REGULATION OF DELAYED TYPE HYPERSENSITIVITY

Cellular and genetic requirements, with emphasis on
the response to histocompatibility antigens

REGULATIE VAN DE VERTRAAGD TYPE
OVERGEVOELIGHEIDSREAKTIE
CELLULAIRE EN GENETISCHE VEREISTEN, MET DE NADRUK OP DE REAKTIE
TEGEN TRANSPLANTATIE ANTIGENEN

PROEFSCHRIFT

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Aan Liezeke
Aan mijn ouders

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VOORWOORD

Doel van het onderzoek

Gewervelde dieren beschikken over een immuunsysteem waarmee zij lichaamsvreemde elementen (antigenen) herkennen en onschadelijk maken. Het immuunsysteem onderscheidt zich van andere afweermechanismen, doordat het in staat is lichaamseigen (zelf) en lichaamsvreemd (niet-zelf) van elkaar te onderscheiden. Het is niet alleen betrokken bij de bescherming tegen ziekteverwekkers als bacteriën, virussen en parasieten welke het lichaam van buitenaf binnendringen, maar het is ook in staat om te reageren tegen bepaalde tumoren, welke binnen het lichaam (zijn) ontstaan.

De lymfocyten, die behoren tot de witte bloedcellen of leukocyten, staan centraal in het immuunsysteem. Lymfocyten laten zich op grond van hun funktionele eigenschappen in twee groepen onderscheiden, de B en T lymfocyten.

De B lymfocyten zorgen voor de z.g.n. humorale immuniteit, d.w.z. immuniteit die wordt veroorzaakt door oplosbare verbindingen, de z.g.n. antistoffen, ook wel antilichamen genoemd. Plasmacellen, de uitgerijpte nakomelingen van B lymfocyten, produceren deze antistoffen welke d.m.v. het bloed en de lymfe door het hele lichaam getransporteerd worden en daardoor op grote afstand van de antistofproducerende plasmacel hun werking kunnen uitoefenen. Chemisch gezien zijn antistoffen eiwitten, die immunoglobulinen (Ig) worden genoemd. Antistoffen spelen een belangrijke rol bij de afweer tegen bacteriën en virussen.

De T lymfocyten zorgen voor de cellulaire immuniteit, d.w.z. immuniteit die veroorzaakt wordt door een direkte interaktie tussen de T lymfocyt en het antigeen. Zo kan de interaktie van bepaalde T lymfocyten (cytotoxische T lymfocyten) leiden tot vernietiging van met virus geïnfekteerde lichaamscellen. Andere T cellen produceren faktoren welke op korte afstand hun werking hebben, zoals faktoren die fagocyten aanzetten tot het doden van intracellulair groeiende bacteriën (b.v. *Listeria monocytogenes*), of faktoren die vertraagd type overgevoeligheidsreakties teweegbrengen (b.v. een positieve Mantoux reactie bij mensen die een infektie hebben doorgemaakt met tuberkelbacillen). In tegenstelling tot de humorale immuniteit, welke met serum naar andere individuen kan worden overgedragen, kan de cellulaire immuniteit alleen via cellen worden overgedragen.

Naast de hierboven beschreven funkties vervullen T lymfocyten ook een belangrijke rol bij de regulatie van de immuunreaktie. T helper cellen zijn noodzakelijk voor de antilichaamvorming tegen bepaalde antigenen. T amplifier cellen

kunnen de immuunreaktie versterken, terwijl T suppressor cellen de immuunreaktie juist kunnen remmen.

Naast het vermogen tot specifieke herkenning van antigenen is een tweede kenmerk van het immuunsysteem het bezit van een 'geheugen'. Wanneer een individu een bepaalde infektie voor de eerste maal doormaakt, zal het enige dagen duren voordat het immuunsysteem specifiek reageert tegen de binnengedrongen ziektekiemen. We spreken dan van een primaire immuunreaktie. Bij een herhaald kontakt met hetzelfde antigeen is de afweerreaktie vaak sterker en komt deze sneller op gang. We spreken dan van een sekundaire immuunreaktie. Door deze snellere en sterkere immuunreaktie kunnen binnendringende ziektekiemen snel en effektief onschadelijk worden gemaakt, waardoor een infektieziekte wordt voorkomen. Op dit verschijnsel, dat meestal wordt aangeduid als immunologisch geheugen, berust de bescherming van vaccinatie tegen infektieziekten. Door de specificiteit van lymfocyten voor één bepaald antigeen of voor nauwverwante antigenen, is ter bescherming tegen verschillende infektieziekten voor elke ziekte een afzonderlijke vaccinatie met het betreffende antigeen nodig. Met andere woorden, het geheugen van het immuunsysteem is antigeen specifiek.

Om lichaamseigen 'antigenen' (z.g.n. 'zelf') te kunnen onderscheiden van andere antigenen ('niet-zelf') worden op de cellen van elk individu een aantal verschillende weefselkenmerken tot expressie gebracht. De genen die voor deze determinanten coderen zijn sterk polymorf. Dat wil zeggen dat van elke determinant die door één van deze genen wordt gecodeerd, verschillende typen bekend zijn. Dit brengt met zich mee dat elk individu een uniek patroon van erfelijk bepaalde weefselkenmerken bezit. Juist om deze reden is het bijzonder moeilijk om tussen individuen van dezelfde species weefsels uit te wisselen. Weefsels van een ander individu worden als 'niet-zelf' van de eigen weefsels onderscheiden, waarna het immuunsysteem wordt geaktiveerd en het transplantaat wordt afgestoten.

De genen welke coderen voor de belangrijkste weefselkenmerken zijn het eerst bij de muis ontdekt door de sterke transplantaat afstotingsreakties die werden waargenomen toen men (tumor-)weefsel transplanteerde van de ene ingeteelde muizestam naar de andere. Men heeft deze weefselkenmerken transplantatieantigenen of histocompatibiliteitsantigenen genoemd.

Bij de mens zijn de weefselkenmerken, die de sterkste afstotingsreakties veroorzaken, gelokaliseerd in één genencomplex. Dit genencomplex wordt aangeduid met de term 'Major Histocompatibility Complex' (MHC), bij de mens ook wel het HLA complex genoemd. Dergelijke MHC complexen zijn ook aangetoond bij de muis (H-2), de rat (Rt1), de hond (DLA) en bij tal van andere gewervelde dieren. Dit genencomplex speelt ook een belangrijke rol bij de afweer tegen allerlei bacteriële, virale en andere antigenen. T lymfocyten reageren namelijk alleen

tegen vreemde antigenen wanneer deze worden herkend in combinatie met 'zelf' (MHC) determinanten. Aan deze voorwaarde van antigeenherkenning in combinatie met 'zelf' herkenning wordt o.a. voldaan na antigeenpresentatie door fagocyterende cellen, die het antigeen presenteren op het celoppervlak in combinatie met MHC determinanten, of door herkenning van virusantigenen in combinatie met MHC determinanten op de membraan van geïnfekteerde lichaamscellen.

Verschillen in een of meerdere loci van het MHC tussen donor en ontvanger vormen bij klinische toepassing van orgaan- en weefseltransplantatie nog steeds het belangrijkste probleem door de sterke afstotingsreakties die deze verschillen veroorzaken. Om afstotingsreakties te voorkomen wordt gebruik gemaakt van verschillende immunosuppressiva. Deze middelen hebben een onderdrukking van het immuunsysteem tot gevolg, die niet beperkt is tot de afweer tegen het getransplanteerde orgaan of weefsel. Hierdoor zijn getransplanteerde patiënten vaak erg vatbaar voor infekties. Bovendien is er, om onduidelijke redenen, een verhoogde kans op het ontstaan van tumoren bij patiënten die met immunosuppressiva worden behandeld. Daarnaast hebben immunosuppressiva nog andere nadelige bijwerkingen. Om deze problemen te vermijden is het wenselijk om de afstotingsreaktie tegen de transplantatie antigenen van de donor selectief te onderdrukken, terwijl de rest van het immuunsysteem ongestoord blijft funktioneren. Hiervoor is een grotere kennis nodig van de regulatie van de immuunreaktie tegen transplantatie antigenen. In dit proefschrift worden experimenten beschreven waarbij gebruik gemaakt is van een muizen-proefdiermodel. Bij veel transplantaatafstotingsreakties speelt de cellulaire immuniteit een centrale rol. Eén van die vormen van cellulaire immuniteit is de vertraagd type overgevoeligheidsreaktie. In dit proefschrift wordt ons onderzoek beschreven naar de regulatie van vertraagd type overgevoeligheidsreakties tegen transplantatie antigenen.

ABBREVIATIONS

ABA	Azobenzenearsonate
APC	Antigen-presenting cell
ATS	Anti-thymocyte serum
ATx	Thymectomy of adult mice
B cell	Bone marrow derived lymphocyte
BCG	Bacillus Calmette Guérin
BSS	Balanced salt solution
CS	Contact sensitivity
CTL	Cytotoxic T lymphocyte
CTL-P	Cytotoxic T lymphocyte precursor
CY	Cyclophosphamide
DLA complex	Major-histocompatibility complex of the dog
DNFB	2,4-dinitro-1-fluorobenzene
DTH	Delayed type hypersensitivity
GvH	Graft-versus-Host
H-antigen	Histocompatibility antigen
H-2 complex	Major histocompatibility complex of the mouse
HLA complex	Major histocompatibility complex of man
HRC	Horse red blood cell
HvG	Host-versus-Graft
H-Y	Male-specific histocompatibility antigen
ip	Intraperitoneal
Igh gene	Gene locus coding for the heavy chain of immunoglobulin molecules
ILT	Immune lymphocyte transfer
Ir gene	Immune response gene
iv	Intravenous
LCMV	Lymphocytic choriomeningitis virus
LDH-B	Lactate dehydrogenase B
MHC	Major histocompatibility complex
MLR	Mixed lymphocyte reaction
Mls-locus	Minor lymphocyte stimulating locus
MW	Molecular weight
NLT	Normal lymphocyte transfer
NRS	Normal rabbit serum
PTL-P	Proliferating T lymphocyte precursor
PPO	2,5-diphenyloxazole
Rt1 complex	Major histocompatibility complex of the rat
sc	Subcutaneous

ShSx	Sham splenectomy
ShTx	Sham thymectomy
SRBC	Sheep red blood cell
SRC	Sheep red blood cell
Sx	Splenectomy
T cell	Thymus derived lymphocyte
Ta cell	Amplifier T cell
Tdth	DTH reactive T cell
Tc cell	Cytotoxic T cell
TDL	Thoracic duct lymphocyte
Th cell	Helper T cell
T'HRC	Horse red blood cell specific helper T cell
Ts cell	Suppressor T cell
TsF	Suppressor T cell factor
T'SRC	Sheep red blood cell specific helper T cell
T1	Sessile T lymphocyte, short-lived after ATx
T2	Recirculating long-lived T lymphocyte, sensitive to ATS *in vivo*

CHAPTER I

GENERAL INTRODUCTION

The failure of successful exchange of tissues and organs between individuals of the same species is mainly due to the expression of histocompatibility (H) antigens on the cell surface of the transplanted tissues. Every individual has a unique set of genetically determined H-antigens.

Gorer and Snell (1) found in their studies with inbred mouse strains that a single locus, situated on chromosome 17, controlled a cell surface antigen (which they called, antigen II) that elicited remarkable rapid allograft rejection. Snell (2) suggested that loci determining the fate of allografts should be referred to as histocompatibility antigens. The locus controling antigen II was called therefore histocompatibility antigen 2 locus or H-2 locus. Later studies (3, 4) showed that H-2 covered a cluster of loci. This complex of loci is called Major Histocompatibility Complex or MHC. Similar MHC's have been described in man (HLA complex) (5), in rat (Rt1 complex) (6), in dog (DLA complex) (7) and in several other vertebrates (8).

Because of their capacity to induce rapid allograft rejection, the MHC coded antigens were called 'strong' or 'major' histocompatibility antigens and the antigens coded for by other loci were referred to as 'weak' or 'minor' histocompatibility (H) antigens (9). A striking quality of most MHCs is their polymorphism. The significance of this polymorphism is still a matter of speculations (10).

The experiments described in this thesis deal with the regulation of the immune response of mice against H-antigens. Therefore the structure and function of the MHC of the mouse will be described in more detail.

1.1. The H-2 complex

Since the discovery of the H-2 complex, a large number of different traits have been associated with this gene complex (11). However, the main natural function, which can be ascribed to the MHC up-till now, is the control or guidance of the immune system (12, 13). Obviously, the fact that MHC molecules are able to function as antigens in case tissues are transplanted from one individual to another is non-physiological.

Very complicated maps and schemes of the H-2 complex have been presented in which all the *in vivo* and *in vitro* immunological traits are indicated. Recently, Klein (12, 14) published a more convenient map (fig. 1) This map shows primarily the well defined loci of the H-2, the molecules coded for by these loci and the

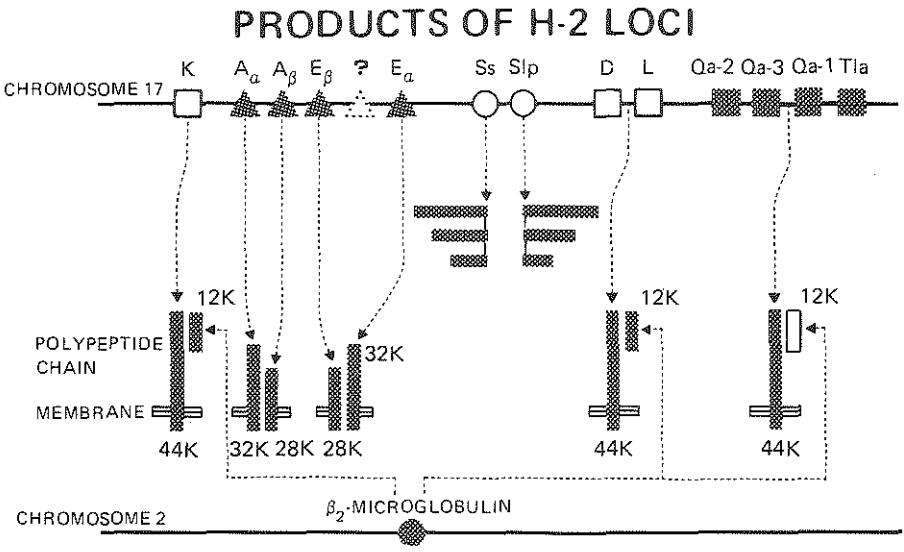

Fig. 1. Loci composing the mouse H-2 complex and their products (adapted from J. Klein (14)).

The various loci of the H-2 complex are divided into four classes. K, D and L, usually indicated as H-2K, H-2D and H-2L, represent class I loci. Each of these loci is coding for a glycoprotein heavy chain of 43.000 to 45.000 molecular weight (MW) (15). The heavy chain is non-covalently bound to a light chain. The light chain, β_2-microglobulin, is a glycoprotein with a MW of about 12.000, which is coded for by a locus on chromosome 2 of the mouse. Class I products are expressed on cells of all somatic tissues. The concentration of these molecules on macrophages and lymphocytes is higher than on other cells (15, 16).

A_α, A_β, E_β and E_α are class II loci. These loci are situated together in one region of the H-2 complex, which is called the H-2I region. The class II molecules A and E are heterodimers composed of an α chain, a 32.000 to 35.000 MW glycoprotein (A_α and E_α), and a β chain, a 28.000 to 31.000 MW glycoprotein (A_β and E_β) (17). The A and E molecules are almost exclusively expressed on macrophages and B lymphocytes (16, 17). Several H-2 maps indicate a J locus in between E_β and E_α. The products coded for by the J locus have been mainly serologically defined. The existence of a J locus inside the H-2I region is still a matter of debate (18). The genetic code between E_β and E_α appeared to be too small for all the traits of the J locus (17). The J locus has been claimed to code for one or more soluble T cell factors and cell surface antigens on macrophages and T cells, which may be involved in T cell suppression (16).

Class III loci cover two S loci situated in the H-2S region, which are coding for some serum proteins (C4) (16), while class IV loci are situated in the H2-Tla region (19). The Tla locus is coding for an antigenic determinant on thymocytes and certain leukemias. Other loci of the Tla region are Qa-1, Qa-2 and Qa-3 which are coding for antigenic determinants on different lymphocyte subpopulations. Some molecules like the products of Tla and Qa-2 are strikingly similar to the products of the K and D loci. All have a MW of approximately 44.000 dalton and are associated with β_2-microglobulin.

association between these molecules or the association of H-2 molecules with molecules coded for outside the H-2 complex when they are expressed on the cell surface. The most important loci are situated in the K, I and D region of the H-2 complex. The K and D loci are coding for so-called class I molecules and the I loci for class II molecules.

The products of two different alleles of a single locus, which are inherited from both parents, are codominantly expressed on the cell surfaces of the offspring.

1.2. Genetic aspects of immune regulation

1.2.1. H-2 restricted recognition

One of the main functions of the MHC molecules is to serve as markers of self in the recognition of non-self by T lymphocytes. Zinkernagel and Doherty (20) described this function of H-2 molecules for the first time. T lymphocytes from lymphocytic choriomeningitis virus (LCMV) infected mice could lyse LCMV infected targets only if they expressed the same H-2 molecules as the donor of the effector cells. H-2K and/or H-2D molecules were found to be the restriction elements for the cytotoxic T lymphocytes (21). *In vitro* studies also showed that helper activity by helper T cells needed for antibody formation depends on presentation of antigen by macrophages which express the appropriate H-2IA restriction element (22). Delayed type hypersensitivity (DTH) experiments showed that the *in vivo* immune response is also subject to H-2 restriction. DTH reactions against virus infected cells were H-2K/H-2D restricted (23) and DTH against *Listeria monocytogenes* was found to be restricted by H-2I coded molecules (24).

The need for self recognition can be explained from a teleological viewpoint (25). Cytotoxic T cells lyse virus infected cells to prevent viral replication and do not interact with free virus particles, because the virus infected cells are the most important threat for survival. Interaction with free virus would be very inefficient. Viruses are able to infect different cell types. As class I molecules are expressed on all cell types, recognition by cytotoxic T cells of virusantigen in combination with K and/or D restriction molecules on a cell's surface is an appropriate signal to lyse an infected cell (23, 25).

For intracellularly growing bacteria and parasites the situation is different. When cells, expressing the antigens of intracellularly growing bacteria and class I molecules, would be lysed by cytotoxic T cells, the bacteria would not be eliminated. Intracellularly growing pathogens are only internalized by phagocytic cells like macrophages. These cells bear next to class I molecules, other restriction elements, namely class II molecules (25, 26). Recognition of bacterial antigens in

combination with class II restriction molecules, is sufficient for activation of helper type T cells, which in turn can activate macrophages to kill the intracellular bacteria.The requirement of class II restricted recognition of bacterial antigens by helper type T cells prevents inefficient interaction of these T cells with free bacteria.

Restricted recognition has also been described for 'non physiological' immune responses like *in vivo* responses against H-antigens. DTH effector cells specific for minor H antigens appeared to be H-2K/D restricted (27), while Korngold and Sprent (28) described H-2 restricted recognition of minor H antigens in a Graft-versus-Host (GvH) mortality assay.

1.2.2. Immune response genes

The magnitude of the T cell responses to a number of antigens, including T cell dependent humoral immune responses, has been shown to be regulated by the H-2 complex. Benacerraf and McDevitt (29) showed that the phenomenon of antigen-specific high and low responsiveness in guinea pigs and mice was genetically determined. The immune response (Ir) genes responsible for this phenomenon have been mapped within the MHC (30, 31).

Two major mechanisms are proposed for the Ir gene function (26, 32, 33). The Ir genes could operate by controlling the T cell receptor repertoires or by controlling antigen presentation to T cells. These hypotheses explain the non-responder status by a gap in the T cell repertoire and an inadequate association between MHC molecules and foreign antigens on the surface of antigen-presenting cells, respectively. No conclusive evidence for either hypothesis has been published as yet (34 – 36).

Other studies showed that the non-responder status of certain mouse strains depends on suppressor T cells (37, 38). The suppressor T cells, which suppress the antibody formation against the antigen lactate dehydrogenase B (LDH-B), are only induced by LDH-B in the context of H-2E molecules of the k or b haplotype.

1.3. Cellular aspects of immune regulation

1.3.1. Memory lymphocytes

Immunization not only induces the generation of antigen specific effector lymphocytes, but also the generation of antigen specific immunological memory.

In general, immunological memory is defined by a more vigorous and faster immune response after secondary contact with the specific antigen. B cell as well as T cell memory occur (39).

B cell memory is characterized by quantitative as well as qualitative differences as compared to the naive situation (40). T cell memory, on the other hand, is characterized by a quantitative change only (41, 42). T cell memory is the result of

an increased frequency of antigen specific T lymphocytes. In contrast to virgin lymphocytes, memory lymphocytes are long-lived small lymphocytes. For secondary type humoral immune responses (43, 44) and for a memory phenomenon of cellular immunity like resistance against *Bacillus Calmette Guérin* (45), it has been shown that the memory T cells have the capacity to recirculate.

