex. For the K-region, this view is strengthened by experiments performed with the mouse strain CBA.M523, which bears a mutation in the H-2K locus (Blandova et al., 1975). Spleen cells derived from this mutant strain, elicited lower DTH responses than spleen cells obtained from the non-mutant CBA/J strain (Table 1, Exp. Vb and Vd).

The influence of H-2 restriction on the effector function of DTH-reactive cells directed against minor H-antigens was also investigated with the ILT-assay. Swiss, C3Hf and BALB/c mice were immunized s.c. with 1 x 10^7 allogeneic H-2 compatible spleen cells derived from A.SW, B10.Br and DBA/2 mice, respectively. Five days later, 5 x 10^6 cells, obtained from the draining inguinal lymph nodes of the immunized mice, were assayed for ILT-reactivity to minor H-antigens of recipient mice, which were either identical with, or congenic to the immunizing mouse strain (Table 2, Exp. I and II). In a similar set-up, H-2 congenic mouse strains of a different background than the original immunizing strain were used as recipients (Table 2, Exp. III). The responses at 24 hr after transfer clearly indicated that ILT-reactivity to minor H-antigens was mainly dependent on the compatibility of the K-region (Table 2, Exp. Ic) and D-region (Table 2, Exp. IIIb) coded antigens of the donor of reactive T lymphocytes and the recipient's H-2 antigens. In these experiments, no significant ILT-reactivity was found, when the immunized donor strain and the recipient strain were either fully H-2 incompatible (Table 2, Exp. Id, IIc and IIIb), or only showed similarity in parts of the I-region (Table 2, Exp. IIb).

Immunization for primary and secondary DTH-responsiveness to minor H-antigens by s.c. injections of H-2 incompatible spleen cells.

To compare the priming capacity of H-2 compatible and H-2 incompatible spleen cells, groups of C3Hf mice were immunized s.c. with either 1 x 10^7 H-2 compatible B10.Br derived spleen cells, or with 1 x 10^7 H-2 incompatible B10.ScSn derived spleen cells. The latter two mouse strains share most minor H-antigens. On day 5 after immunization the primary DTH-reactivity to minor H-antigens was tested by a challenge injection of B10.A derived spleen cells. Since a part of the H-2 complex (a.o. the K-region) in this latter strain is identical to that in the responding C3Hf mouse strain, a DTH-response to the minor H-antigens was evoked (Table 1, Exp. III). Both B10.Br and B10.ScSn derived spleen cells were able to induce primary DTH-reactivity, but the H-2 incompatible B10.ScSn derived cells did so to a lesser extent (Table 3, Exp. I). Similarly, s.c. immunization of BALB/c mice with

TABLE 2

H-2 RESTRICTED ILT-REACTIVITY TO NON H-2 ALLOANTIGENS

Exp.		Immunizing[1] strain	Immunized donor strain	Recipient strain	Response[2] (ILT-NLT)	Shared region of the H-2 complex
I	a	A.SW	Swiss	A.SW	32.5 ± 13.8	K, I, S, D
	b	A.SW	Swiss	A.TH	14.5 ± 7.6	K, I, S
	c	A.SW	Swiss	A.TL	17.5 ± 8.5	K
	d	A.SW	Swiss	A	-1.3 ± 6.5	-
II	a	B10.Br	C3Hf	B10.A	26.2 ± 8.9	K, I_{A-E}
	b	B10.Br	C3Hf	B10.AQR	0.7 ± 6.3	I_{A-E}
	c	B10.Br	C3Hf	B10.T(6R)	-0.6 ± 6.3	-
III[3]	a	DBA/2	BALB/c	B10.T(6R)	30 ± 5.8	D
	b	DBA/2	BALB/c	B10.ScSn	3 ± 7.1	-

1. The indicated mice were s.c. immunized with 1 x 10^7 H-2 compatible allogeneic spleen cells. Five days later the draining lymph nodes from these mice were taken out, and cell suspensions were made. These cells were s.c. transferred into the indicated recipient mice.

2. At 24 hr after s.c. transfer of 5 x 10^6 lymph node cells from the immunized donor strain into the right hind foot of the recipient mice the foot thickness was determined. The response was measured as indicated in the Materials and Methods section. The figures represent the mean response at 24 hr after transfer: (ILT-NLT) ± 1 SD (n=4), in 10^{-2}cm.