In vitro assays for mixed lymphocyte reactions and cytotoxic T cell responses demonstrate more vigorous responses after secondary than after primary contact with the antigen (46, 47). Limiting dilution analyses show a considerable frequency raise of proliferating T cell precursors (PTL-P) and cytotoxic T cell precursors (CTL-P) (47). However, the results from *in vitro* studies are not necessarily representative for the *in vivo* situation. Ryser *et al.* (47) have shown that *in vivo* immunization with H-2I alloantigens hardly affects the frequency of the allo-antigen specific PTL-P, while the 3 to 4 times increase of the H-2K and H-2D specific CTL-P upon *in vivo* immunization with these antigens is relatively small as compared with the increase under *in vitro* conditions.

1.3.2. Enhancing T-T cell interactions

Synergism between T cells has been first described by Cantor and Asofsky (48, 49). They found that two populations of T lymphocytes synergize in GvH reactions, measured with a splenomegaly assay. One class, T1 cells, is found mainly in spleen and thymus and is resistant to *in vivo* treatment with anti-thymocyte serum (ATS) and sensitive to adult thymectomy (ATx). The second class, T2 cells, is susceptible to ATS treatment *in vivo* and is ATx resistant.

T1—T2 cell synergism has also been demonstrated *in vitro* for the helper function in anti-hapten antibody formation (50) and in mixed lymphocyte reactions (51, 52) and *in vivo* in alloantigen specific DTH assays (53, 54). In the DTH studies, the T1 and T2 cells were activated against alloantigens under GvH conditions. It could be demonstrated that DTH effector T cells specific for alloantigens coded for by genes of the H-2I region (53) or the Mls locus (54) were the progeny of long-lived, recirculating T2 cells. The responses could be amplified by short-lived, sessile T1 cells. Activation of amplifier cells was induced by H-2K or minor-H coded alloantigens.

1.3.3. Suppressor T lymphocytes

The induction of immune reactivity and tolerance depends on the dose of antigen (55, 56), on the route of antigen administration (56, 58) and on the antigen form (57 – 59). Tolerance may depend on either shortage of antigen reactive cells or on active suppression mediated by suppressor T cells (58, 60, 61).

The first description of suppressor T (Ts) cell activity was given in 1970 by Gershon *et al.* (62). From that moment on, Ts cell activity has been established

in a variety of assays, concerning different immune functions.

Suppression of cellular immunity of mice by intravenous (iv) preimmunization is a well-known phenomenon. Most of the studies deal with suppression of DTH reactivity against heterologous erythrocytes (63, 64) and haptens (56, 58 – 60). Some of them have shown that the development of suppressor cell activity depends upon complex interactions of three subsets of Ts cells in a regulatory circuit under control of Igh and H-2 genes (61). The first subset of suppressor cells, Ts1, affects the induction phase of the immune response, while Ts2 and Ts3 are involved in suppression of the expression phase. These suppressor cells are acting by means of suppressor factors (TsF). Since T cell hybridomas have been made from the three different subsets of Ts cells, producing TsF1, TsF2 and TsF3, respectively (65), the function and composition of the suppressor factors can be studied (66).

Iv induction of Ts cells have also been described in assays dealing with cellular immunity against H-antigens, e.g., suppression of skin and heart allograft rejection (67 – 69). However, in these experiments, in addition to iv preimmunization with donor antigen, pretreatment with antilymphocyte serum (67) or cyclophosphamide (68) is necessary to reveal the suppressive effect by the Ts cells.

1.4. Anti-allograft reactivity

Antibody as well as cellular mechanisms may account for allograft rejection. Generally, antibody plays a minor role. Most attempts to reproduce organ graft rejection by adoptive transfer of immune sera have been unsuccessful (70, 71). Administration of antibodies specific for the alloantigens of the graft can even result in a prolonged survival (72, 73). This phenomenon has been called *enhancement* (74), and is highly dependent on the Ig (sub)class of the specific antibodies, IgG1 and IgG2 being the most effective (75). An oversimplification should not be made, because antibodies can also cause 'hyperacute' allograft rejection, e.g., in the case of kidney allografts (72).

Medawar *et al.* (76) described already in 1958 the correlation between DTH reactivity and skin graft rejection. Govaerts (77) suggested in 1960 the involvement of cytotoxic lymphocytes in allograft rejection.

For a long period the cytotoxic T cell was supposed to be the major effector of graft rejection (78). However, recent immunogenetic studies showed a strict correlation between (H-Y specific) DTH and skin graft rejection. Both events appeared to be controlled by the same Ir genes, while H-Y specific cytotoxic T cells are controlled by other Ir genes (79, 80). Transfer studies with different T cell subpopulations in mouse (81) and rat (82) showed that skin and organ allograft rejection is mainly dependent on lymphoid cells of the T helper/DTH phenotype. Moreover, these studies also showed a close correlation between skin graft rejection and DTH reactivity after reconstitution of the irradiated recipients

with lymphoid cells of the T helper/DTH phenotype. Thus, helper/DTH reactive T cells play an important role in allograft responses.

1.5. Clinical transplantation

After blood transfusion, kidney transplantation is the most common application of clinical transplantation. Also in the human situation the MHC coded antigens (HLA) induce the strongest allograft reactions. Therefore, HLA typing is done of the kidney donor and prospective recipients, in order to be able to choose the most compatible combination (83, 84). Because of the polymorphism of the HLA loci, incompatibilities between donor and recipient are, with some exceptions, inevitable. The anti-allograft response of the recipient is still the major problem of clinical organ transplantation. All patients, who receive allogeneic transplants, are treated with immunosuppressive drugs like cyclophosphamide, azathioprine and cortison to prevent or minimize allograft rejections (85). Another example of immunosuppression is treatment of patients with anti-thymocyte serum (ATS) (86). This is a xenogeneic antiserum specific for the recipients' lymphocytes, particularly T lymphocytes. These agents nonspecifically affect the immune system. As a consequence, they decrease the general body resistance against infections. Furthermore, they increase the incidence of malignancies and have several other undesirable side effects (85). More recently a promising immunosuppressive drug, the antibiotic cyclosporin A, has been introduced. The first randomized clinical trials on the effect of cyclosporin A as compared to conventional immunosuppressive therapy seem to show an up to 20% increased survival after 1 year in patients receiving cyclosporin A (87). Among other effects cyclosporin A has been claimed to interfere with the acquisition of responsiveness to growth factors by T lymphocytes (88, 89).

In 1973 Opelz *et al.* (90) described the beneficial effect of preoperative blood transfusion on kidney transplant survival. Initially, the report about the beneficial effect of blood transfusion was a matter of disagreement. Since other studies confirmed the original observation, the beneficial effect of blood transfusion by itself has become generally accepted (83, 91). Several explanations for the phenomenon have been given (92). The possibility that the transfusion effect may depend on selection of 'nonresponder' patients has been rejected by most of the investigators who are studying the transfusion effect. Several studies suggest that specific anti-idiotypic antibodies and/or enhancing antibodies play a role in the transfusion effect. Many investigators suggest that Ts cells are involved in the blood transfusion effect.

Recently, Terasaki (93) presented the hypothesis that the blood transfusion effect is due to priming for a secondary anti-allograft response. A patient primed by preoperative blood transfusions should respond with a strengthened and accelerated anti-allograft response. An earlier appearing anti-allograft response

should be more vulnerable for the immunosuppressive drugs which are applicated in highest dosages during the period just after transplantation. This hypothesis is attractive by its simplicity. However, it bypasses the dominant suppression by blood transfusion, which has been demonstrated in several studies (67 – 69, 92).

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CHAPTER II

INTRODUCTION TO THE EXPERIMENTAL WORK

Delayed type hypersensitivity reactions can be elicited in sensitized individuals by local application of antigen. The expression of a local DTH response is characterized by infiltration of inflammatory cells and accumulation of edematous fluid at the site of antigen application (1). The onset of this inflammatory reaction is strictly dependent on the activation of T lymphocytes by the specific antigen. The activated lymphocytes release vasoactive factors which cause influx of other lymphocytes, mononuclear cells and granulocytes to the site of antigen application (2, 3). DTH responses are maximal about 24 to 96 hr after elicitation. The phenomenology of DTH varies considerably and depends on the species tested and the antigen used (4).

In mice, DTH can be elicited easily by subcutaneous (sc) injection of a 'challenge' dose of the specific antigen in a hind foot of sensitized mice. The increase of foot thickness of the sensitized animals is corrected for the non-specific swelling caused by antigen injection in the foot of naive animals. The corrected response is the *antigen specific* DTH response. DTH responses in mice have been induced with a variety of antigens e.g., fungal, protozoal and viral components, chemically defined molecules, serum proteins, heterologous erythrocytes and H-antigens (1). The DTH assay is a quick and reliable assay for *in vivo* studies on cellular immunity.

In this thesis the genetic and cellular regulation of *in vivo* cellular immunity of mice against conventional- and H-antigens was studied by means of the DTH assay. The existence and the availability of a large number of different congenic mouse strains and of different H-2 recombinant mouse strains make the mouse very suitable for these studies (5). DTH constitutes one aspect of the *in vivo* cellular immune response, and plays an essential role in anti-allograft reactions (viz. 1.4). Earlier studies on our laboratory were focussed a.o. on the role of T cell subsets in DTH against SRBC (6) and H-antigens (6-8), on the requirements for H-2 restricted recognition of DTH against minor H-antigens (9) and on the induction and expression of secondary type DTH against SRBC (10, 11) and minor H-antigens (12).

The studies described in this thesis throw light upon new aspects like the relation between helper T cells and DTH reactive T cells, the recirculation properties of memory cells involved in secondary type DTH responses, the genetic requirements for the induction of primary and secondary DTH reactivity against

H-2 coded alloantigens, and upon the specificity of and the cellular and genetic requirements for iv induced suppression of alloantigen specific DTH.

A better understanding of the regulation of cellular immunity against H-antigens will be useful for a better understanding of immune reactivity against 'natural' antigens. Furthermore, it will, in the long run, be helpful to develop more specific immunosuppression therapies for clinical transplantation.

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CHAPTER III

CLONES OF HELPER T CELLS MEDIATE ANTIGEN-SPECIFIC, H-2-RESTRICTED DELAYED TYPE HYPERSENSITIVITY

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It is now well established that in the mouse, helper T cells and killer T cells are two distinct thymus-derived lymphocyte subpopulations, differing from each other in Lyt phenotype and H-2 restriction, among other parameters. Helper T cells are Lyt-1$^+$ and their action in immune responses involves restriction at the H-2I region of the major histocompatibility complex (MHC) (1, 2). Killer cells, on the other hand, are Lyt-23$^+$ and their activity is restricted by H-2K/D (1, 3). In most instances, T cells mediating delayed-type hypersensitivity (DTH) responses share the Lyt phenotype and H-2 restriction of the helper T cell (4-7). This raises the question of whether or not helper activity and DTH can be mediated by the same activated T cell. Arguments for both views have been reported (8-12). We analysed this question using clones of specific helper T cells, which were obtained by long-term culture in vitro *of* in vivo *primed T cells, followed by single-cell cloning (13-15). Here we show that these clones of helper T cells mediate antigen-specific and fully H-2-restricted DTH. The restricting element lies to the left of the I-B region in genetic maps of the mouse MHC.*

Long-term cultured, cloned and subcloned sheep or horse red blood cell-specific helper T cells (T'_{SRC}, T'_{HRC} respectively), which have been maintained for 5-24 months in culture and previously shown to function as helper T cells *in vitro* (13-15) and *in vivo* (15), were examined for DTH activity. This DTH responsiveness was assayed by means of the immune lymphocyte transfer assay (16). As few as 1,000 cloned helper T cells, injected together with SRC into syngeneic recipients, caused a significant DTH response which increased in severity as the dose of T cells increased (Fig. 1). In comparison with *in vivo* activated lymph node cells, the cloned helper T cells were far more effective on a per cell basis. At least 10^3 times more *in vivo* activated cells were required to induce a response of similar magnitude (Fig. 1, right). As controls, the other footpad of the mice was injected with cloned helper T cells without antigen or with an irrelevant antigen – in neither case was a DTH response induced. DTH responses were mediated equally well by other clones of T'_{SRC} and T'_{HRC} and by four different clones of egg albumin-specific helper T cells when injected together with the relevant antigen.

The H-2 restriction of cellular interactions involved in DTH responses (17) was examined using a clone of T'_{SRC} and a subclone of T'_{HRC}. Absolute H-2 restriction was observed in both instances. Results obtained with T'_{SRC} (clone 26-14) are shown in Fig. 2. The C57BL/6J-derived cloned helper T cells mediated DTH responses only in the C57BL/6J and B10.A(5R) recipients. B10.HTT mice (incompatible with C57BL/6J mice for the whole H-2 complex) and B10.A(4R) mice (incompatible at H-2K and I-A) did not show any DTH reactivity on injection of the cloned helper T cells plus antigen. It should be stressed that this results was found after transfer of 3.3×10^4 viable T cells, which is about 30

times the number of cells required to induce a significant response in a syngeneic combination. This result shows that the T cell-macrophage interactions which underlie these DTH responses (17) are restricted by either H-2K or I-A. Appropirate congeneic strains were not available at the time of these experiments for distinguishing between K- or I-A-region restriction. However, the action of helper T cells in immune responses *in vivo* has been demonstrated to involve restriction only at I-A (2).

The antigen-specific and H-2-restricted induction of DTH response with cloned helper T cells in normal mice does not exclude the participation of host T cells at the effector level. We have therefore the DTH response induced by cloned helper T cells in congeneic *nu/nu* mice (Fig. 2). The DTH response in these T cell-deficient animals showed the same kinetics as and was of similar magnitude to that observed in thymus-bearing, I-A-compatible mice. This result indicates that host T cells are not essential for the response and that the cloned helper T cells are the DTH effector cells.

Many investigations have attempted to establish the relationship between helper T cells and DTH effector cells (8-12). Although some studies pointed to an inverse relationship between the *in vivo* occurrence of activated helper T cells and DTH effector cells (8-10), others have found that helper activity and DTH can coincide (11, 12). These experiments were carried out with uncloned T-cell populations and failed to prove that both responses can be mediated by the same cell. However, our experiments show unequivocally that helper T cells can mediate DTH responses which were observed not only in the local immune lymphocyte transfer assay, but also after intravenous injection of these cells. Mice infused with 1-3 x 10^6 cloned helper T cells responded to antigen challenge to the hind footpad with a smaller but significant DTH response. This requirement for much higher numbers of cells may be due to changes in recirculation and homing properties brought about by long-term *in vitro* propagation. Similar observations were made in clonally reconstituted *nu/nu* mice where 1-5 x 10^6 of the same cloned helper T cells were required to induce specific antibody responses comparable with or higher than those observed in thymusbearing mice (15).

Obviously, our results do not argue against the possibility that T cells other than helper T cells may also mediate DTH reactivity. Several studies have shown that DTH to allogeneic cells can be transferred by Ly-2^+ cells (7) and that contact sensitivity to dinitrofluorobenzene (6) and DTH to non-H-2 alloantigens (18) and viral antigens (19) can be H-2K/D restricted. Indeed, it has been reported that T cells mediating DTH and cytotoxic T cells could not be separated by size, density or antigen-binding properties (20).

The clones of helper T cells used for these studies have been shown to release

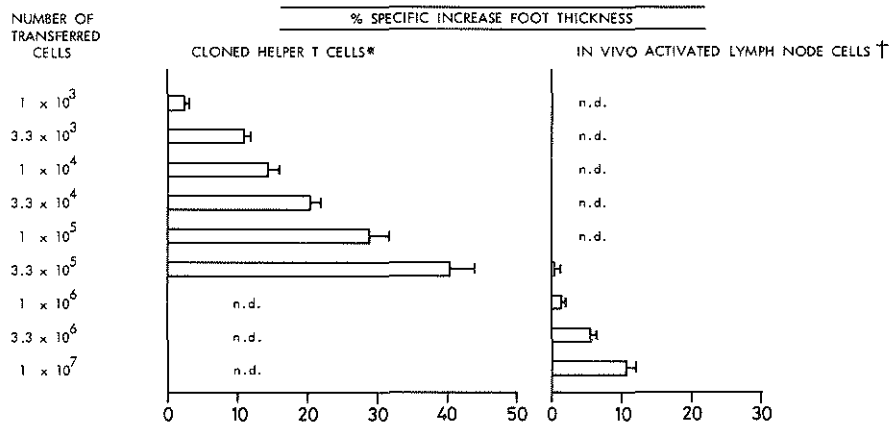

Fig. 1. DTH sensitivity by different numbers of long-term cultured C57BL/6J-derived, SRC-specific helper T cells (clone S26-14) (left), and by different numbers of lymph node cells which were activated *in vivo* by subcutaneous immunization of C57BL/6J mice with 2 x 10^7 SRC (right). The cells to be tested were injected together with 5 x 10^7 SRC in a volume of 50 μl into the instep of the right hind leg of C57BL/6J female mice (Institut für Biologisch-Medizinische Forschung AG). Recipient mice injected with cloned SRC-specific helper T cells plus SRC in the right hind leg received the same number of HRC-specific helper T cells (subclone 18-33-3) together with 5 x 10^7 SRC in the left hind leg. Recipient mice which received *in vivo* activated lymph node cells plus SRC in the right hind leg received the same number of non-activated lymph node cells and 5 x 10^7 SRC in the left hind leg. At 24 h after injection the thickness of the hind feet was measured. DTH responses are expressed as the specific % increase in foot thickness, and were calculated as (thickness right foot – thickness left foot)/thickness left foot x 100%. Horizontal bars represent 1 s.e.m. (n = 7). ND, not determined. *, The establishment and maintenance of the long-term cultured specific helper T cells has been described previously (13-15). †, The activated lymph node cells were obtained by collecting the inguinal and axillary lymph nodes of C57BL/6J mice 4 days after subcutaneous immunization with 2 x 10^7 SRC, equally distributed over the inguinal and axillary areas. The non-activated lymph node cells were obtained from naive C57BL/6J mice.

several biologically active mediators, the induction of which is strictly antigen specific and H-2 restricted (13, 14, 21-23). So far, factors affecting B-cell (21, 22) and T-cell (14) growth and *in vitro* colony formation by granulocytic, macrophage and erythroid precursor cells (23) have been discerned. Soluble factors have been indicated in the induction of the DTH lesion (24). Histologically, the DTH response mediated by cloned helper T cells in *nu/nu* and normal mice was indistinguishable from other Jones-Mote-type DTH responses.

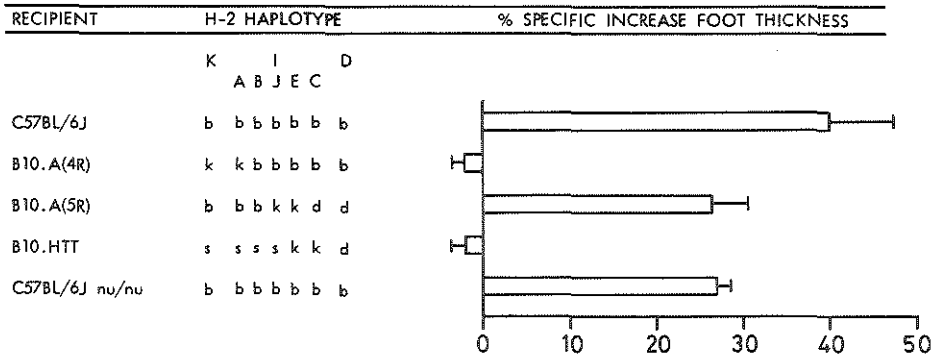

Fig. 2. DTH reactivity mediated by cloned SRC-specific helper T cells was tested for H-2 restriction. Recipient mice (all from the Institut für Biologisch-Medizinische Forschung AG) were injected with 3.3 x 10^4 SRC-specific helper T cells (clone S26-14) and 5 x 10^7 SRC in a volume of 50 μl in the instep of the right hind foot, and with 3.3 x 10^4 HRC-specific helper T cells (subclone 18-33-3) and 5 x 10^7 SRC in the instep of the left hind foot. At 24 h after transfer the thickness of the hind feet was measured. DTH responses are expressed as the specific percentage increase in foot thickness and calculated as described in Fig. 1 legend. Horizontal bars represent 1 s.e.m. (n = 7).

The nature of the cellular infiltration strongly indicates the release of chemotactic factors. Clones of helper T cells may help to characterize these activities. In addition, the DTH response is the most rapid assay to date for screening cloned and uncloned helper T cells for specificity and H-2 restricted activity. The assay is as sensitive and reproducible as the *in vitro* assay for specific helper activity. Moreover, the cellular nature of the I-A-bearing, antigen-presenting cells may be identified by their simultaneous injection with helper T cells into I-A-incompatible hosts.

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CHAPTER IV

SECONDARY DELAYED TYPE HYPERSENSITIVITY TO SHEEP RED BLOOD CELLS AND MINOR HISTOCOMPATIBILITY ANTIGENS IN MICE: TRANSFER OF MEMORY BY RECIRCULATING THORACIC DUCT LYMPHOCYTES

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ABSTRACT

Secondary delayed-type hypersensitivity (DTH) in mice to sheep red blood cells (SRBC) and minor histocompatibility (H) antigens is dependent on long-lived memory T cells. In this paper we investigated whether these memory T cells recirculate. It was shown that late phase 'immune' thoracic duct lymphocytes (TDL) from mice which were immunized with SRBC or non-H-2-incompatible spleen cells several weeks previously could adoptively transfer secondary DTH to these antigens. Passing the immune TDL through intermediate recipients demonstrated that these SRBC- or minor H-antigen-reactive memory T cells recirculate from blood to lymph. In contrast to mice immunized with minor H antigens, no secondary type DTH reactivity could be demonstrated in mice immunized with H-2-incompatible spleen cells. Also after adoptive transfer of TDL from mice immunized with H-2 alloantigens, it was impossible to demonstrate an accelerated DTH reactivity.

INTRODUCTION

Previous studies from our laboratory have shown that the secondary type responsiveness for delayed-type hypersensitivity (DTH) to sheep red blood cells (SRBC) (1) and to minor histocompatibility (H) antigens (2) in mice is a long-lived memory phenomenon. The secondary type DTH is characterized by an accelerated reappearance of the state of DTH after booster injection of the antigen. Adoptive transfer experiments with spleen cells from mice primed with SRBC or minor H antigens indicate that this memory phenomenon is dependent on Thy-1.2$^+$ cells (1, 2). Parabiosis of nonprimed mice and mice primed with SRBC indicate that these DTH-related memory T cells are long-lived potentially circulating cells (3).

Studies by others investigating as to whether the long-lived memory cells responsible for secondary type cellular immune responses recirculate or mainly belong to a resident population suggest that memory T cells can have a variable nature.

Sprent and Miller (4, 5) have shown by means of thoracic duct cannulation that the progeny of T cells activated *in vivo* against H-2 antigens were highly enriched in long-lived recirculating T lymphocytes reactive to determinants expressed by particular tumor allografts. However, the same population of recirculating thoracic duct lymphocytes was unable to mediate increased graft-versus-host (GvH) or mixed lymphocyte reactivity. Hall *et al.* (6) have confirmed the data of Sprent and Miller (4, 5) that memory T cells involved in second-set allograft rejection can occur in the lymph of sensitized donors but, in contrast, showed that the bulk of these long-lived memory T cells are non-recirculating. Other memory phenomena in cell-mediated immunity were also found to be heterogeneous with respect to the characteristics of the cells involved (7, 8).

In this paper we investigate by means of thoracic duct drainage whether the long-lived memory T cells responsible for secondary DTH to SRBC and minor H antigens occur in the lymph of sensitized mice and whether these memory T cells recirculate.

MATERIALS AND METHODS

Animals

Female BALB/c (H-2^d) mice were purchased from the Laboratory Animals Centre of the Erasmus University, Rotterdam, The Netherlands. Female B10.ScSn (H-2^b) and B10.D2 (H-2^d) mice were purchased from Olac Ltd., Bicester, U.K. The age of the responder mice varied between 12 and 24 weeks.

Preparation of cell suspensions

Mice were killed by carbon dioxide. Spleens or lymph nodes were removed, brought into a balanced salt solution (BSS), and squeezed through a nylon gauze filter to give single cell suspensions. Bone marrow was collected from femora and tibiae and prepared for a single cell suspension as described previously (9). Nucleated cells were counted with a Coulter counter Model B. Viable nucleated cells were counted in a hemocytometer using 0.2-% trypan blue in BSS as a diluent.

Antigen and immunization

Sheep red blood cells were kindly provided by the Central Veterinary Institute, Rotterdam, The Netherlands. The cells were stored and washed as described in a previous paper from our laboratory (10). Primary and secondary immunization was done by i.v. injection of 3×10^4 and 10^5 SRBC in 0.5 ml of saline, respectively. Primary and secondary immunization with H antigens was performed by subcutaneous (s.c) injection of 10^7 and 10^6 allogeneic spleen cells from the appropriate mouse strain in 0.1 ml BSS, respectively. These doses have previously been shown to be optimal (1). The antigen dose was equally distributed over both inguinal areas. The spleen cells used for primary immunization were ultrasonically disrupted to prevent proliferation of the injected allogeneic cells and, thereby, to reduce the persistence of the antigen.

Selective depletion of B lymphocytes

The method described by Julius, Simpson and Herzenberg (11) for the depletion of B lymphocytes was used. After incubation for 45 min at 37°C on prewashed nylon wool (Fenwall Laboratories, Morton, Grove, Illinois U.S.A.), approximately 90% of the recovered lymphoid cells were Thy-1.2-positive (12).

Thoracic duct drainage

Thoracic duct fistulas were established essentially according to techniques previously described (13). The surgery of the mice was done under Avertin (Merck-Schuchardt) anesthesia (14). During drainage, the mice were infused with saline at a flow rate of 0.5 ml/hr. Thoracic duct lymphocytes (TDL) were collected in 10 ml of Dulbecco's BSS that was supplemented with 50 to 100 IU preservative-free heparine. The mice were cannulated for 24 or 48 hrs, and the lymph was stored at 4°C. The yield of TDL after 24 hrs drainage of BALB/c mice varied between 5 and 10×10^7 cells, and 72% of these cells were Thy-1.2-positive (15). The viability of the collected cells was usually 95%.

Irradiation

For adoptive transfer, the recipient mice received 5.0 to 6.0 Gy whole body irradiation, generated in a Philips-Müller MG 300 X-ray machine (1). The recipient

mice were i.v.-injected with 3 x 10^6 bone marrow cells in 0.5 ml BSS within 4 hrs after irradiation. One day later, the mice were i.v.-injected with the appropriate number of TDL suspended in 0.5 ml BSS.

Assay for DTH

DTH reactions were determined by measuring the difference in thickness of the hind feet 24 hrs after s.c. injection of 1 x 10^8 SRBC or 2 x 10^7 allogeneic spleen cells suspended in 0.05 ml BSS into the dorsum of the right hind leg. As a control for background DTH reactivity, naive responder mice were used which only received the challenge dose. The specific DTH response was calculated as the relative increase in foot thickness of the immune mice minus the relative increase of the control mice. The foot swelling of the control mice ranged between 10 and 15%.

RESULTS

Adoptive transfer of secondary DTH to SRBC and H antigens by thoracic duct lymphocytes

The occurrence of memory T cells for DTH to SRBC and minor H antigens in the lymphs of immunized mice was investigated by means of adoptive transfer of thoracic duct lymphocytes. In the case of SRBC, female BALB/c mice were primed with 3 x 10^4 SRBC i.v. Three months later, thoracic duct fistulas were established, and the 'immune' TDL were collected during 24 hrs. A number of 1 x 10^7 viable TDL was adoptively transferred to lethally irradiated BALB/c mice. Furthermore, one group of recipients received 1 x 10^7 viable TDL from non-immunized donors ('naive' TDL). Both groups of recipients were reconstituted with 3 x 10^6 BALB/c bone marrow cells and immunized with 1 x 10^5 SRBC i.v.

On days 2, 3 and 4 after immunization of the recipients, separate groups of mice were challenged. The DTH response was measured 24 and 48 hrs later (Fig. 1). The difference in time course of the DTH reactivity between the recipients of 'immune' TDL and 'naive' TDL indicates that DTH-related memory to SRBC can be transferred with TDL derived from immunized donors.

Essentially the same results were obtained with BALB/c mice that were s.c.-immunized with 1 x 10^7 sonicated B10.D2 spleen cells. These cells are H-2-compatible with BALB/c, but non-H-2-incompatible. Three months later, TDL were collected from these immunized mice. A number of 1 x 10^7 viable 'immune' TDL was adoptively transferred to irradiated and with syngeneic bone marrow cells reconstituted BALB/c mice. A control group which received 1 x 10^7 'naive' TDL was included. On days 3, 4, and 5 after s.c. immunization with 1 x 10^6 B10.D2 spleen cells, separate groups of mice were challenged, and the DTH

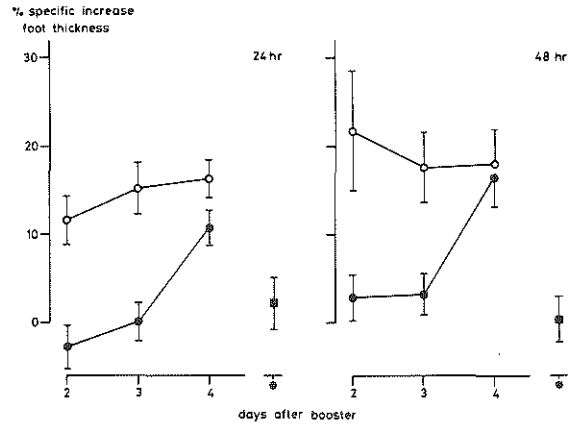

Fig. 1. Adoptive transfer of secondary DTH to SRBC by TDL. Lethally irradiated BALB/c recipient mice were i.v. injected with 3 x 10^6 BALB/c bone marrow cells and one day later with 1 x 10^7 viable 'immune' (○) or 'naive' TDL (●). 'Immune' TDL were obtained from BALB/c mice i.v. primed with 3 x 10^4 SRBC 3 months previously. The recipients were i.v. immunized with 1 x 10^5 SRBC immediately after adoptive transfer, and were challenged 2, 3 or 4 days later. The asterix indicates the persistent DTH reactivity after passive transfer of 1 x 10^7 viable 'immune' TDL (■). These latter recipients were challenged one day after the passive transfer of TDL. In all recipients the specific increase of foot thickness was determined at 24 and 48 hr after challenge. Vertical bars represent SE (n=6).

response was measured 24 and 48 hrs later (Fig. 2).

The inability of 'immune' TDL induced by SRBC or minor H antigens to passively transfer DTH reactivity shows that the 'immune' TDL mediate a typical secondary type DTH response after adoptive transfer (Figs. 1 and 2). However, TDL from BALB/c mice immunized with B10.ScSn spleen cells, which are incompatible for non-H-2 as well as H-2 antigens, did not transfer secondary DTH responsiveness to naive recipients (Fig. 3). This is in line with the inability of mice to mount secondary type DTH to H-2 alloantigens after multiple immunization (data not shown; 2).

Recirculation of DTH-related memory T cells specific for SRBC or non-H-2 antigens

The capacity of DTH-related memory T cells to recirculate was investigated by adoptive transfer of TDL from naive recipients inoculated either with spleen and lymph node cells from immune mice, or with TDL from such immune mice.

In the case of inoculation of spleen and lymph node cells, the total cell yield from spleen, inguinal, axillary, and mesenteric lymph nodes from a single with

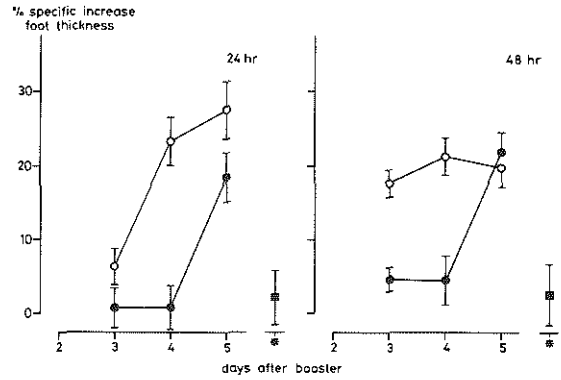

Fig. 2. Adoptive transfer of sedondary DTH to minor H antigens by TDL. Lethally irradiated BALB/c recipient mice were i.v. injected with 3 x 10^6 BALB/c bone marrow cells and one day later with 1 x 10^7 viable 'immune' (○) or 'naive' TDL (●). 'Immune' TDL were obtained from BALB/c mice s.c. primed with 1 x 10^7 sonicated B10.D2 spleen cells 3 months previously. The recipients were s.c. immunized with 1 x 10^6 B10.D2 spleen cells immediately after adoptive transfer, and were challenged 3, 4 or 5 days later. The asterix indicates the persistent DTH reactivity after passive transfer of 1 x 10^7 viable immune TDL (▣). These latter recipients were challenged one day after the passive transfer of TDL. In all recipients the specific increase of foot thickness was determined at 24 and 48 hr after challenge. Vertical bars represent SE (n=6).

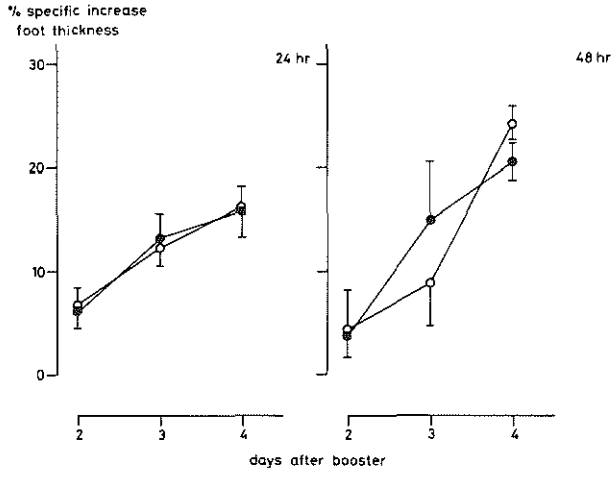

Fig. 3. Inability of secondary DTH to major and minor H antigens by adoptive transfer of 'immune' TDL. 'Immune' TDL were obtained from BALB/c mice s.c. primed with 1 x 10^7 sonicated B10.ScSn spleen cells 3 months previously. The other experimental details are the same as in Figure 2.

SRBC immunized donor was transferred to a naive intermediate recipient. After one day, TDL were collected from such intermediate recipients during 48 hrs. At 24 and 48 hrs, the collected TDL were transferred to irradiated and with syngeneic bone marrow cells reconstituted BALB/c mice. Each recipient received a total number of 4×10^7 'immune' TDL from the intermediate hosts. A control group which received 4×10^7 'naive' TDL was included. Both groups were immunized with 1×10^5 SRBC i.v. and challenged 3 days later. The DTH response was measured 24 and 48 hrs later. It was found that the 'immune' TDL mediated a clear-cut secondary type DTH response (Table 1, exp. A). This indicates that these 'immune' TDL include DTH-related memory T cells.

Subsequently, we investigated whether the memory T cells among TDL from immune mice recirculate. Therefore 1×10^8 TDL from immunized donors were i.v.-transferred into naive intermediate recipients. Subsequently, the intermediate recipients were also cannulated. These 'double-drained' TDL were transferred into irradiated and with syngeneic bone marrow cells reconstituted BALB/c mice. Each recipient received 4×10^7 'double-drained' 'immune' TDL. A control group received 4×10^7 'double-drained' 'naive' TDL. Both groups were immunized with 1×10^5 SRBC i.v. and challenged 3 days later. The DTH response was measured 24 and 48 hrs later. Again, a clear-cut secondary type DTH response

Table 1. Recirculation of DTH-related memory T cells specific for SRBC

	DTH response[b]	
Adoptive transfer [a]	**24 hr**	**48 hr**
A. 'immune' TDL	11.1 ± 3.1	16.7 ± 3.9
'naive' TDL	4.5 ± 2.9	2.9 ± 3.6
B. 'immune' TDL	20.3 ± 3.4	37.1 ± 5.4
'naive' TDL	8.6 ± 5.4	24.4 ± 6.7

a. Lethally irradiated BALB/c recipient mice were i.v. injected with 3×10^6 BALB/c bone marrow cells and one day later with 4×10^7 viable 'immune' or 'naive' TDL. 'Immune' TDL were obtained from intermediate BALB/c mice which had been i.v. injected with the spleen and lymph node cells of one immunized donor each (A) or with 1×10^8 TDL of immunized donors (B). The immunized donors had been i.v. primed with 3×10^4 SRBC 3 months previously. The secondary recipient mice were i.v. immunized with 1×10^5 SRBC immediately after adoptive transfer, and were challenged 3 days later.

b. The DTH response was determined at 24 and 48 hr after challenge. Figures represent mean DTH responses ± SE (n=6).

was found (Table 1; exp. B), indicating that the memory T cells that mediate this response do recirculate.

In subsequent experiments we investigated whether DTH-related memory T cells induced by minor H antigens also recirculate. Therefore BALB/c mice were s.c.-immunized with 1 x 10^7 sonicated B10.D2 spleen cells. Three months later, naive intermediate recipients were i.v.-inoculated with the nylon-wool-purified T cells from spleens and lymph nodes of these immune donors. Each recipient received a number of purified T cells equivalent to the number isolated from two donors. The TDL from these recipients were transferred to irradiated and with syngeneic bone marrow cells reconstituted BALB/c mice. Each recipient received a total number of 1 x 10^8 'immune' TDL from the intermediate hosts. A control group which received 1 x 10^8 'naive' TDL was included. Similarly, 2 x 10^8 'immune' TDL were transferred to intermediate BALB/c mice, from these mice, 1 x 10^8 'double-drained' TDL were transferred to irradiated and with syngeneic bone marrow cells reconstituted BALB/c mice. Again, a control group was included that received 1 x 10^8 'naive' TDL. All groups were s.c. immunized with

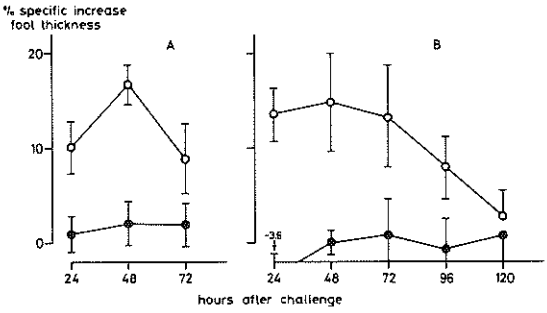

Fig. 4. Recirculation of DTH-related memory T cells specific for minor H antigens. Lethally irradiated BALB/c recipient mice were i.v. injected with 3 x 10^6 BALB/c bone marrow cells and one day later with 1 x 10^8 viable 'immune' (○) or 'naive' TDL (●). 'Immune' TDL were obtained from intermediate BALB/c mice which were either i.v. injected with the nylon-wool-purified T cells from the spleens and lymph nodes of two immune donors each (A) or with 2 x 10^8 TDL from immunized donors (B). The immune BALB/c donor mice had been s.c. primed with 1 x 10^7 sonicated B10.D2 spleen cells 3 months previously. The secondary recipient mice were s.c. immunized with 1 x 10^6 B10.D2 spleen cells immediately after adoptive transfer, and were challenged 3 days later. In all recipients the specific increase of foot thickness was determined at different intervals after challenge. Vertical bars represent SE (n=6).

1×10^6 B10.D2 spleen cells and challenged 3 days later. The DTH response was measured at various intervals after challenge. It was found that in both experimental systems 'immune' TDL were able to transfer secondary DTH to non-H-2 antigens (Fig. 4). Thus DTH-related memory T cells induced by minor H antigens do recirculate.

DISCUSSION

This paper shows that a substantial portion of the memory cells, capable of adoptive transfer of secondary type DTH to SRBC and minor H antigens, occur in the thoracic lymph of primed mice during the late phase of the response. TDL from primed mice were found to be incapable of passive transfer of DTH reactivity. After immunization of recipients of 'immune' TDL, a faster sensitization for DTH occurred than in recipients of 'naive' TDL. Passing the 'immune' TDL through intermediate recipients showed that these memory cells recirculate from blood to lymph. Previous studies from our laboratory (2, 3) have shown that such memory T cells are vinblastine-resistant, while after booster immunization, the passive transfer of DTH-mediating T cells can be completely abolished by vinblastine treatment. Thus the memory T cells involved in secondary DTH to SRBC and minor H antigens have the same characteristics as the memory cells involved in secondary type humoral immune responses (6, 16-18).

Memory phenomena of cellular immunity to intracellularly growing organisms are heterogeneous with respect to the characteristics of the cells involved. Memory cells involved in the cellular resistance to Bacillus Calmette Guérin (BCG) infection have been isolated from the lymphs of immunized rats (17). These cells have the capacity to recirculate and are vinblastine-resistant. Thus they are very similar to the memory cells described in this paper. In addition to cellular resistance, BCG-infected rats display persisting anti-tuberculin DTH. However, passive transfer of anti-tuberculin DTH by TDL was not possible (19). It has been stated that secondary type tuberculin DTH can be adoptively transferred with 'immune' TDL (7), although there is, as yet, only limited evidence concerning this.

In contrast to BCG, the acquired resistance to intracellular growth of *L. monocytogenes* is mainly dependent on a resident population of immune lymphocytes (2, 20-23). This cellular resistance is associated with DTH, but the DTH wanes more rapidly than the resistance (24). This DTH could not be passively transferred with 'late phase' TDL. It has been suggested that in *Listeria*-infected mice, non-dividing DTH effector cells with a long life span account for the ongoing, but gradually decreasing DTH reactivity, and that the state of DTH

represents the state of T-cell memory (22). Our previous and present studies show that persistence of DTH and DTH-related memory are two independent phenomena, because immunization of mice with SRBC or non-H-2-incompatible sonicated spleen cells does not induce persisting DTH (2), while secondary DTH to these antigens (2), and transfer of this secondary DTH by 'immune' TDL, is easily achieved.

Van der Kwast *et al.* (3) have shown that ongoing DTH reactivity and the capacity of secondary type DTH reactivity are based upon two separate populations of T cells with different characteristics. Ongoing DTH reactivity is based upon proliferating T cells with a short functional life span that require specific antigenic stimulation for their generation (25). The capacity of secondary-type DTH, on the other hand, is dependent upon hardly or not proliferating T cells that persist for long periods, even in the apparent absence of antigen (3). Van der Kwast *et al.* (2) explain the persistence of DTH reactivity to minor H antigens by proposing a continuous activation, by the persisting antigen, of a small portion of the memory cell population. This might account for an ongoing production of DTH effector cells. This view is supported by the observation that persistent DTH to minor H antigens does occur when living cells, which survive for a long period (26), are used for immunization (2).