3. In this experiment the congenic recipient strains and the immunizing strain did not have an identical genetic background, but they share a large number of minor H-antigens.

TABLE 3

INDUCTION OF PRIMARY DTH-REACTIVITY TO NON H-2 ALLOANTIGENS WITH H-2 COMPATIBLE AND H-2 INCOMPATIBLE ALLOGENEIC SPLEEN CELLS

Exp.	Recipient[1]	Donor of the immunizing spleen cells	Shared region of the H-2 complex	Challenge	Response[2]
I	C3Hf	B10.Br	K, I, S, D	B10.A	26 ± 4
	C3Hf	B10.ScSn	-	B10.A	14 ± 1
II	BALB/c	B10.D2	K, I, S, D	B10.A	32 ± 3
	BALB/c	B10.ScSn	-	B10.A	11 ± 3
	BALB/c	BALB.B+B10.D2	K, I, S, D	B10.A	27 ± 2

1. Recipients were s.c. immunized with the indicated allogeneic H-2 compatible or H-2 incompatible spleen cells. The DTH reactions were elicited with B10.A ($H\text{-}2^a$) derived spleen cells. The B10.A strain shares the K, I_A, I_B, I_J and I_E region of the H-2 complex with C3Hf ($H\text{-}2^K$) mice, and the I_C and D region of the H-2 complex with BALB/c ($H\text{-}2^d$) mice.

2. Five days after s.c. immunization the mice were challenged. At 24 hr after challenge the specific increase of foot thickness was determined. Figures represent the mean responses ± 1 SEM (n=5).

1 x 10^7 H-2 incompatible B10.ScSn derived spleen cells led to a less intense primary DTH-reactivity to B10.A spleen cells than was observed after s.c. immunization with H-2 compatible B10.D2 derived spleen cells (Table 3, Exp. II). Suppression of the DTH-reactivity to minor H-antigens by the accompanying immune response to H-2 coded antigens was excluded by simultaneous s.c. injection of BALB/c mice with both 1 x 10^7 H-2 compatible B10.D2 derived spleen cells and 1 x 10^7 H-2 incompatible BALB.B spleen cells at separate sites in both inguinal areas. The injection of BALB.B and B10.D2 spleen cells was carried out at separate sites to prevent the occurrence of a mixed lymphocyte reaction between the injected B10.D2 and BALB.B spleen cells. The accompanying anti-BALB.B reaction caused only a slight, non-significant reduction in primary DTH-reactivity to the minor H-antigens (Table 3, Exp. II).

The capacity for induction of secondary DTH-responsiveness to minor H-antigens by s.c. injection of H-2 incompatible cells was investigated in C3Hf mice. These mice were primed with B10 minor H-antigens by injection of either 1 x 10^7 H-2 compatible B10.Br spleen cells or 1 x 10^7 H-2 incompatible B10.ScSn spleen cells. Six weeks later all mice were boosted s.c. with 1 x 10^7 B10.Br spleen cells. On day 3 after booster the mice were challenged with B10.A spleen cells. This latter interval was chosen, since in a previous paper it was demonstrated that the DTH-reaction elicited on the third day after booster is a good parameter for the level of secondary DTH-responsiveness to multiple minor H-antigens (Van der Kwast et al., in press). As a negative control for secondary DTH-responsiveness, one group of C3Hf mice was included which was challenged with B10.A spleen cells on day 3 after a single injection of 1 x 10^7 B10.Br spleen cells. The results show that the H-2 incompatible B10.ScSn spleen cells can indeed induce secondary DTH-responsiveness to minor H-antigens, but to a lesser extent than the H-2 compatible B10.Br spleen cells (Table 4), in spite of the common B10 background.