The finding that in BCG and *Listeria*-infected animals DTH reactivity cannot be passively tranferred with 'late phase' TDL possibly depends on the number of DTH effector cells in the lymph, which might be too small to detect in a passive transfer system. In *Listeria*-infected mice the antigen is rapidly eliminated in contrast to BCG-infected animals (23). This correlates with the decline of anti-*Listeria* DTH (24). In BCG-infected rats, on the other hand, it was found that the persisting tuberculine DTH reactivity remained at the same level for a long period (27), which might be due to persisting antigen. This is supported by the observation that in man, tuberculine hypersensitivity disappeared after elimination of viable BCG by antimycobacterial drugs (28-30). This effect was not apparent in rats (27). However, it cannot be excluded that killed BCG persist for long periods.

In contrast to mice immunized with minor H antigens, no secondary type DTH reactivity could be demonstrated in mice immunized with H-2-incompatible spleen cells (2). Also after adoptive transfer of 'immune' TDL to irradiated recipient mice, it was impossible to demonstrate an accelerated DTH reactivity as compared to recipients of naive TDL. Apparently, reactivity to non H-2 alloantigens is low in naive cells and elevated in primed cells, whereas reactivity to H-2 alloantigens is relatively high already in naive cells and not detectably enhanced in immune cells. In studies concerning graft rejection (31, 32) and graft-versus-host (33, 34), it has also been shown that there is no marked acceleration of the response by prior immunization when major H antigens are

involved. However, Hall *et al.* (6) have shown that in rats, demonstration of a dramatic and specific increase of allograft reactivity after immunization with major H antigens depends on the use of an adoptive transfer system. The effect of booster immunization of mice primed with class-I or class-II histocompatibility antigens is subject of our present studies and will be discussed in a following paper.

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CHAPTER V

SECONDARY DELAYED TYPE HYPERSENSITIVITY TO H-2 SUBREGION CODED ALLOANTIGENS

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ABSTRACT

Secondary type delayed type hypersensitivity (DTH) in mice against class I alloantigens or non-H-2 alloantigens is characterized by an earlier appearance of DTH reactivity after booster immunization compared with the development of DTH reactivity after primary immunization. In contrast to the primary and secondary DTH against class I or non-H-2 alloantigens, the development of secondary DTH against class II alloantigens or a set of alloantigens that includes class II alloantigens is *not* faster than the development of primary DTH. We conclude that priming with class II alloantigens has a dominant effect which prevents secondary DTH reactivity to class I alloantigens and non-H-2 alloantigens.

INTRODUCTION

The *in vivo* immune response of mice against histocompatibility (H) antigens has been extensively studied by skingraft experiments (1-3). These investigations have shown that the effect of priming on a second set skingraft is inversely related to the immunogenicity of the presented alloantigens (3-5). Thus, the difference in survival time between first- and second set minor-H incompatible skingrafts in H-2 compatible mice is much greater than the difference in survival time of first- and second set H-2 incompatible skingrafts.

Some aspects of the *in vivo* cellular immune response against H-antigens can be studied in rather detail by the delayed type hypersensitivity (DTH) assay. DTH against alloantigens can be readily induced by allogeneic skin and tumor grafts (6, 7) and by subcutaneous (sc) injection of allogeneic lymphoid cells (8, 9).

In previous studies we have extensively investigated DTH reactivity in mice sc injected with allogeneic cells and cell fragments (8, 10, 11). We have shown, a.o., that priming with alloantigens not only can lead to the accelerated rejection of second set minor-H-incompatible skingrafts, but also to a state of secondary DTH responsiveness (10). The state of immunological memory is characterized by an accelerated reappearance of the DTH reactivity after booster immunization. The sc route of priming is obligatory as intravenous (iv) immunization induces a state of antigen specific suppression (11, 12).

It appeared that the secondary DTH responsiveness against minor-H-antigens depended on the induction of a population of long-lived non-dividing T memory cells (10). Transfer of thoracic duct lymphocytes from immunized mice to naive recipients has shown that a substantial portion of these T memory cells recirculate (13).

In this paper we extensively investigated the kinetics of the secondary DTH response of mice to H-2 and non-H-2 alloantigens and, furthermore, investigated secondary DTH responsiveness after immunization with class I (H-2K and/or H-2D) and class II alloantigens (H-2I) separately.

MATERIALS AND METHODS

Mice

Female A.SW (H-2^s), BALB/c (H-2^d), B10.A (H-2^a) and B10.ScSn (H-2^b) mice were purchased from the Laboratory Animals Centre of the Erasmus University, Rotterdam, The Netherlands. Female DBA/2 (H-2^d) mice were purchased from the Radiobiological Institute TNO, Rijswijk, The Netherlands. Female A.TL (H-2^{t1}), A.TH (H-2^{t2}), B10.D2 (H-2^d), B10.AQR (H-2^{y1}), B10.T(6R) (H-2^{y2}), B10.A(2R) (H-2^{h2}) and DBA/1 (H-2^q) were purchased from OLAC Ltd.,

Bicester, U.K. Female A.AL(H-2^{a1}) mice were purchased from Yeda Research and Development Co. Ltd., Weizmann Institute, Rehovot, Israel. The age of all responder mice varied between 12 and 24 weeks.

Preparation of cell suspensions

Mice were killed by carbon dioxide vapour exposition. Spleens were removed, brought into balanced salt solution (BSS), minced with scissors and squeezed trough a nylon gauze filter to give a single cell suspension. Nucleated cells were counted with a Coulter counter Model BZI.

Antigen and immunization

Primary and secondary immunization with allogeneic histocompatibility (H) antigens was performed by sc injection of 10^7 allogeneic spleen cells from the appropriate mouse strain in 0.1 ml BSS. This dose has previously been shown to be optimal (10). The antigen dose was equally distributed over both inguinal areas. The spleen cells used for primary immunization were ultrasonically disrupted to prevent proliferation of the injected allogeneic cells and, thereby, to reduce the persistence of the antigen. Mice used for comparison of primary and secondary DTH were of the same delivery and the same age.

Assay for DTH

DTH reactions were determined by measuring the difference in thickness of the hind feet 24 hr after sc injection of 2×10^7 allogeneic spleen cells suspended in 0.05 ml BSS into the instep of the right hind foot of appropriately immunized mice. As a control for background DTH reactivity, naive responder mice were used, which only received the challenge dose. The specific DTH response was calculated as the relative increase in foot thickness of the immune mice minus the relative increase of the control mice. The foot swelling of the control mice ranged between 15 and 20%.

RESULTS

Effect of booster immunization with non-H-2 or H-2 coded alloantigens

In order to investigate the effect of booster immunization of mice primed with multiple minor-H-antigens, groups of DBA/2 and BALB/c responder mice were sc immunized for primary DTH or primed and boosted for secondary DTH by injection with minor-H incompatible B10.D2 spleen cells. Priming for secondary DTH was always performed with ultrasonically disrupted spleen cells, while for booster immunization and for induction of primary DTH viable spleen cells were used. The interval between priming and booster immunization for secondary DTH was 3 to 5 months. In order to compare the kinetics of primary

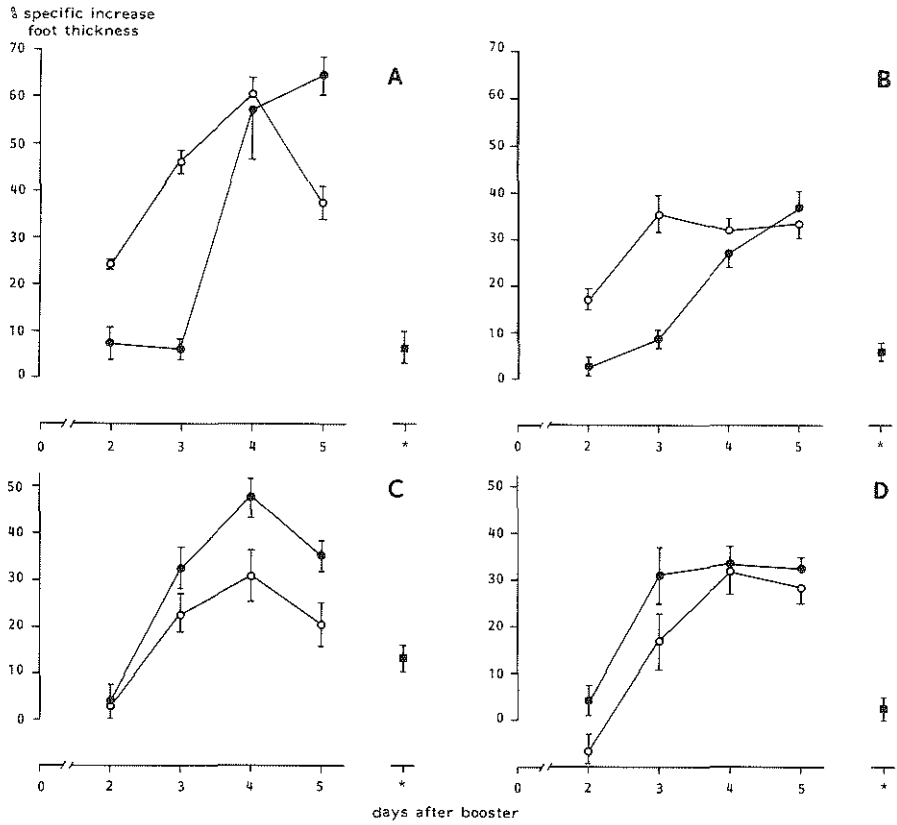

Fig. 1. Primary and secondary DTH responsiveness to allogeneic non-H-2 (A,B) and H-2 (C, D) incompatible spleen cells. The graphs respresent the time course of the primary DTH (●) and secondary DTH (○) response of DBA/2 mice to the non-H-2 incompatible B10.D2 spleen cells (A), of BALB/c mice to the non-H-2 incompatible B10.D2 spleen cells (B), of B10.ScSn mice to the H-2 incompatible B10.D2 spleen cells (C) and of DBA/2 mice to the H-2 incompatible DBA/1 spleen cells (D). In all strain combinations the mice were sc immunized for primary DTH with 1×10^7 viable spleen cells or sc primed with 1×10^7 sonicated spleen cells and sc boosted with 1×10^7 viable spleen cells for secondary DTH. The persistent primary DTH reactivity (■) due to priming with 1×10^7 sonicated spleen cells was determined at the moment of booster, 3 to 5 months after priming. Vertical bars represent SE (n = 6).

and secondary DTH, the booster immunization for secondary DTH was done on the same day as the immunization for primary DTH. At different days after immunization for primary DTH and booster immunization, groups of mice were

challenged with B10.D2 spleen cells. In both strain combinations it appeared that the time course of the primary and secondary DTH response was different (Fig. 1A and 1B). At 2 days after booster immunization, the mice showed a clear DTH response, while primary DTH was hardly detectable by day 3. Secondary DTH was found to be maximal 3 of 4 days after booster immunization, while maximal primary DTH was found on day 5 or later. The kinetics of the foot-swell reaction after challenge did not differ for primary and secondary DTH (data not shown). As a control for persistent DTH responsiveness due to the primary immunization, one group of mice was challenged for DTH reactivity 3 months after priming. In both strain combinations no significant persistent DTH response could be measured.

Different results were obtained in mice immunized with H-2 coded alloantigens. Therefore, DBA/2 and B10.ScSn responder mice were tested for primary and secondary DTH against H-2 incompatible DBA/1 and B10.D2 spleen cells, respectively. Significant primary and secondary DTH responses against H-2 alloantigens were found on day 3 and maximal responses were found on day 4 after primary or secondary immunization (Fig. 1C and 1D). In contrast to the primary and secondary DTH against non-H-2 alloantigens, the development of secondary DTH against H-2 alloantigens was not faster than the development of primary DTH. Furthermore, in both strain combinations secondary DTH reactivity was weaker or equal to primary DTH reactivity to H-2 alloantigens.

Effect of booster immunization with class I of class II alloantigens.

We have also investigated whether class I or class II H-2 alloantigens separately are able to induce a secondary type DTH response. The same immunization protocol was used as described above. At first we tested the effect of immunization with class I alloantigens only in the strain combinations B10.A-B10.AQR (Fig. 2A) and A.TL-A.AL (Fig. 2B), which are both H-2K incompatible, and in the H-2K/D incompatible combination of B10.AQR-B10.A(2R) mice (Fig. 2C). In all three combinations a secondary type DTH response could be elicited. Significant primary DTH responses were found on day 4 or later after immunization, while after booster immunization substantial DTH responses were found already on day 3. The kinetics of the foot-swell reaction after challenge, however, did not differ for primary and secondary DTH (data not shown). Mice used for control of persisting DTH responsiveness due to the primary immunization showed no significant DTH response.

The capacity of class II coded alloantigens to induce secondary type DTH responses was studied in the H-2I incompatible strain combinations B10.AQR-B10.T(6R) (Fig. 3A) and A.TL-A.TH (Fig. 3B). Both combinations showed a similar development of primary and secondary DTH responsiveness. Also in the strain combinations B10.A-B10.T(6R) (Fig. 3C) and A.TL-A.SW (Fig. 3D) in

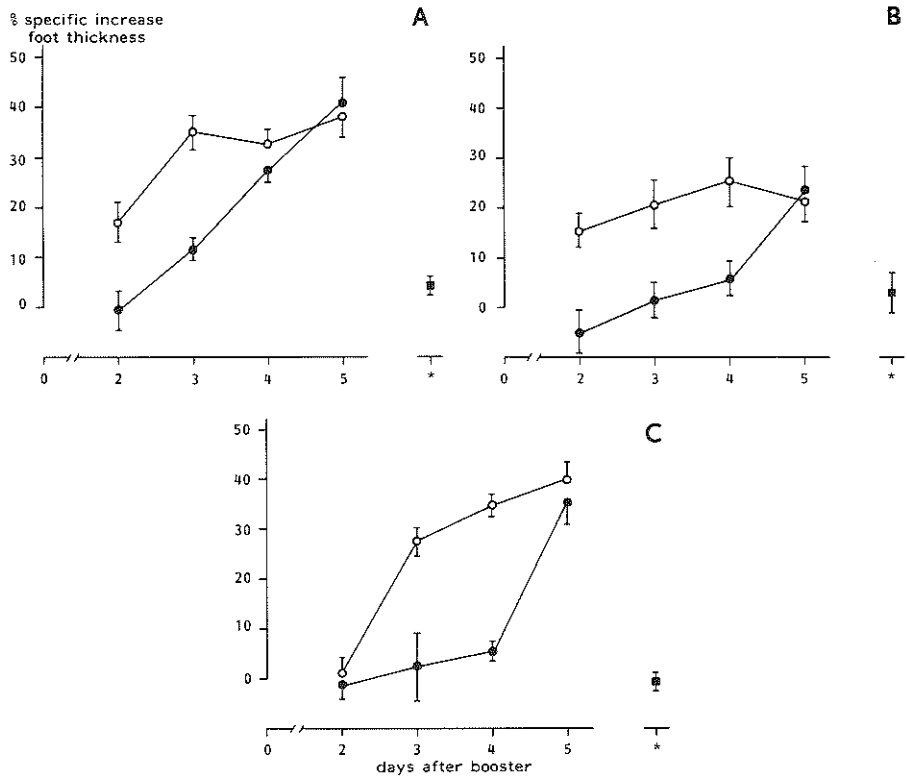

Fig. 2. Primary and secondary DTH responsiveness to allogeneic H-2K and H-2K/D incompatible spleen cells. The graphs represent the time course of the primary DTH (●) and secondary DTH (○) of B10.A mice to H-2K incompatible B10.AQR spleen cells (A), of A.TL mice to H-2K incompatible A.AL spleen cells (B) and of B10.AQR mice to H-2K/D incompatible B10.A(2R) spleen cells (C). The other experimental details are the same as described in the legend to Fig. 1.

which a H-2K and a H-2D incompatibility, respectively, is presented in combination with a H-2 I incompatibility, no significant difference was found between the development of the primary and the secondary DTH responsiveness.

Thus, in donor-recipient combinations in which class II alloantigenic differences are included, it is not possible to induce secondary type DTH against class I alloantigens.

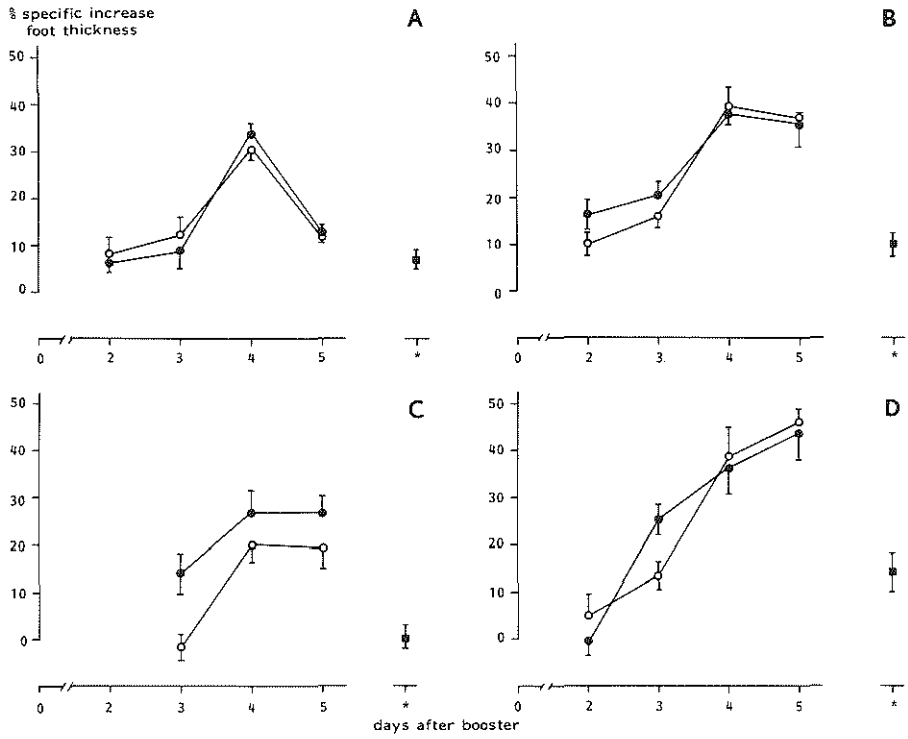

Fig. 3. Primary and secondary DTH responsiveness to allogeneic H-2I, H-2K/I and H-2I/D incompatible spleen cells. The graphs represent the time course of the primary DTH (●) and secondary DTH (○) response of B10.AQR mice to H-2I incompatible B10.T(6R) spleen cells (A), of A.TL mice to H-2I incompatible A.TH spleen cells (B), of B10.A mice to H-2K/I incompatible B10.T(6R) spleen cells (C) and of A.TL mice to H-2I/D incompatible A.SW spleen cells (D). The other experimental details are the same as described in the legend to Fig. 1.

DISCUSSION

The data presented in this paper show that minor H antigens (Fig. 1) and class I alloantigens (Fig. 2) can induce secondary type DTH. No secondary type DTH, however, could be demonstrated in mice primed and boosted with a set of H-antigens that includes class II alloantigens (Fig. 1 and 3). The inability to induce secondary type anti-allograft responses against class II alloantigens was also described by Zacharova *et al.* (14) in skingraft experiments. However, McKenzie

et al. (15) demonstrated an accelerated second set skingraft rejection in the H-2I (class II) incompatible mouse strain combinations B10.AQR-B10.T(6R) and A.TH-A.TL. In precisely the same strain combinations we failed to demonstrate anamnestic DTH. Also for fully H-2 incompatible skingrafts accelerated second set graft rejection has been found (3, 16, 17), which is in contrast to our DTH experiments (Fig. 1C and 1D).

In transplantation experiments with allogeneic, MHC-incompatible heart grafts, Hall *et al.* (18) could not demonstrate an accelerated heart graft rejection in sensitized rats. Transfer of thoracic duct lymphocytes (TDL) from sensitized rats, on the other hand, showed that on a per cell base these "immune" TDL were more potent to restore the capacity of irradiated recipients to reject heart grafts from the relevant allogeneic donor strain than "naive" TDL. In previous studies (13) we failed to demonstrate a memory effect in comparable transfer experiments with "immune" TDL from mice which were primed with spleen cells that were incompatible for H-2 as well as non-H-2 alloantigens.

The above discrepancies between graft rejection and DTH might be due to a more dominant role of class II alloantigens in DTH alloreactivity than in other *in vivo* anti-allograft reactions. I region encoded alloantigens activate in naive animals 10 times more proliferative T cell precursors than do H-2K or H-2D encoded alloantigens (19). Ryser *et al.* (20) showed that *in vivo* immunization hardly affects the frequency of H-2I specific proliferative T lymphocyte precursors. This can explain why priming with class II alloantigens does not lead to secondary type DTH. Immunization with H-2K or H-2D alloantigens, however, causes a 3 to 4 times higher frequency of K/D specific cytotoxic T lymphocyte precursors (20). As cytotoxic T lymphocytes are able to mediate DTH (21, 22), an increase of the frequency of cytotoxic T lymphocyte precursors may account for the observed secondary type DTH to class I alloantigens.

The above explanation, however, does not fit properly with all results we have obtained. In the first place, the relatively high frequency of alloreactive T cells specific for class II alloantigens is not expressed in the magnitude of the maximal DTH response, since immunization with non-H-2 alloantigens is often leading to higher peak DTH responses than immunization with H-2 alloantigens (Fig. 1; refs. 10, 23). Secondly, the height of the primary and secondary DTH against completely H-2 (K, I and D) incompatible spleen cells is not in harmony with the sum of the primary or secondary anti H-2I (Fig. 3A) and anti H-2K/D responses together (Fig. 1C and 1D and Fig. 2C), Furthermore, the secondary anti H-2 response is even somewhat lower than the primary anti H-2 response (Fig. 1C and 1D). The data even suggest that class II alloantigens have a suppressive influence upon the secondary DTH response to other alloantigens.

Enhancing antibodies might play a role in this phenomenon. McKenzie *et al.* (15, 24, 25) have shown that enhancing antibodies are mainly directed against

H-2I alloantigens. Moreover, H-2I induced antibodies can also influence the anti H-2K or anti H-2D allograft response, when the H-2K/D alloantigens are presented in combination with the H-2I alloantigens. However, induction of hyporeactivity in skingraft experiments due to enhancing antibodies requires several injections of allogeneic cells. Furthermore, Capel *et al.* (26) have shown that enhancement requires persisting antigen and enhancing antibodies. In our DTH experiments, on the other hand, the mice were primed by a single injection of allogeneic spleen cell fragments only. By purpose we used allogeneic spleen cell fragments for priming in order to avoid long persistence of the injected alloantigens, and thereby an ongoing immune response.

Just like in the above host versus graft DTH experiments it is impossible to show any effect of priming in graft versus host (GvH) responses (18, 27-28). Ford and Simonsen (29) have shown with bursectomized chickens that enhancing antibodies were not responsible for the absence of a priming effect in GvH reactions. Thus, there is little evidence for the involvement of enhancement in the lack of secondary DTH against H-2 alloantigens that include H-2I.

It is also not likely that the failure to elicit secondary type DTH responses to H-2 alloantigens that include H-2I subregion coded antigens is due to restricted recognition of the alloantigens. Restricted recognition of H-2 coded alloantigens has been demonstrated in GvH (30, 31) and DTH (31) experiments. However, the present data show that H-2 incompatible spleen cells, which do not express restriction elements syngeneic with the responder mice, are still able to elicit a primary DTH response. We have previously shown that allogeneic H-2 molecules can also act as restriction elements for the DTH response to other H-2 alloantigens expressed on the same cell (31). Therefore, the lack of secondary type anti H-2 DTH reactivity cannot be explained by supposing a requirement for syngeneic restriction elements by alloreactive T memory cells. Furthermore, Van der Kwast *et al.* (10) have shown that secondary type anti H-2 DTH reactivity was also not found when the H-2 alloantigens were presented in combination with syngeneic H-2 molecules by using F1 hybrid cells.

Thus, we have to conclude that priming with class II alloantigens has a dominant effect which prevents secondary type DTH reactivity to class I alloantigens and non-H-2 alloantigens. The secondary type DTH response in H-2I incompatible strain combinations is often somewhat lower than primary DTH responses (Fig. 1C and 1D). This seemingly suppressive effect is not comparable with the firm suppression found after iv preimmunization with alloantigens (32), which is not H-2I dependent. The mechanism underlying this H-2I dependent suppressive effect on the secondary DTH response against H-antigens is subject of our present studies.

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CHAPTER VI

RESTRICTED RECOGNITION OF H-2 SUBREGION CODED ALLOANTIGENS IN DELAYED TYPE HYPERSENSITIVITY

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ABSTRACT

Subcutaneous (sc) immunization of mice with H-2K, I or D incompatible spleen cells induces a state of host-versus-graft (HvG) delayed-type hypersensitivity (DTH). The DTH reaction is elicited by challenging the immunized mice in a hind foot with similar allogeneic spleen cells and is measured as the subsequent foot swelling. DTH effector T cells specific for H-2I coded alloantigens, but not for H-2K/D coded alloantigens, can be induced in a Graft-versus-Host (GvH) model as well. In this paper we report that under HvG as well as under GvH conditions the recognition of class II antigens by DTH effector T cells is restricted by class I molecules. Furthermore, DTH effector T cells induced by sc immunization with class I antigens appear to be restricted by class II molecules.

INTRODUCTION

Since the original observation of Zinkernagel and Doherty (1974) that T lymphocytes from lymphocytic choriomeningitis virus (LCMV)-infected mice lysed LCMV-infected target cells only if they expressed the same H-2 haplotype as the donor of the effector cells, many data have been gathered concerning major histocompatibility complex (MHC) restricted immune responses. In several other viral systems, restricted recognition of infected target cells has been shown (Gardner *et al.*, 1974; Koszinowski and Thomssen, 1975; Doherty *et al.*, 1976). In some of these systems the H-2 subregion involved in the MHC restriction was identified, e.g., H-2K and/or H-2D were found to be the restriction elements for LCMV-specific cytotoxic T lymphocytes (Zinkernagel and Doherty, 1975). The cytotoxic response to trinitrophenyl-modified syngeneic cells (Shearer *et al.*, 1975) as well as the cytotoxic response to minor histocompatibility (H) antigens (Bevan, 1975) was also found to be restricted by products coded for by the H-2K and/or H-2D subregions.

The *in vivo* relevance of the phenomenon of H-2 restriction has also been shown. DTH reactions against virus-infected cells were H-2K/D restricted (Zinkernagel, 1976) and DTH against *Listeria monocytogenes* was found to be restricted by H-2I coded molecules (Zinkernagel *et al.*, 1977). Furthermore, transfer of DTH effector T cells specific for fowl-γ-globulin appeared to be H-2IA restricted (Miller *et al.*, 1975), while H-2K, I or D subregion coded molecules functioned equally well as restriction element in the dinitrofluorobenzene induced DTH response (Miller *et al.*, 1975; Vadas *et al.*, 1977). DTH reactivity can also be induced by H-2 and minor H antigens. DTH effector T cells specific for minor H antigens appeared to be H-2K/D restricted (Smith and Miller, 1979: Van der Kwast, 1980). Restricted recognition of minor H antigens *in vivo* was also shown by Korngold and Sprent (1981, 1982) in a GvH mortality assay.

Most literature data indicate that recognition of H-2 alloantigens is an exception to the general rules of H-2 restricted recognition of antigen by T cells (Klein *et al.*, 1977; Smith and Miller, 1979; Weiss and Dennert, 1981; Swain, 1981: Vadas and Greene, 1981). However, recently a few papers appeared suggesting that under certain conditions the recognition of H-2 subregion coded antigens *in vitro* (Minami and Shreffler, 1981; Rock *et al.*, 1983) and *in vivo* (Kindred, 1981, 1983a,b) can be restricted by other H-2 coded molecules.

In this paper we present data which show that the recognition of H-2 subregion coded alloantigens by DTH effector T cells *in vivo* is H-2 restricted. Using both a Host-versus-Graft (HvG) and a Graft-versus-Host (GvH) assay, we show that DTH effector T cells specific for class II antigens are restricted in their antigen

recognition by class I molecules. On the other hand, DTH effector T cells activated in a HvG assay by class I antigens, are restricted by class II molecules.

MATERIALS AND METHODS

Mouse strains

B10.A (H-2^a) and A.SW (H-2^s) mice were purchased from the Laboratory Animals Centre of the Erasmus University, Rotterdam, The Netherlands. B10.AQR (H-2^{y1}), B10.T(6R) (H-2^{y2}), B10.A(2R) (H-2^{h2}), B10.BR (H-2^k), A.TL (H-2^{t1}) and A.TH (H-2^{t2}) mice were purchased from OLAC Ltd., Bicester, United Kingdom. A/J (H-2^a) mice were purchased from Bomholtgard Ltd, Ry, Denmark. A.AL (H-2^{a1}) mice were purchased from YEDA Research and Development Co. Ltd. at the Weizmann Institute of Science, Rehovot, Israel. B10.BYR (H-2^{by1}), (B10.A x B10.T(6R))F1 (H-$2^{a/y2}$), (B10.T(6R) x B10.A(2R))F1 (H-$2^{y2/h2}$), and (A/J x A.TH)F1 (H-$2^{a/t2}$) mice were bred at our own department. B10.BYR breeding pairs were kindly provided by Prof. J. Klein, Max-Planck-Institute für Biologie, Tübingen, W.-Germany. The age of the responder mice varied between 10 and 24 weeks. Only female mice were used.

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.

Irradiation

The recipient mice received 7.5 Gy whole body irradiation, generated in a Philips Müller MG 300 X-ray machine. Radiation control mice died in 14 to 21 days.

Host-versus-Graft reactions

Induction of DTH reactivity was done by subcutaneous (sc) immunization with 1 x 10^7 of the appropriate allogeneic spleen cells, suspended in a volume of 0.1 ml. A total volume of 50 μl of this spleen cell suspension was injected in both inguinal areas.

Acute Graft-versus-Host reactions

Acute GvH reactions were elicited by intravenous (iv) injection of 2 x 10^7 nucleated spleen cells into lethally irradiated allogeneic recipients within 4 h after irradiation. The cells to be injected were suspended in a volume of 0.5 ml BSS.

Assay for delayed type hypersensitivity

The DTH assays for measuring HvG and GvH immune reactivity have been described in detail in previous papers (Van der Kwast and Benner, 1978; Wolters and Benner, 1978). HvG DTH responses were elicited by sc injection of a challenge dose of 2×10^7 allogeneic spleen cells into the dorsum of the right hind foot, five or six days after the sc immunization in the inguinal area. The DTH response to this challenge was measured as the difference in thickness of the hind feet 24 h 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 received only the challenge. The swelling of these control mice varied between 12 and 20%. For measuring GvH-related DTH reactivity we transferred iv a number of cells equivalent to the total cell yield obtained from spleen, inguinal, axillary and mesenteric lymph nodes from an irradiated and reconstituted mouse into a normal secondary recipient five days after reconstitution. The secondary recipients were syngeneic to the original spleen donors. Twenty million spleen cells, syngeneic with the irradiated recipients were administered as a challenge into the dorsum of the right hind foot of the secondary recipient mice. The subsequent DTH response was measured and calculated as described above for the HvG DTH response.

Assay for immune lymphocyte transfer reactivity

The immune lymphocyte transfer (ILT) reactivity in lymph node cells from immunized donors, directed against H antigens of a particular recipient, was determined by sc injection of 5×10^6 of these lymph node cells into the dorsum of the right hind foot of the recipients to be tested. The recipients received a sc injection into the left hind foot consisting of 5×10^6 lymph node cells from non-immune mice, syngeneic with the immunized donor mice. This latter injection results in a normal lymphocyte transfer (NLT) reaction. For ILT and NLT the cells were injected in a volume of 50 μl. A control group consisting of recipient mice syngeneic to immunized donor mice was included. These mice were similarly injected with immune and non-immune lymph node cells in 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-NLT)test-(ILT-NLT)control, and was expressed in 10^{-2}mm.

RESULTS

H-2 restricted recognition of H-2 subregion coded alloantigens during the expression of primary DTH reactivity

To investigate whether the recognition of H-2 subregion coded alloantigens

by DTH effector T cells is restricted by other H-2 molecules, B10.A responder mice were sc immunized with (H-2) K incompatible B10.AQR spleen cells, or with (H-2) K+I incompatible B10.T(6R) spleen cells. Five days later the responder mice were challenged with B10.AQR (K), B10.T(6R) (K+I) or (B10.A x B10.T)6R)F1 (K+I) spleen cells. The results show that DTH reactions to K and K+I coded alloantigens can be demonstrated (Fig. 1, lines 1, 4 and 6). However, when responder mice were immunized to K antigens only and subsequently challenged with B10.T(6R) spleen cells, which express the same K antigens in a context of allogeneic I molecules, only a marginal reaction occurred (Fig. 1, line 2). On the other hand, mice immunized with K+I alloantigens and challenged with K alloantigens only, displayed a clear DTH response (Fig. 1, line 5). In combinations of B10 congenics involving B10.AQR there is the possibility of reactivity to non-H-2 antigens on the B10.AQR strain, which has been insufficiently backcrossed onto B10 (K. Fischer-Lindahl, personal communication). To investigate whether the B10.A anti-B10.AQR DTH response is directed against other alloantigens than K coded alloantigens, B10.A mice primed with B10.AQR spleen cells were also challenged for anti-K reactivity with (B10.A x

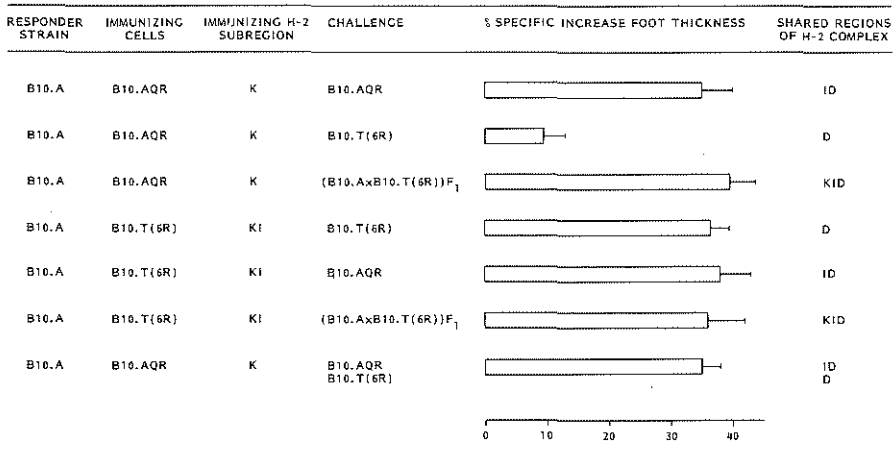

Fig. 1. H-2 restricted recognition of H-2K coded alloantigens in DTH. B10.A responder mice were sc immunized with 1 x 10^7 B10.AQR or B10.T(6R) spleen cells and challenged with 2 x 10^7 B10.AQR, B10.T(6R), (B10.A x B10.T(6R))F1 or a mixture of B10.AQR and B10.T(6R) spleen cells 5 days later. DTH responses were measured 24 h later. Each column represents the mean response ± SE (n=6). 'Shared regions of H-2 complex' relates to the responder-challenge combinations.

B10.T(6R))F1 spleen cells. These F1 spleen cells bear both the self I molecule and the allogeneic K molecule against which the responder is putatively immunized. The results show that the response against the F1 spleen cells is equivalent to the response against B10.AQR spleen cells (Fig. 1, lines 1 and 3, respectively). Thus, in this DTH assay B10.AQR spleen cells do immunize B10.A mice for anti-K reactivity. To exclude any suppressive influence of K+I incompatible B10.T(6R) spleen cells, these cells were mixed with K incompatible B10.AQR cells in the challenge inoculum of K primed mice. As can be seen from Fig. 1, line 7, no suppressive influence could be detected. In conclusion DTH effector T cells from mice primed with K coded alloantigens recognize these alloantigens in the context of syngeneic I molecules irrespective of whether they are induced with K alloantigens in the context of syngeneic or allogeneic I molecules. The induction of a sufficient number of DTH effector T cells which recognize the K alloantigens in the context of the allo I molecules occurs only when the responder mice are primed with K antigens in a context of allogeneic I molecules.

The recognition of I alloantigens by DTH effector T cells was also found to be restricted by other H-2 subregion coded molecules. When B10.T(6R) responder mice were immunized with I incompatible B10.AQR spleen cells and subsequently challenged with K+I+D incompatible B10.A(2R) spleen cells, only a marginal reaction occurred (Fig. 2, line 2). This indicates that DTH effector T cells primed for I alloantigens in the context of syngeneic K+D molecules do not recognize these I alloantigens in the context of allogeneic K+D molecules. To verify the induction of anti-I reactivity by sc immunization of B10.T(6R) responder mice with B10.AQR spleen cells, the primed responders were challenged for anti-I reactivity with (B10.T(6R) x B10.A(2R))F1 spleen cells. The results show that challenge with (B10.T(6R) x B10.A(2R))F1 spleen cells was as effective as challenge with B10.AQR spleen cells (Fig. 2, lines 3 and 1, respectively). Responder mice activated to I alloantigens in the context of allogeneic K+D molecules recognized the I alloantigens on a background of the syngeneic K+D molecules or the original allogeneic K+D molecules (Fig. 2, lines 5 and 4, respectively). Again no suppressive influence of K+I+D incompatible B10.A(2R) spleen cells could be detected on the DTH reaction to I alloantigens on a syngeneic K+D background (Fig. 2, line 7).

H-2 restricted recognition of H-2I alloantigens during the expression of GvH related DTH

We investigated whether the recognition of H-2 subregion coded alloantigens under GvH conditions is also restricted by other H-2 subregion coded molecules. The GvH assay used is based upon iv transfer of GvH-activated lymphoid cells to secondary recipients, which are syngeneic with the spleen cells donors to

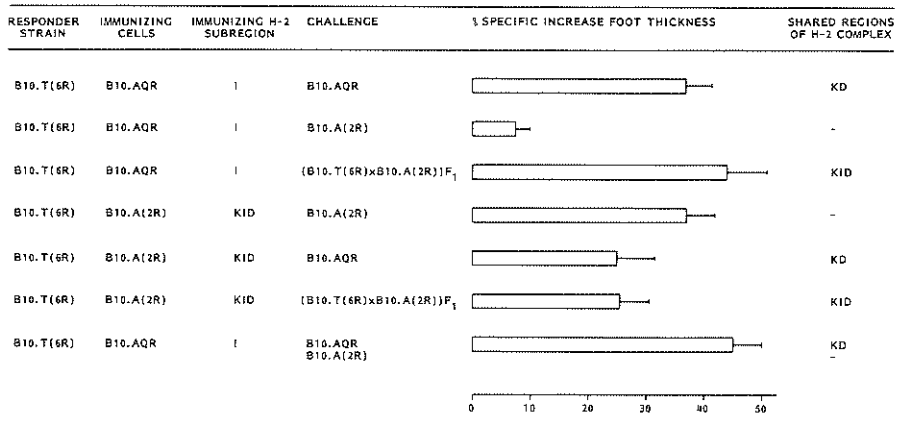

Fig. 2. H-2 restricted recognition of H-2I subregion coded alloantigens in DTH. B10.T(6R) responder mice were sc immunized with 1 x 10^7 B10.AQR or B10.A(2R) spleen cells and 5 days later challenged with 2 x 10^7 B10.AQR, B10.A(2R), (B10.T(6R) x B10.A(2R))F1 or a mixture of B10.AQR and B10.A(2R) spleen cells. DTH responses were measured 24 h later. Each column represents the mean response ± SE (n=6).

reconstitute the irradiated allogeneic recipients, and measurement of their DTH reactivity. Therefore, after transfer the secondary recipients were challenged in the right hind foot with spleen cells syngeneic to the irradiated primary recipients. The subsequent footpad swelling was determined 24 h after challenge. In this assay, only DTH reactions to H-2I and Mls-coded alloantigens can be determined (Wolters and Benner, 1979; Wolters *et al.*, 1981). For the present studies, B10.T-(6R) responder cells were used to reconstitute irradiated I incompatible B10.AQR mice. Five days later, spleen and lymph node cells form these irradiated and reconstituted mice were iv transferred to naive B10.T(6R) secondary recipients. These recipients were subsequently challenged with either B10.AQR (I incompatible), B10.BR (K+I+D incompatible), B10.A (K+I incompatible) or B10.BYR (I+D incompatible) spleen cells. When B10.AQR spleen cells were used, i.e. when the I alloantigens were presented in the context of syngeneic K+D molecules, a significant response occurred (Fig. 3, line 1). However, when the I alloantigens were presented in the context of foreign K+D, K or D molecules, only marginal DTH reactions were found (Fig. 3, lines 2, 3 and 4). The same phenomenon occurred in the A.TH-A.TL-A/J combinations: when the I alloantigens were presented on a background of foreign K molecules, no reaction was found. Thus,

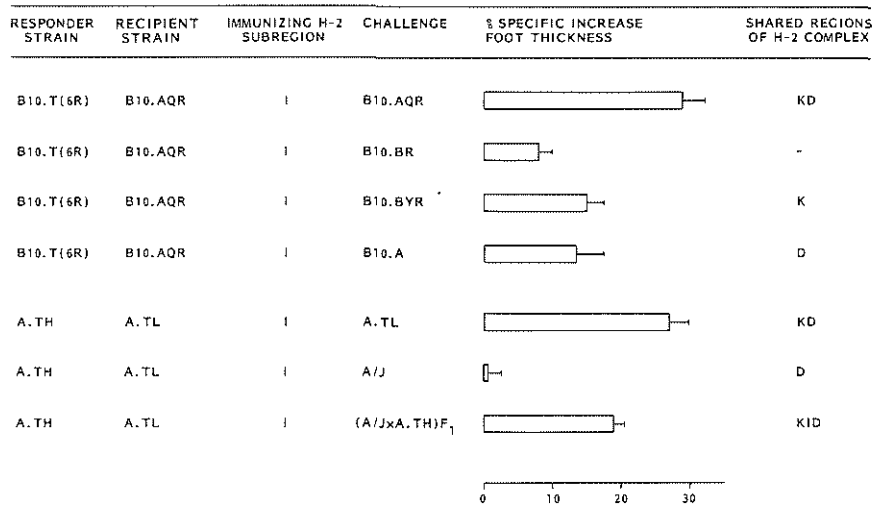

Fig. 3. H-2 restricted recognition of H-2I coded alloantigens by DTH reactive T cells in a GvH-related DTH assay. Lethally irradiated B10.AQR and A.TL mice were reconstituted with 2 x 10^7 spleen cells from B10.T(6R) and A.TH donors, respectively. Five days later, spleen and lymph node cells were transferred iv to naive B10.T(6R) and A.TH secondary recipients, respectively. Immediately after transfer, these mice were challenged with 2 x 10^7 B10.AQR, B10.BR, B10.BYR, B10.A, A.TL, (A/J x A.TH)F1 or A/J spleen cells, which differ in the indicated H-2 subregions from B10.T(6R) and A.TH responder mice. DTH responses were measured 24 h after challenge. Each column represents the mean response ± SE (n=6).