Booster of minor H-antigen primed mice by s.c. injection of H-2 incompatible spleen cells

The role of the H-2 haplotype of the cells used for booster injection in secondary DTH to minor H-antigens was studied at first in BALB.B mice, which had been primed 4 months previously with 1 x 10^7 H-2 compatible viable B10.ScSn spleen cells. The primed BALB.B mice were boosted either with 1 x 10^7 H-2 compatible B10.ScSn spleen cells or with 1 x 10^7 H-2 incompatible B10.Br spleen cells. Significant secondary DTH-reactivity was observed both after booster injection with H-2 compatible B10.ScSn spleen cells (Table 5, Exp. Ia) and with H-2 incompatible B10.Br spleen cells (Table 5, Exp. Ib). However, the B10.ScSn derived spleen

TABLE 4

PRIMING FOR SECONDARY DTH RESPONSIVENESS BY ALLOGENEIC H-2 COMPATIBLE AND H-2 INCOMPATIBLE SPLEEN CELLS

Recipient	Donor of the[1] priming cells	Shared region[2] of the H-2 complex	Booster	Challenge	Response
C3Hf	B10.Br	K, I, S, D	B10.Br	B10.A	38 ± 2
C3Hf	B10.ScSn	-	B10.Br	B10.A	20 ± 3
C3Hf	-	-	B10.Br	B10.A	4 ± 1

1. C3Hf (H-2^k) mice were s.c. immunized for DTH-related memory cells with B10.Br (H-2^k) or B10.ScSn (H-2^b) derived spleen cells, and boosted 3 months later.

2. Three days after s.c. immunization or booster the mice were challenged. At 24 hr after challenge the specific increase of foot thickness was determined. Figures represent the mean responses ± 1 SEM (n=5).

TABLE 5

BOOSTING OF MINOR H-ANTIGEN-PRIMED MICE WITH H-2 COMPATIBLE SPLEEN CELLS

Exp.		Responder[1]	Donor of the priming spleen cells	Donor of the boosting spleen cells	Shared region of the H-2 complex	Challenge (day of challenge)	Response[2]
I	a	BALB.B	B10.ScSn	B10.ScSn	K, I, S, D	B10.ScSn(3)	26 ± 2
	b	BALB.B	B10.ScSn	B10.Br	-	B10.ScSn(3)	19 ± 1
	c	BALB.B	B10.ScSn	-		B10.ScSn(3)	-1 ± 2
	d	BALB.B	B10.ScSn	-		B10.ScSn(90)	12 ± 2
II	a	C3Hf	AKR	B10.Br	K, I, S, D	B10.A (3)	30 ± 2
	b	C3Hf	AKR	B10.ScSn	-	B10.A (3)	38 ± 2
	c	C3Hf	B10.Br	-		B10.A (3)	-2 ± 1
	d	C3Hf	AKR	-		B10.A(120)	13 ± 4

1. Mice were primed by 10^7 allogeneic H-2 compatible spleen cells, boosted 3 months later with 1 x 10^7 allogeneic H-2 compatible or H-2 incompatible spleen cells and challenged 3 days after the booster injection.

2. Figures represent the mean response as measured 24 hr after challenge. ± 1 SEM (n=5).

cells induced a somewhat stronger secondary DTH-reactivity than did the B10.Br derived spleen cells.

In a similar set up, C3Hf mice which had been primed 4 months previously with 1 x 10^7 viable H-2 compatible AKR spleen cells, were boosted with either H-2 compatible B10.Br spleen cells or H-2 incompatible B10.ScSn spleen cells. Again, significant secondary DTH-reactivity was induced by both the H-2 compatible and H-2 incompatible spleen cells (Table 5, Exp. IIa and b).

DISCUSSION

The experimental data reported in this paper demonstrate that the expression of primary DTH-reactivity to minor H-antigens is restricted by H-2 coded products on the cells used for challenge. For primary DTH-reactivity this H-2 restricted recognition seemed only partial (Table 1), but in the less sensitive ILT-assay the H-2 restricted effector function was rather absolute (Table 2). The capacity of allogeneic H-2 incompatible spleen cells to elicit a DTH-reaction in mice, immunized with non H-2 alloantigens, might be explained by the assumption that host-derived macrophages phagocytized some of the H-2 incompatible cells. Thereby, these macrophages can present the minor H-antigens of the donor cells, together with the MHC-coded antigens of the host, to the activated T cells. This latter view might be supported by the complete H-2 restriction found in the ILT-assay i.e. the inoculum of immune lymph node cells contains relatively few macrophages, and phagocytosis of the surrounding tissue cells would thus not occur.