I alloantigens have to be presented with syngeneic K+D molecules to ensure optimal GvH-related DTH reactivity.

For the combinations of B10 congenic lines it has been shown already in the experiments described above (Figs. 1 and 2) that non-H-2 incompatibilities of the B10.AQR are not involved in the DTH responses observed. Moreover, as we stated earlier, in GvH related DTH only reactions to H-2I and MIs coded antigens occur (Wolters and Benner, 1979; Wolters *et al.*, 1981). Nevertheless, we verified whether the Qa/Tla region incompatibilities between A.TH and A.TL might account for the GvH related DTH response. Therefore, the anti-I reactivity of A.TH against A.TL was determined by challenging the responders with (A/J x A.TH)F1 spleen cells (Fig. 3, line 7). These F1 cells do express the same I molecules as A.TL cells but not the Qa/Tla region incompatibilities of A.TL. The results show that a clear anti-I response is induced by A.TL.

H-2 restricted recognition of H-2 subregion coded alloantigens during the expression of ILT-reactivity

The influence of H-2 restriction on the effector function of DTH-reactive T cells directed against H-2 subregion coded alloantigens was also investigated with the ILT assay, which can be viewed as a local GvH assay. This assay, which was originally developed by Brent *et al.*, (1962) is based upon local transfer of 5 x 10^6 immunized lymphoid cells to naive recipients, which are either identical or partially identical to the original spleen cell inoculum used for immunization. The lymphoid cells from immunized donors were transferred into the dorsum of a hind foot of naive recipients five days after sc immunization. The foot swelling of the secondary recipients was measured 24 h after transfer. Fig. 4, line 1, shows that B10.A responder cells, immunized to K incompatible B10.AQR spleen cells, produced a significant ILT response when transferred to K incompatible B10.AQR recipients, whereas after transfer to K+I incompatible B10.T-

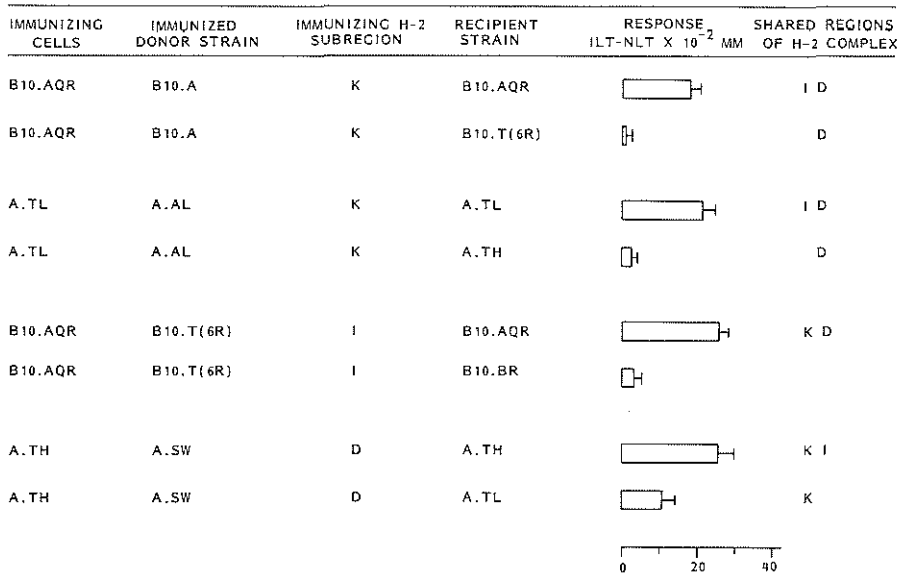

Fig. 4. H-2 restricted recognition of H-2K, I or D subregion coded alloantigens by DTH reactive T cells in the immune lymphocyte transfer assay. B10.A, A.AL, B10.T(6R) and A.SW mice were immunized with 1 x 10^7 B10.AQR, A.TL, B10.AQR and A.TH spleen cells, respectively. Five days later the draining lymph nodes from these mice were taken out, and cell suspensions were made. A number of 5 x 10^6 of these cells were transferred into the right hind foot of the indicated recipient mice. After 24 hr the foot thickness was measured. The response was calculated as indicated in the *Materials and Methods* section. The columns represent the mean response (ILT-NLT) ± SE in 10^{-2} mm (n=6).

(6R) recipients no reaction was observed (Fig. 4, line 2). The same was found for the A.TL-A.AL-A.TH combination (Fig. 4, lines 3 and 4). Furthermore, when B10.T(6R) responder mice were immunized with I incompatible B10.AQR spleen cells and lymphoid cells from these mice were transferred to I incompatible B10.AQR naive recipients, a clear ILT response was found, whereas transfer to K+I+D incompatible B10.BR recipients did not result in a significant ILT response (Fig. 4, lines 5 and 6). Moreover, when lymphoid cells from A.SW mice, immunized to D alloantigens from A.TH mice, were transferred to D incompatible A.TH naive recipients, they produced a significant ILT response, whereas after transfer to I+D incompatible A.TL naive recipients a much lower ILT response was found (Fig. 4, lines 7 and 8).

DISCUSSION

In this paper we report that DTH reactions to H-2 subregion coded alloantigens are restricted by other H-2 subregions, i.e., reactions to class I (H-2K/D) alloantigens are restricted by class II (H-2I) molecules and reactions to class II alloantigens are restricted by class I molecules. This can be demonstrated in a Host-versus-Graft, a local Graft-versus-Host and a systemic Graft-versus-Host assay. Thus, DTH reactions to MHC subregion coded alloantigens do not basically differ from DTH reactions to *Listeria monocytogenes* (Zinkernagel *et al.*, 1977), lymphocytic choriomeningitis virus (Zinkernagel *et al.*, 1976), fowl-γ-globulin (Miller *et al.*, 1975), dinitrofluorobenzene (Vadas *et al.*, 1977), sheep red blood cells (Bianchi *et al.*, 1981), and minor histocompatibility antigens (Van der Kwast, 1980), which all have been shown to be MHC restricted.

However, there are several reports in which the authors come to opposite conclusions with regard to the MHC restricted recognition of H-2 subregion coded alloantigens. Klein *et al.* (1977) have shown that cytotoxic lymphocytes, primed *in vivo* to IA alloantigens and subsequently restimulated *in vitro* with the same IA alloantigens, were able to lyse target cells bearing these IA alloantigens without a concomitant need for the target cells to bear the same K/D molecules as the killer T cells. Thus, K/D region compatibility was not needed for I region specific cell-mediated lymphocytotoxicity. In contrast, Kindred (1981, 1983a,b) has shown that T cells from radiation chimeras cause a lethal GvH reaction in irradiated bone marrow protected recipients only if the recipient shares a restriction element with the T cell donor. In her model, K and D molecules were found to act as restriction elements in lethal GvH induced by I coded alloantigens.

Restricted recognition of K and D alloantigens by I molecules has been investigated by Swain (1981). She has found that primed helper T cells directed

against allogeneic K + D alloantigens are not restricted by syngeneic I molecules. However, studies in which Ia positive cells were eliminated (Minami and Shreffler, 1981) and Ia blocking studies (Rock *et al.*, 1983) have shown that mixed lymphocyte responses to class I alloantigens are Ia restricted.

The conclusions from studies on restricted recognition of H-2 alloantigens from different laboratories seem to be mutually exclusive: the reaction to H-2 subregion alloantigens is either restricted or unrestricted. However, one must bear in mind that the results from Klein *et al.* (1977) and Swain (1981) were obtained with assay systems in which secondary type reactions are elicited, while the studies of Minami and Shreffler (1981) and Kindred (1981, 1983a,b) deal with primary responses. Moreover, Kindred showed by using different mouse strain combinations and by using T cells from normal mice and from radiation chimeras, that different H-2I specific T cell populations causing lethal GvH exist, which are either K/D restricted or unrestricted. Thus, it might be that the assay systems of Klein *et al.* (1977) and Swain (1981) select for unrestricted T cells.

In contrast to our conclusion, Smith and Miller (1979) and Weiss and Dennert (1981) have stated that H-2 specific DTH effector cells are unrestricted. These authors have shown that *in vivo* activated H-2 specific DTH effector T cells and Ia specific T cell lines can express DTH reactivity after transfer to H-2 incompatible naive recipients. The allogeneic cells used for elicitation of the DTH response in their experiments were similar to the allogeneic cells used for activation of the DTH effector T cells.

Our opinion is that these results do not exclude the possibility that these responses were actually H-2 restricted. We shall explain this in the following hypothetical model (Fig. 5). During the induction of DTH effector T cells to H-2 subregion coded alloantigens, these antigens are recognized in the context of other H-2 molecules on the surface of the same allogeneic cells or, after processing by antigen-presenting cells (APC), in the context of the H-2 molecules of the APC. When the injected alloantigens occur on cells from which the other H-2 molecules are syngeneic with the responder mouse, only *syn*restricted DTH effector T cells will be induced. On the other hand, when the particular H-2 alloantigens are presented on an allogeneic H-2 background, the DTH effector T cells induced by direct interaction with the allogeneic cells will be *allo*restricted, while the DTH effector T cells induced by alloantigen processing and presentation by APC will be *syn*restricted.

Van der Kwast (1980) has shown in studies concerning H-2 restricted recognition of minor H antigens in DTH that macrophage processing of antigen is only important during the induction of DTH reactivity and not during the expression phase. Thus, when H-2 subregion coded alloantigens are presented during the sc induction of the DTH reactivity on cells which are syngeneic to the

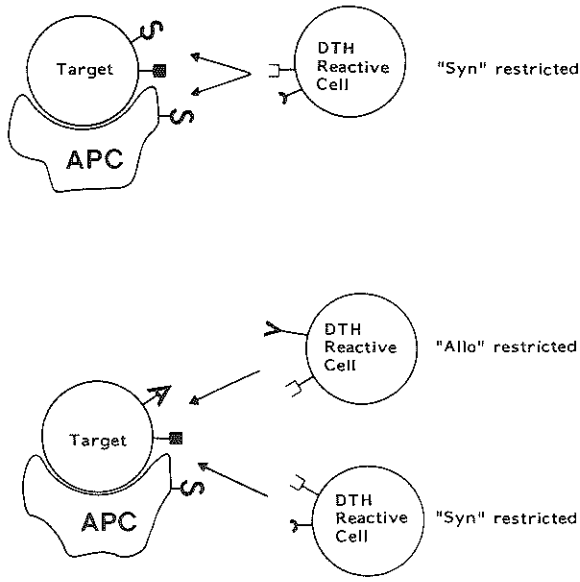

Fig. 5. Model for syngeneically and allogeneically H-2 restricted recognition of H-2 subregion coded alloantigens.

responder mouse strain for the other H-2 molecules, the activated T cells will not recognize these H-2 subregion coded alloantigens in combination with other H-2 alloantigens during the expression phase of DTH (Fig. 1 and 2, line 2). However, induction of DTH reactivity by allogeneic cells incompatible for several H-2 subregions (viz. Figs. 1 and 2, lines 3 and 4) will lead to activation of two subsets of DTH reactive T cells, one *syn*restricted (due to APC-processing) and one *allo*-restricted. Consequently, the mice will respond to a challenge of these alloantigens on the original allogeneic H-2 background as well as on a syngeneic H-2 background, which is precisely what we found (Figs. 1 and 2, lines 3 and 4). This model of generation of *'allo'* and *'syn'* restricted DTH effector T cells, can thus explain all the data presented in this paper. Furthermore, it can explain the data from Smith and Miller (1979) and Weiss and Dennert (1981) in that their DTH reactive T cells recognized the H-2 coded alloantigens in an allorestricted manner.

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CHAPTER VII

SUPPRESSION OF ANTIGRAFT IMMUNITY BY PREIMMUNIZATION I. KINETIC ASPECTS AND SPECIFICITY

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SUMMARY

Intravenous injection with 2,000 rad irradiated allogeneic cells can suppress the development of antigraft delayed type hypersensitivity (DTH) to major and minor histocompatibility (H) antigens which normally arises after s.c. immunization. Secondary type DTH responses to minor H antigens were also largely suppressed by an i.v. injection of irradiated allogeneic cells 1 week preceding the s.c. priming injection. The extent of suppression of primary DTH to allogeneic H-2-incompatible cells depended on the dose of i.v. injected irradiated cells. After a dose of 1×10^7 irradiated spleen cells i.v., the suppression persisted for at least 40 days. Intravenous injection of cells incompatible for minor H antigens could not suppress the DTH to H-2 alloantigens and vice versa. Suppression of DTH to H-2 alloantigens was haplotype specific.

Proliferation studies indicated that the immunosuppressed mice do not respond upon s.c. immunization with an increased proliferative activity in the draining lymph nodes, in contrast to nonsuppressed mice.

The data suggest that i.v. preimmunization with allogeneic cells induces specific suppression of antigraft immunity acting at the induction stage of the immune response.

INTRODUCTION

Suppression of immune responsiveness has been demonstrated *in vivo* for T helper function (1), DTH (2, 3), allograft rejection (4), graft-versus-host reactivity (5, 6), and *in vitro* for mixed lymphocyte reactivity (7, 8) and cell-mediated lympholysis (9, 10). Suppression may act upon the afferent as well as upon the efferent limb of the immune response (11). Suppression of alloreactivity can be antigen specific as well as nonspecific and may be mediated by humoral factors (e.g., antibodies or antigen-antibody complexes) as well as by T suppressor cells and macrophages (12, 13).

Immunization of mice for DTH to alloantigens is highly dependent on the route of antigen administration. Thus, some authors demonstrated that i.v. or i.p. immunization with allogeneic cells induces a poor state of DTH (14, 15), whereas s.c. immunization results in a good and stable DTH response to both major and minor H antigens (15, 16). Similarly, graft rejection seems to depend on the site of the graft, i.e., skin allografts are more prone to rejection than kidney allografts (17). Intravenous preimmunization of mice with allogeneic lymphoid cells could specifically prolong skin allograft rejection in a proportion of mice, if they were pretreated with either antithymocyte serum and procarbazine hydrochloride vaccine or cyclophosphamide (4, 18, 19). In the former model it was shown that T suppressor cells were present in mice which carried a skin allograft for a long time.

In experiments on DTH to hapten-modified syngeneic cells and on contact sensitivity to haptens, it was demonstrated that i.v. injection of hapten-modified cells to mice could inhibit the development of DTH after subsequent s.c. immunization or skin painting with the specific antigen (20, 21).

This paper deals with the suppressive effect of i.v. preimmunization with allogeneic lymphoid cells on allograft immunity to major and minor H antigens as determined with the DTH assay. The kinetics, specificity, and mode of action of suppression was investigated.

MATERIALS AND METHODS

Animals

(C57BL/Rij x CBA/Rij)F_1 (H-$2^{b/q}$), BALB/c (H-2^d), DBA/2 (H-2^d), C3H/Lw (H-2^k), and AKR (H-2^k) mice were purchased from the Radiobiological Institute TNO, Rijswijk, The Netherlands. C3Hf (H-2^k), B10.A (H-2^a), and A.SW (H-2^s) mice were purchased from the Laboratory Animal Centre of Erasmus University, Rotterdam, The Netherlands. B10.G (H-2^q), BALB.K (H-2^k), and BALB.B (H-2^b) mice were purchased from OLAC Ltd., Bicester, United Kingdom. Swiss

(H-2^s) and B10.ScSn (H-2^b) mice were purchased from the Central Institute for the Breeding of Laboratory Animals, TNO, Zeist, The Netherlands. CWB (H-2^b) mice were obtained from the Institut für Biologisch-Medizinische Forschung AG, Füllinsdorf, Switzerland. The age of the responder mice varied between 12 and 24 weeks. All mice used were females.

Preparation of cells suspensions

Spleens and lymph nodes were removed, placed in a balanced salt solution, and squeezed through a nylon gauze filter to provide a single-cell suspension. Nucleated cells were counted with a Coulter Counter model B.

Antigen and immunization

Primary and secondary immunization were performed with the appropriate allogeneic spleen cells, suspended in a volume of 0.1 ml. The priming and boosting dose was always 1 x 10^7 spleen cells. These cells were injected s.c., equally distributed over both inguinal areas. In a previous paper, it was shown that this dose induces maximal DTH responses in primary and secondary DTH to H-2 and non-H-2 alloantigens. In primary DTH to minor H antigens, peak DTH reactivity is generally found on day 5 after immunization (16). Suppressive injections of allogeneic cells suspended in a volume of 0.5 ml of balanced salt solution were given i.v., in doses as stated in the experiments. Immediately before the i.v. injection, the cell suspensions were irradiated *in vitro* with 2,000 rad, generated in a Phillips-Müller MG 300 X-ray machine as described in detail prevoiously (22).

Estimation of cell proliferation in vivo

For estimation of the cell proliferation in inguinal lymph nodes, the method described by North et al. (23) was used. Briefly, at varying intervals after s.c. immunization with 1 x 10^7 allogeneic spleen cells, the mice were given i.v. injections of 20 μc of [methyl-^{3}H]thymidine (specific activity, 5 c/mM). Thirty minutes later their inguinal lymph nodes were taken out and suspended in 5% ice cold trichloroacetic acid. Each cell suspension was extracted twice for 1 hr with 20 ml of cold 5% trichloroacetic acid. Thereafter, the suspension was extracted in 6 ml of 5% trichloroacetic acid at 90 C for 1 hr. After cooling, 1 ml of the supernatant was added to 9 ml of scintillant consisting of 3 ml of Triton X-100 and 6 ml of toluene containing 4 mg of 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 30 to 35 cpm.

Assay for DTH

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 with a 0.05 mm accuracy. During measurement the mice were anesthetized with ether.

A control group consisting of nonimmune mice challenged with the same number of 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 control mice. The swelling in control mice ranged between 18 and 26%.

RESULTS

Suppression of the capacity of DTH reactivity by i.v. injection of allogeneic spleen cells

The influence of i.v. injection of allogeneic spleen cells upon subsequent induction of DTH reactivity by s.c. immunization with spleen cells from the same donor strain was investigated. Groups of (C57BL x CBA)F_1 mice were given i.v. injections of either 1×10^7 of 2,000 rad of irradiated allogeneic H-2-incompatible BALB/c spleen cells or of the same number of syngeneic spleen cells. For comparison, groups of (C57BL x CBA)F_1 mice were given s.c. injections of 1×10^7 irradiated BALB/c or (C57BL x CBA)F_1 spleen cells. Seven days later all mice were immunized with 1×10^7 BALB/c spleen cells s.c. and another 6 days later a challenge injection of BALB/c spleen cells was given. At 24 hr the DTH reactivity was determined. It seemed that i.v. preimmunization with irradiated BALB/c spleen cells caused a significant immunosuppression of DTH reactivity, whereas no suppression was found in the other groups of mice (Table 1). The extent of suppression of DTH induced by i.v. preimmunization was not affected by the age of the responder mice used (data not shown).

The determination of the optimal dose of i.v. injected spleen cells was done with different groups of (C57BL x CBA)F_1 mice that were injected with 10^4, 10^5, 10^6, or 10^7 irradiated BALB/c or (C57BL x CBA)F_1 spleen cells. Seven days later all mice were immunized s.c. with 1×10^7 BALB/c spleen cells and tested for DTH again 6 days later. The immunosuppression appeared to be dose dependent and maximal when induced by 1×10^7 or more irradiated allogeneic spleen cells (Fig. 1A).

The optimal dose of i.v. injected spleen cells was also determined in a combination differing for minor H antigens only. For this purpose Swiss mice were given i.v. injections of 1×10^6, 3×10^6, 1×10^7, 3×10^7, or 1×10^8 irradiated A.SW or Swiss spleen cells, s.c. immunized with 1×10^7 A.SW spleen

TABLE 1. Preimmunization with allogeneic cells

Preimmunization[a]		% specific increase[b]
Inoculum	Route	
(C57BL x CBA)F_1	s.c.	28 ± 1
BALB/c	s.c.	22 ± 2
(C57BL x CBA)F_1	i.v.	25 ± 2
BALB/c	i.v.	5 ± 3

[a] (C57BL x CBA)F_1 mice were given i.v. or s.c. injections of 1 x 10^7 irradiated syngeneic or H-2-incompatible allogeneic BALB/c spleen cells. Seven days later all mice were immunized s.c. with 1 x 10^7 BALB/c cells and challenged another 6 days later.
[b] Mean responses ± 1 SE (n = 5).

cells 7 days later, and tested for anti-A.SW DTH reactivity again 5 days later. Also, in this combination i.v. preimmunization by 1 x 10^7 or more irradiated allogeneic spleen cells induced maximal suppression (Fig. 1B). The same result was obtained with DBA/2 responder mice immunized with BALB/c spleen cells, another combination only differing for minor H antigens (data not shown).