On the other hand, it is possible that the observed incomplete H-2 restriction in primary DTH to minor H-antigens is inherent in primary cell-mediated immunity to minor H-antigens. Experiments on CML to minor H-antigens suggest this possibility. Thus, spleen cells from normal mice are not capable of inducing primary in-vitro CML to minor H-antigens, but spleen cells derived from unprimed, autoimmune disease susceptible, New Zealand Black (NZB) mice are able to kill H-2 compatible cells, bearing non H-2 alloantigens, after primary in-vitro sensitization (Botzenhardt et al., 1978). It was found that these NZB-derived cytotoxic T cells, generated in vitro after stimulation with allogeneic H-2 compatible spleen cells cross-react to a considerable extent with H-2 incompatible target cells. In CML to minor H-antigens, induced by in-vivo priming of normal mice and in-vitro restimulation with the same antigens, it was shown that about 1% of the induced effector T cells could kill H-2 incompatible target cells (Bevan, 1977). The possibility thus exists that in primary DTH to minor

H-antigens, a number of the generated effector T cells is able to react to H-2 incompatible cells.

The H-2 restriction of T helper function in the humoral response to proteins is encoded in the I-region of the H-2 complex (Katz & Benacerraf, 1975), as well as DTH to thymus-dependent antigens (Vadas et al., 1977; Miller et al., 1977). In-vitro cytotoxicity both to cells bearing multiple minor H-antigens and to virus-infected target cells is restricted by K- and D-region coded antigens of the MHC (Bevan, 1975; Doherty et al., 1976). Similarly, the K- and D-region of the H-2 complex imposes restriction on DTH to virally infected target cells (Zinkernagel, 1976) and to cells bearing minor H-antigens (Table 1 and 2). On the basis of these findings it may be postulated that DTH may be mediated by T cells, belonging either to the cytotoxic T cell line (Lyt-$1^-2^+3^+$) or to the T helper cell line (Lyt-$1^+2^-3^-$), depending on the antigen to which the DTH-reactivity was induced.

Allogeneic H-2 incompatible cells, bearing non H-2 alloantigens, could induce both primary DTH-reactivity and DTH-related memory for these non H-2 allo-antigens. These findings are in harmony with the earlier in-vivo experiments on second set graft rejection by Snell (1957), and Gasser & Silvers (1972), who showed that allogeneic H-2 incompatible skin grafts could accelerate rejection of H-2 compatible grafts, presenting identical minor H-antigens to those of the first (set) graft. More recently, it was also demonstrated that H-2 incompatible male derived cells can presensitize female mice in vivo for CML to the male specific H-Y-antigen (Gordon et al., 1976). The capacity of H-2 incompatible spleen cells to prime for primary DTH and DTH-related memory and to boost for secondary DTH-reactivity may be due to processing of the allogeneic H-2 incompatible cells by host-derived macrophages. The present results show that priming with H-2 incompatible spleen cells is less effective for induction of primary DTH reactivity and DTH-related memory to minor H-antigens, than priming with allogeneic H-2 compatible spleen cells (Table 3). This difference in immunogenicity of H-2 compatible and H-2 incompatible spleen cells is probably not due to an immune response to the H-2 alloantigens. This can be deduced from the finding that simultaneous immunization with allogeneic H-2 compatible spleen cells and allogeneic H-2 incompatible, non H-2 compatible spleen cells hardly affected the response to the non H-2 alloantigens (Table 3).

It can be concluded that the expression of DTH reactivity to multiple minor H-antigens is certainly restricted by K- and D-region coded antigens, but a role of I-region coded antigens in the restriction of DTH could not be excluded. The immunogenicity

of minor H-antigens presented to unprimed animals was decreased when the immunizing cells were H-2 incompatible with the responder strain. This finding may point to a preferential recognition of minor H-antigens on H-2 compatible cells by the unprimed T cells. The residual capacity of H-2 incompatible spleen cells to induce primary DTH-reactivity and DTH-related memory and the booster capacity of H-2 incompatible spleen cells cannot be interpreted further in terms of H-2 restriction on the basis of the experiments reported in this paper.

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