Effect of i.v. preimmunization upon the kinetics of the DTH reactivity

To assess the suppressive effect of i.v. preimmunization upon the kinetics of the DTH response to H-2-incompatible allogeneic cells, groups of (C57BL x CBA)F1 mice were given i.v. injections of either 1 x 10^7 irradiated BALB/c or 1 x 10^7 irradiated (C57BL x CBA)F_1 spleen cells. Seven days later all mice were s.c. immunized with 1 x 10^7 normal BALB/c spleen cells and challenged on day 1, 3, 5, 6, 8, or 15 after immunization. The immunosuppressed group of mice did not show significant DTH reactivity at any of the tested time points. The nonsuppressed mice reached peak levels of antigraft DTH reactivity between days 5 and 6 after immunization (Fig. 2). The same results were obtained in a similar set up with another strain combination, in which C3Hf mice were immunized with C57BL/Rij spleen cells (data not shown).

Time course of the immunosuppression induced by i.v. preimmunization

The duration of the suppressive effect induced by i.v. injection of irradiated allogeneic spleen cells was investigated in the combination of BALB/c ($H\text{-}2^d$) and BALB.K ($H\text{-}2^k$) mice, which have distinct H-2 haplotypes, and in the

combination of C3H/Lw (H-2^k) and AKR (H-2^k) mice, which only differ for minor H antigens.

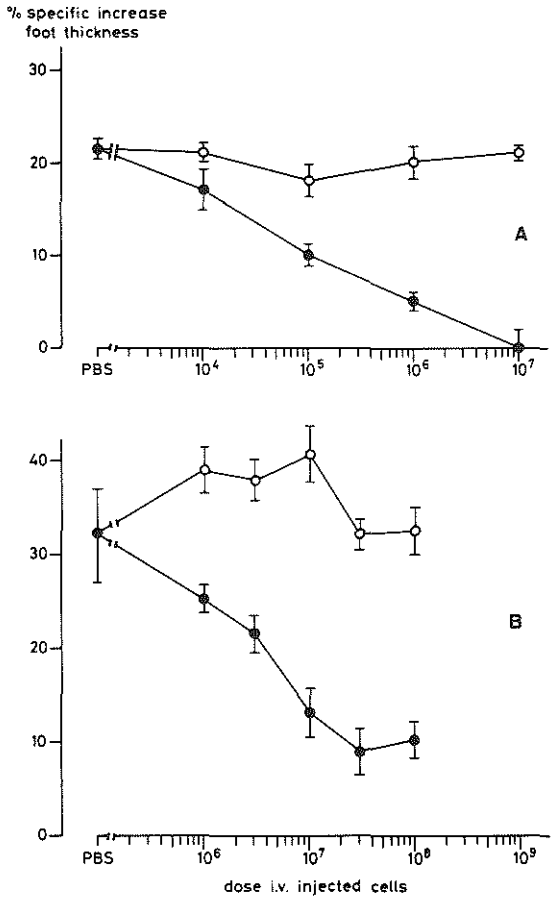

Fig. 1. Determination of the optimal dose of i.v. injected spleen cells for suppression of DTH reactivity to allogeneic spleen cells. A: (C57BL x CBA)F_1 mice were given injections of 1 x 10^4, 1 x 10^5, 1 x 10^6, or 1 x 10^7 irradiated BALB/c (●) or (C57BL x CBA)F_1 spleen cells (○). Seven days later all mice were s.c. immunized with 1 x 10^7 BALB/c spleen cells, and another 6 days later tested for DTH. B: Swiss mice were given injections of 1 x 10^6, 3 x 10^6, 1 x 10^7, 3 x 10^7, or 1 x 10^8 irradiated A.SW (●) or Swiss spleen cells (○). Seven days later all mice were immunized s.c. with 1 x 10^7 A.SW spleen cells, and another 5 days later tested for DTH. Each experimental group represents the arithmetic mean ± 1 SE (n = 5).

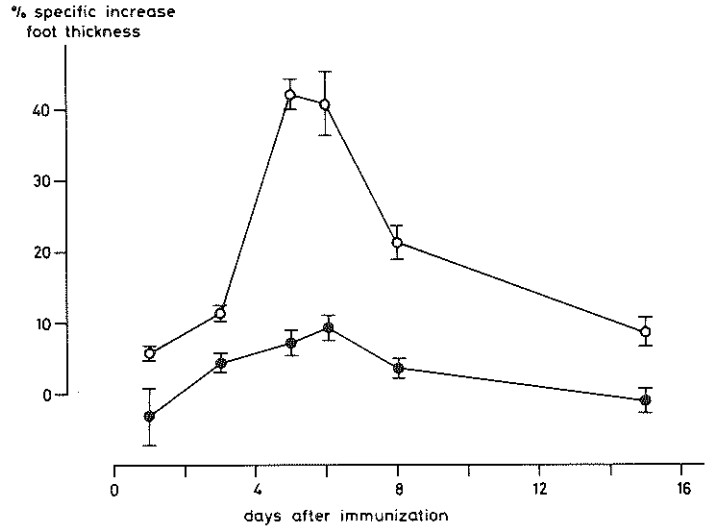

Fig. 2. Effect of i.v. preimmunization upon the kinetics of the DTH reactivity to allogeneic spleen cells. (C57BL x CBA)F_1 mice were given i.v. injections of 1 x 10^7 irradiated BALB/c (●) or (C57BL x CBA)F_1 spleen cells (○). Seven days later all mice were s.c. immunized with 1 x 10^7 BALB/c spleen cells, and challenged on day 1, 3, 5, 6, 8, or 15 after immunization. Each experimental group represents the arithmetic mean ± 1 SE (n = 5).

Groups of BALB/c mice were given i.v. injections of 1 x 10^7 irradiated BALB.K or BALB/c spleen cells on days -43, -23, -16, -7, and -3. On day 0 groups of these mice were s.c. immunized with BALB.K spleen cells, and on day 6 all mice were challenged. It seemed that the suppression of DTH reactivity by i.v. preimmunization lasted for at least 43 days (Fig. 3A). The data tend to indicate that the suppression slightly diminished in course of time.

For the suppression of DTH to non-H-2 alloantigens, C3H/Lw mice were given i.v. injections of 1 x 10^7 irradiated AKR or C3H/Lw spleen cells on days -41, -27, -14, -8, and -5. On day 0 groups of these mice were s.c. immunized with AKR spleen cells, and on day 5 all mice were challenged. It was found that the suppression did not diminish during the experimental period of 41 days (Fig. 3B).

Effect of i.v. preimmunization upon the secondary DTH response to non-H-2 alloantigens

To investigate whether i.v. preimmunization could also lead to suppression of secondary type DTH reactivity to minor H antigens, groups of DBA/2 mice

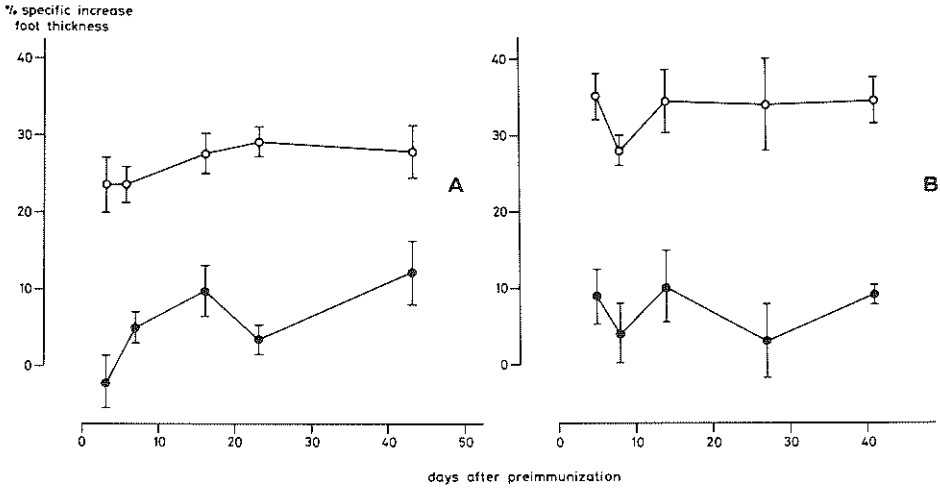

Fig. 3. Time course of the immunosuppression induced by i.v. preimmunization. A: Groups of BALB/c mice were given i.v. injections of 1 x 10^7 irradiated BALB.K (●) or BALB/c spleen cells (○) on days -43, -23, -16, -7, and -3. On day 0, groups of these mice were s.c. immunized with BALB.K spleen cells, and on day 6 all mice were challenged. B: Groups of C3H/Lw mice were given i.v. injections of 1 x 10^7 irradiated AKR (●) or C3H/Lw spleen cells (○) on days -41, -27, -14, -8, and -5. On day 0, groups of these mice were s.c. immunized with AKR spleen cells, and on day 5 all mice were challenged. Each experimental point represents the arithmetic mean ± 1 SE (n = 5).

were given i.v. injections of irradiated H-2-compatible allogeneic BALB/c spleen cells or syngeneic DBA/2 spleen cells. Seven days later all mice were s.c. immunized with 1 x 10^7 BALB/c spleen cells and boosted with similar cells 6 weeks later. Three, 5 and 7 days after the booster immunization, all mice were challenged to measure the secondary DTH reactivity. Primary DTH reactivity was determined as well on days 3, 5, and 7 after immunization. BALB/c mice that were given i.v. injections of irradiated syngeneic spleen cells showed a clear secondary type DTH response to the DBA/2 minor H antigens, in contrast to the BALB/c mice preimmunized with irradiated DBA/2 cells (Fig. 4). Thus, secondary type DTH reactivity to minor H antigens was largely suppressed in the mice that received an i.v. injection of allogenic irradiated cells 1 week before s.c. priming.

Specificity of suppression of DTH to H-2 and minor H-incompatible spleen cells

Different groups of BALB/c (H-2^d) mice were given i.v. injections of either 1 x 10^7 irradiated H-2-incompatible BALB.K (H-2^k), 1 x 10^7 minor H-

incompatible DBA/2 (H-2^d), or 1 x 10^7 syngeneic BALB/c spleen cells. Seven days later all mice were s.c. immunized with BALB.K or DBA/2 spleen cells and challenged with similar cells another 6 days later. Intravenous preimmunization with the H-2-incompatible BALB.K cells did not interfere with the development of DTH after s.c. injection with minor H-incompatible DBA/2 spleen cells.

Similarly, i.v. preimmunization with DBA/2 cells did not significantly affect the anti-BALB.K DTH reactivity. Suppression of anti-BALB.K or anti-DBA/2

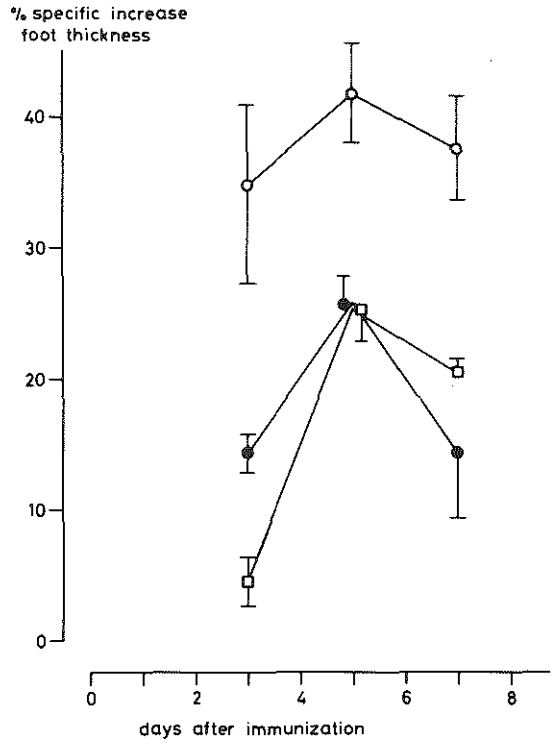

Fig. 4. Effect of i.v. preimmunization upon the secondary DTH response to non-H-2 alloantigens. Groups of DBA/2 mice were given i.v. injections of 1 x 10^7 irradiated BALB/c (●) or DBA/2 spleen cells (○). Seven days later all mice were s.c. immunized with 1 x 10^7 BALB/c spleen cells, and another 6 weeks later boosted with similar cells. Three, 5 and 7 days after booster immunization, different groups of mice were challenged with BALB/c spleen cells to measure the secondary DTH reactivity to these cells. Primary anti-BALB/c DTH reactivity (□) was determined as well on days 3, 5, and 7 after immunization. Each experimental point represents the arithmetic mean ± 1 SE (n = 5).

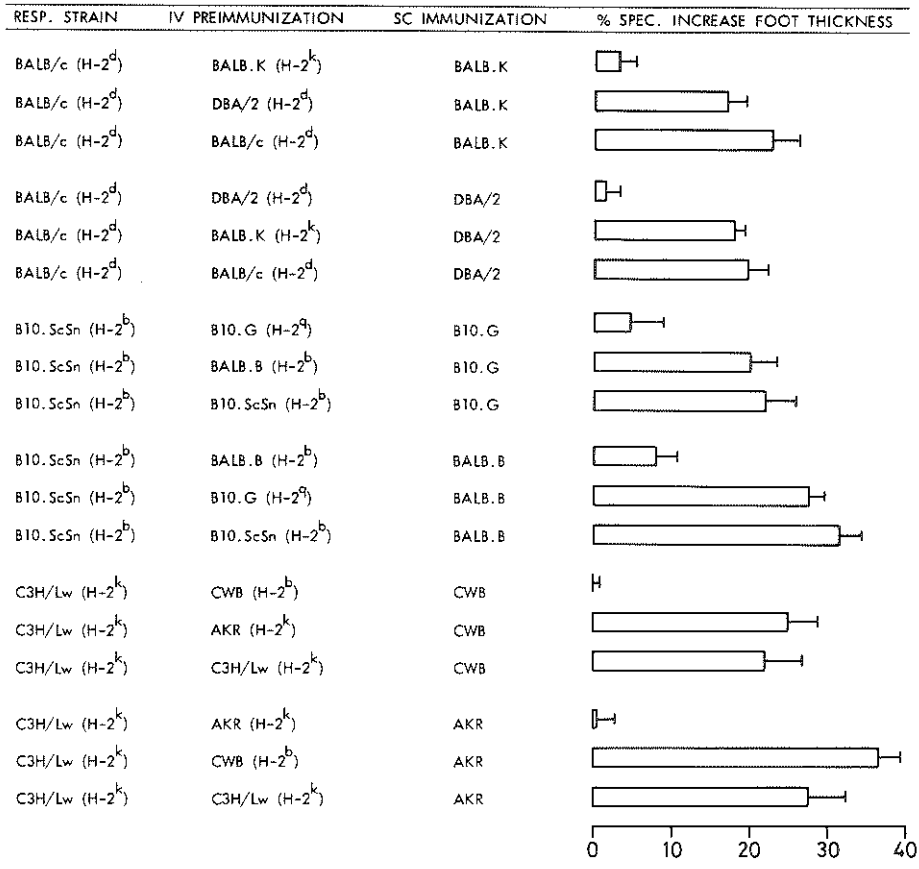

Fig. 5. Specificity of i.v. induced suppression for H-2 and non-H-2 alloantigens. Responder mice were given i.v. injections of 1 x 10^7 irradiated allogeneic or syngeneic spleen cells and s.c. immunized with 1 x 10^7 allogeneic spleen cells 7 days later. Challenge for DTH was performed on day 6 after s.c. immunization. Each column represents the mean response ± 1 SE of five mice.

DTH reactivity only occurred when the i.v. and s.c. injections were done with identical spleen cells (Fig. 5).

Similar experiments were done with B10.ScSn (H-2^b) responder mice given i.v. and s.c. injections of either H-2-incompatible B10.G (H-2^q) spleen cells or minor H-incompatible spleen cells. These experiments also revealed that suppression of anti-B10.G or anti-BALB.B DTH reactivity only occurred when the i.v. and s.c. injections were done with identical spleen cells (Fig. 5). The same results were

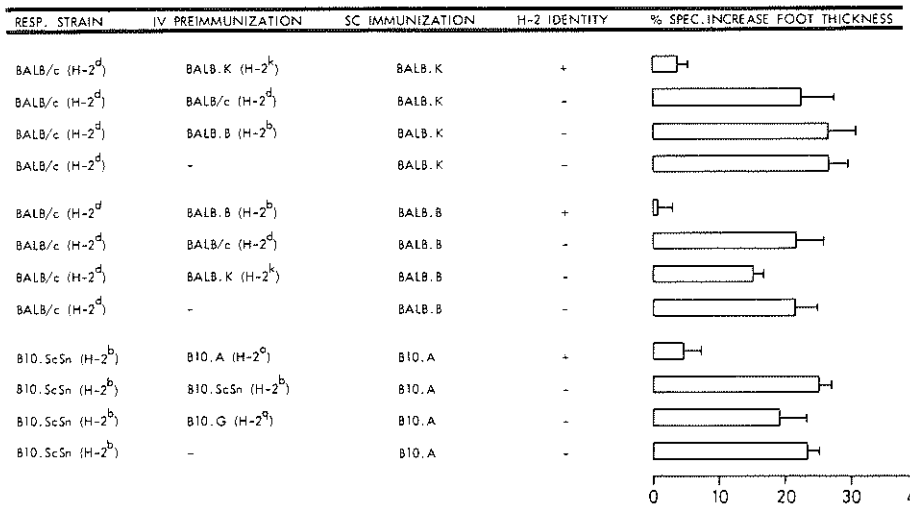

Fig. 6. Specificity of i.v. induced suppression for different H-2 haplotypes. Responder mice were given i.v. injections of 1 x 10^7 irradiated allogeneic or syngeneic spleen cells and s.c. immunized with 1 x 10^7 allogeneic spleen cells 7 days later. Challenge for DTH was performed on day 5 after s.c. immunization. Each column represents the mean response ± 1 SE of five mice.

obtained in a similar protocol with C3H/Lw (H-2^k) responder mice, CWB (H-2^b) spleen cells, and AKR (H-2^k) spleen cells (Fig. 5).

Specificity of suppression of DTH to H-2 alloantigens

After having established that it is impossible to suppress the induction of DTH reactivity to H-2-incompatible cells by i.v. preimmunization with minor H-incompatible spleen cells and vice versa, we investigated whether or not the suppression of the DTH reaction to H-2 alloantigens is haplotype specific. Therefore, groups of BALB/c (H-2^d) mice were given i.v. injections of either 1 x 10^7 irradiated H-2-incompatible BALB.K (H-2^k), 1 x 10^7 H-2-incompatible BALB/B (H-2^b), or 1 x 10^7 syngeneic BALB/c spleen cells. Seven days later all of these mice and a group of BALB/c mice that had not been preimmunized were s.c. immunized with 1 x 10^7 BALB.K or 1 x 10^7 BALB.B spleen cells. Another six days later the mice were challenged with similar spleen cells as used for the s.c. immunization. Significant suppression was only found in the groups of BALB/c mice given i.v. and s.c. injections of H-2-incompatible cells of the same haplotype (Fig. 6).

According to the same protocol, B10.ScSn (H-2^b) mice were given i.v.

injections of either B10.A (H-2^a), B10.G (H-2^q), or B10.ScSn (H-2^b) spleen cells. Seven days later all of these mice and a control group of untreated B10.ScSn mice were s.c. immunized with B10.A spleen cells. Another six days later all mice were challenged with B10.A cells. Again, suppression seemed to be specific for the H-2 haplotype of the i.v. injected cells. Thus, the DTH reactivity to B10.A spleen cells could only be suppressed by i.v. preimmunization with B10.A cells (Fig. 6).

Proliferative activity in lymph nodes of immunosuppressed mice after s.c. immunization

Groups of (C57BL x CBA)F_1 mice (H-$2^{b/q}$) were given i.v. injections of either 1 x 10^7 irradiated H-2- and minor H-incompatible BALB/c (H-2^d) or syngeneic (C57BL x CBA)F_1 spleen cells on day -7. On day 0, the mice of both

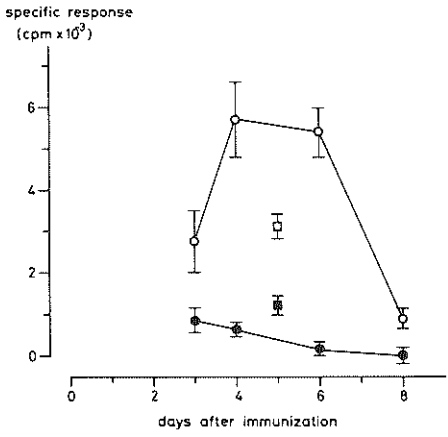

Fig. 7. Proliferative activity in the draining lymph nodes of immunosuppressed mice after s.c. immunization. Groups of (C57BL x CBA)F_1 mice were given i.v. injections of 1 x 10^7 irradiated BALB/c (●) or (C57BL x CBA)F_1 (○) spleen cells on day -7. On day 0, the mice of both groups were s.c. immunized with BALB/c spleen cells, and on days 1, 3, 4, 6, and 8 the proliferative activity in the draining inguinal lymph nodes was determined. Similarly, proliferation was determined in lymph nodes of BALB/c mice i.v. preimmunized with 1 x 10^7 irradiated DBA/2 (■) or BALB/c spleen cells (□). On day 0, the mice of both groups were s.c. immunized with DBA/2 spleen cells and on day 5 the proliferative activity in the draining inguinal lymph nodes was determined. For each experimental point there was a control group included which was not i.v. and s.c. immunized. The proliferative activity in the lymph nodes of these mice was the same as in the immunosuppressed mice. Each point represents the arithmetic mean ± 1 SE (n = 5).

groups were s.c. immunized with BALB/c spleen cells, and on days 3, 4, 6, and 8 the proliferative activity in the draining inguinal lymph nodes of the different groups of mice was determined. (C57BL x CBA)F_1 mice given i.v. injections of syngeneic cells showed peak proliferative activity at 4 to 6 days after s.c. immunization with BALB/c spleen cells. However, in the immunosuppressed mice no apparent increase in proliferative activity of lymph node tissue was observed at any of the tested intervals after s.c. immunization (Fig. 7). Similarly, proliferation was determined in lymph nodes of BALB/c mice i.v. preimmunized with minor H-incompatible DBA/2 or syngeneic BALB/c spleen cells, and s.c. immunized with allogeneic H-2-compatible DBA/2 (H-2^d) spleen cells. Again, significant inhibition of proliferative activity was observed in the lymph nodes of the immunosuppressed mice (Fig. 7).

DISCUSSION

This paper demonstrates that a single i.v. injection of mice with irradiated (2,000 rad) allogeneic spleen cells can induce an antigen-specific suppression of primary and secondary antigraft DTH responsiveness. The extent of suppression can vary between different experiments, but usually accounts for a reduction of the response by 70 to 90%. In some experiments a complete suppression of the antigraft DTH reactivity was found. The i.v. route of preimmunization is obligatory for the suppression (Table 1). This suggests that the spleen is directly involved, which is supported by experiments involving splenectomy (Bianchi *et al.,* to be published). Since the suppression can be induced by heavily irradiated cells as well as by crude membrane preparations (data not shown), the suppression of DTH to alloantigens must be mediated by the host. The suppression cannot be explained as a recruitment phenomenon, because recruitment is a short-lasting process (24) and the suppression described here lasts for at least 40 days (Fig. 3). From the reduced proliferative activity in the regional lymph nodes of s.c. immunized immunosuppressed mice (Fig. 7), it can be concluded that the suppressive mechanism affects the "afferent" limb of the immune response. Recently, it was found that Thy-1.2^+ spleen cells from immunosuppressed mice were also capable to suppress the "efferent" limb of the DTH response (Bianchi *et al.,* to be published). Experiments that will reveal whether or not similar suppressor T cells are also responsible for the suppression of the "induction" phase of the DTH response are in progress.

A number of studies have revealed antigen-nonspecific T cell-mediated suppression of mixed lymphocyte reaction and cell-mediated lympholysis to H-2-incompatible cells. The nonspecific suppressor T cells were induced *in vivo* by s.c. or i.v. injection of alloantigens, and could only be recovered from the spleen

(7, 25). Recently, however, it was found that injection of heat-treated allogeneic cells could induce alloantigen-specific suppression of cytotoxic T cell responses in a large proportion of mice (26). In this latter study the i.v. route of injection was obligatory for induction of suppression.

A DTH model with some similarity to the one presented here is that of Miller *et al.* (20, 27, 28), who induced suppression of contact sensitivity to haptens by i.v. preimmunization with hapten-modified syngeneic or allogeneic lymphoid cells. Both in their model and in the experiments reported here, suppression of DTH reactivity could be induced for antigenic determinants presented on H-2-compatible or H-2-incompatible lymphoid cells. Intravenous injection of hapten-modified syngeneic cells induced a clonal inhibition of immune reactive cells directed against the hapten as well as induced the generation of antigen-specific suppressor T cells (27). These suppressor T cells affected the efferent limb of the DTH immune response. Contrarily, i.v. injected hapten-modified H-2-incompatible lymphoid cells induced suppressor T cells acting at the afferent limb of the immune response only. These suppressor T cells exerted their suppressive effect only upon transfer to the mouse strain from which the hapten-modified allogeneic cells were derived (28). Furhter investigations are required to fit these data of contact sensitivity into our antigraft DTH model.

Others have reported that i.v. injection of alloantigens can also prolong graft survival. However, such results were only found when the preimmunization was done in combination with treatment with immunosuppressive drugs or anti-lymphocyte serum (4, 18, 19), and only in certain donor-host strain combinations. Even under these conditions only a proportion of the treated mice showed a delayed graft rejection. This is in clear contrast to our studies on suppression of DTH reactivity to histocompatibility antigens where no significant recovery of DTH responsiveness arose at any time interval after the s.c. immunization. Thus, suppression of DTH is not based on a mere shift of the moment of peak responsiveness (Fig. 2).

From the foregoing it is clear that i.v. preimmunization more easily induces suppression of antigraft DTH reactivity than suppression of allograft rejection. This is probably related to the different types of T effector cells mediating these immune responses and may point to the complexity of the process of graft rejection.

DTH responses are mediated by lymphokine-producing T cells that require proliferation for full development of their reactivity (29). Skin graft rejection, on the other hand, is mainly dependent upon cytolytic T lymphocytes (CTLs). This may be inferred from the experiments of Rouse and Wagner (30) showing that CBA CTLs, activated by BALB/c stimulator cells *in vitro*, specifically rejected BALB/c allografts upon transfer into thymectomized, lethally irradiated, bone marrow-reconstituted CBA mice. Although generation of active CTLs from

their Lyt-2,3$^+$ precursors is dependent upon the presence of an amplifying factor derived from antigen-activated Lyt-1$^+$ T cells (31), the production of this factor does not require proliferation (32). This might explain why a suppressor system that inhibits proliferation (Fig. 7) can account for complete inhibition of the development of DTH reactivity, although it hardly affects transplant rejection. Alternatively, the i.v. preimmunization might lead to a selective stimulation of the precursors of CTLs.

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CHAPTER VIII

SUPPRESSION OF ANTIGRAFT IMMUNITY BY PREIMMUNIZATION II. CHARACTERIZATION OF THE SUPPRESSOR CELLS

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SUMMARY

Immunization of mice with irradiated (20 Gy) or *non*-irradiated allogeneic spleen cells iv induces delayed type hypersensitivity (DTH) reactive T cells, as well as suppressor T cells, against histocompatibility (H) antigens. The suppressor T cells are unable to suppress the induction and functional activity of the simultaneously activated DTH reactive T cells. However, the suppressor T cells do suppress the generation of DTH reactive T cells after subsequent sc immunization of the same mice and after transfer into secondary recipients.

Systemic transfer of suppressor T cells is effective the first few days after their induction only, and affects the afferent limb of the DTH response. The population of suppressor T cells which is essential for the systemic transfer of suppression, appeared to by Lyt-$1^{+}2^{+}$. Splenectomy experiments showed that the spleen is not essential for induction of the suppressor T cells. The precursors of the suppressor T cells belong to the pool of recirculating T lymphocytes; they are insensitive to adult thymectomy and can be depleted by anti thymocyte serum treatment.

INTRODUCTION

Induction of immune reactivity and tolerance *in vivo* has been shown to depend on the dosage of antigen (1-3), on the route of antigen administration (2, 4, 5) and on the antigen form (4-6). As far as cell-mediated immune reactions are concerned, most studies deal with the induction or suppression of delayed type hypersensitivity (DTH) or contact sensitivity (CS) against haptens (2, 6, 7). These studies suggest that unresponsiveness may depend on a shortage of antigen reactive cells or on an active suppression mediated by suppressor T cells (5, 8, 9). Some of the suppressor T cell systems were found to affect either the induction phase (10, 11) or the effector phase (12, 13) of the immune response, but other systems affect both (5, 8).

Studies aimed at manipulation of the immune response against histocompatibility (H) antigens are of special interest in view of their potential application in influencing the anti-graft immune response of transplant recipients. Several studies with mice have shown that infusion of blood cells or lymphoid cells can prolong skin (14, 15) or heart (16, 17) transplant survival. In most of these studies additional immunosuppression (e.g., by antilymphocyte serum or cyclophosphamide) appeared to be required in order to reveal the suppression by the suppressor T cells (14-16, 18).

In previous studies we have shown that sc induction of host-vs-graft DTH reactivity of mice against H antigens can be prevented by iv preimmunization of the responder mice with similar irradiated allogeneic spleen cells (3). Preimmunization iv with alloantigens can also suppress the development of graft-vs-host (GvH) related DTH effector T cells after inoculation of irradiated, allogeneic recipients with the preimmunized donor cells (19).

The iv induced suppression appears to be antigen-dose dependent and the suppressive effect is a long-lasting phenomenon (3). Specificity studies have shown that the iv induced suppression can only be elicited by the antigen(s) originally used for induction of the state of suppression (19, 20). However, after elicitation of the suppressive effect with the relevant antigen, the immune response against third party antigens is also suppressed (19, 20). Preliminary studies (20, 21) have shown that suppressor T cells account for the iv induced suppression of DTH to H-antigens. Antigen specific suppression can be induced by minor H antigens as well as by H-2K, H-2I or H-2D antigens (19, 22, 23). By using our protocol for iv induction of suppression there is, in contrast to the protocol of Liew (23), no special need for allo-I-J antigens for iv induction of suppression to H-2 coded alloantigens (24).

The requirements for the induction and expression of the suppressive effect, the requirements for transfer of the suppressive effect to syngeneic recipients and the characterization of the T cells involved in this type of suppression are discussed here.

MATERIALS AND METHODS

Mice

(C57BL/Rij x CBA/Rij)F1 (H-$2^{b/q}$), BALB/c (H-2^{d}) and A.SW (H-2^{s}) mice were purchased from the Laboratory Animals Centre of the Erasmus University, Rotterdam. DBA/2 (H-2^{d}) mice were purchased from the Radiobiological Institute TNO, Rijswijk, The Netherlands. Swiss (H-2^{s}) mice were purchased from the Central Institute for the Breeding of Laboratory Animals TNO, Zeist, The Netherlands. C3H/Tif (H-2^{k}) mice were purchased from Bomholtgard, Ry, Denmark. B10.D2 (H-2^{d}) and B10.BR (H-2^{k}) mice were purchased from OLAC Ltd., Bicester, United Kingdom. The age of the responder mice varied between 10 and 24 weeks. All mice used were females.

Preparation of cell suspensions

Mice were killed using carbon dioxide. The spleens or lymph nodes were removed, placed in 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. The viability of the cell suspensions obtained was at least 90%.

Immunization

Suppression was induced by iv preimmunization with 5 x 10^{7} irradiated (20 Gy) allogeneic spleen cells, unless indicated otherwise. In general, DTH was induced by sc immunization with 1 x 10^{7} nonirradiated allogeneic spleen cells. There was an interval of 7 days between iv induction of suppression and sc immunization for DTH. Five or six days after the sc immunization the mice were tested for DTH reactivity by injection of a challenge dose into the dorsum of the right hind foot. The H-2 and non-H-2 compatibilities and incompatibilities of the mouse strain combinations used in this study are listed in Tabel 1.

Transfer of suppression

The state of suppression was transferred to recipient mice by iv injection of spleen and lymph node cells from mice which were iv suppressed several days previously. The interval between iv suppression and transfer is given in the *Results* section for each experiment separately. A few hours after transfer, the recipients were sc immunized, unless indicated otherwise.

Splenectomy

Splenectomy (Sx) and sham-splenectomy (ShSx) were performed 1 month before iv suppression. Mice were anesthetized by an ip injection of 0.1 ml/10 g body weight of a 1:40 diluted stock solution of Avertin (25). The incision was

TABLE 1. H-2 and non-H-2 compatibilities and incompatibilities of the mouse strain combinations used for induction of delayed type hypersensitivity reactions

Responder	Donor	H-2 compatible	non-H-2 compatible
Swiss	A.SW	+[1)]	–
(C57BL x CBA)F1	BALB/c	–	–
B10.BR	B10.D2	–	+
BALB/c	B10.D2	+	–
(C57BL x CBA)F1	DBA/2	–	–
DBA/2	BALB/c	+	–
C3H/Tif	(C57BL x CBA)F1	–	–

1) The plus sign means that that particular donor-recipient combination is compatible, whereas a minus sign means that that combination is incompatible with regard to the H-2 or non-H-2 histocompatibility antigens.

made in the left upper abdomen. For splenectomy, the splenic blood vessels were tied in a single suture, then cut and the spleen was removed. The incision was closed in two layers. There was no postoperative mortality.

Adult thymectomy

Adult thymectomy (ATx) and sham-thymectomy (ShTx) were performed when the mice were 6 weeks old. The operation was performed as described by Miller (26). Avertin was used for anesthesia. The ATx and ShTx mice were rested for 10 weeks before experimental use. Mice used at least 6 weeks after ATx are considered to be depleted of short-lived, sessile T cells (T1 cells).

Anti-thymocyte serum

Anti-thymocyte serum (ATS) was prepared in New Zealand White rabbits by two iv injections of 5×10^8 BALB/c thymocytes, according to the method of Jooste *et al.* (27). Before use *in vivo*, the ATS and normal rabbit serum (NRS) were absorbed once with an equal volume of mouse red blood cells. For the elimination of long-lived recirculating T cells (T2 cells) a total volume of 0.2 ml ATS was subcutaneously injected, equally distributed over the inguinal and axillary areas. These injections were given 5 and 2 days before iv suppression. We have previously shown that the above procedure depletes the T2 cells (28).

Selective elimination of Thy-1.2, Lyt-1.1 and Lyt-2 positive cells

Monoclonal IgM anti-Thy-1.2 antibodies (clone F7D5) were purchased from OLAC Ltd., Bicester, U.K. Monoclonal IgG2a anti-Lyt-1.1 (clone 7-20.6/3) was purchased from Cedar Lane Laboratories Ltd., Hornby, Ontario, Canada. Monoclonal IgM anti-Lyt-2 was obtained by the *in vitro* culture of an IgM anti-Lyt-2 producing hybridoma, which was kindly provided by Dr. F.W. Fitch, Department of Pathology, University of Chicago. Cell suspensions were treated for 30 min at 4 C with anti-Thy-1.2, anti-Lyt-1.1 or anti-Lyt-2 antibodies. After incubation the cells were centrifuged, resuspended in BSS, and incubated with guinea pig complement (Flow Laboratories, Irvine, Scotland) for 15 min at 37 C. The cells were then washed three times and resuspended in BSS. The applied procedure eliminated at least 90% of the viable lymphocytes that were positive for the marker detected by the monoclonal antibody used.

Estimation of cell proliferation in vivo

For estimation of the cell proliferation in the inguinal lymph nodes, the method described by North *et al.* (29) was used. Briefly, 5 days after sc immunization with 1×10^7 allogeneic spleen cells, the mice were given iv injection of 20 μCi of methyl-^{3}H-thymidine (specific activity: 5 Ci/mM). Thirty minutes later their inguinal lymph nodes were taken out and suspended in 5% ice cold trichloroacetic acid. Each cell suspension was extracted twice for 1 hr with 20 ml cold 5% trichloroacetic acid and then once with 6 ml 5% trichloroacetic acid for 1 hr at 90 C. After cooling, 1 ml of the supernatant was added to 9 ml scintillant consisting of 3 ml of Triton X-100 and 6 ml of toluene containing 4 mg PPO per liter, and counted in a liquid scintillation counter (Packard, model B375). Radioactivity was corrected for background and quenching and expressed as cpm.

Assay for DTH

DTH reactions were determined by measuring the difference in thickness of the hind feet 24 hr after sc injection of a challenge dose of 2×10^7 of the appropriate allogeneic spleen cells into the dorsum of the right hind foot. As a control for background DTH reactivity, naive syngeneic mice were used which only received the challenge dose. Foot thickness was measured with a footpad meter with a 0.05 mm accuracy and a 0.05 mm reproducibility. The specific DTH response was calculated as the relative increase in foot thickness of the immune mice minus the relative increase in foot thickness of the control mice. The swelling of the control mice ranged between 15 and 25%.

RESULTS

Induction and suppression of DTH against histocompatibility antigens

Immunization of mice with allogeneic spleen cells sc induces a state of DTH reactivity (30). In order to investigate whethter iv immunization with allogeneic spleen cells also induces DTH reactivity, different groups of Swiss responder mice were immunized with various doses of irradiated or nonirradiated A.SW spleen cells and tested for DTH reactivity 5 days later. Increasing doses of irradiated and nonirradiated A.SW spleen cells induced an increasing DTH reactivity. Maximal DTH responses were found after iv injection of 1×10^7 irradiated and 3×10^7 nonirradiated spleen cells, respectively (Fig. 1A). Nonirradiated cells were found to be more effective in the induction of DTH reactivity than the same dose of irradiated cells. Similar data were obtained in other combinations involving H-2 alloantigens.

In previous studies (3, 19, 20, 22) suppression of DTH was found when sc immunized mice had been iv preimmunized with similar, but irradiated allogeneic cells. Now we investigated whether suppression can also be found when non-

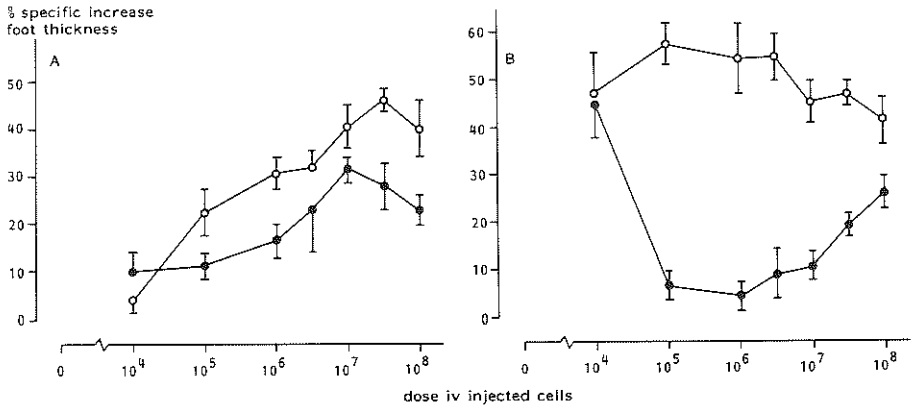

Fig. 1. (A) Induction of DTH reactivity by iv immunization with allogeneic cells. Swiss mice were iv injected with either 1×10^4, 1×10^5, 1×10^6, 3×10^6, 1×10^7, 3×10^7 or 1×10^8 irradiated (●) or nonirradiated (○) A.SW spleen cells. Five days later all mice were challenged for DTH. (B) Suppression of DTH reactivity to allogeneic spleen cells by iv preimmunization with nonirradiated spleen cells. Swiss mice were iv preimmunized with either 1×10^4, 1×10^5, 1×10^6, 3×10^6, 1×10^7, 3×10^7 or 1×10^8 nonirradiated A.SW (●) or Swiss (○) spleen cells. Seven days later all mice were sc immunized with 1×10^7 A.SW spleen cells and another 5 days later challenged for DTH. Each experimental point respresents the arithmetic mean of the DTH response ± SE (n=6).

irradiated cells are used for iv preimmunization. Therefore, groups of Swiss responder mice were iv preimmunized with various doses of nonirradiated allogeneic A.SW or syngeneic Swiss spleen cells. Seven days later all mice were sc immunized with A.SW spleen cells and tested for DTH another 5 days later. Indeed, the nonirradiated cells also induced suppression. The suppression appeared to be dose dependent and maximal when induced by about 1×10^6 allogeneic cells (Fig. 1B). In contrast to the suppression induced by irradiated allogeneic spleen cells (3), higher doses of non-irradiated cells induced less suppression.

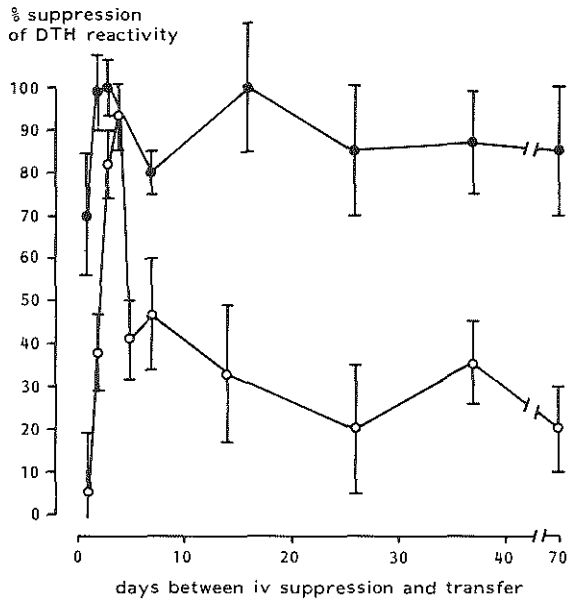

Fig. 2. Systemic transfer of iv induced suppression. Groups of (C57BL x CBA)F1 mice were iv preimmunized with 5×10^7 irradiated BALB/c spleen cells. At each of the indicated intervals after iv preimmunization, one group of mice was sc immunized with 1×10^7 BALB/c spleen cells (●), while spleen and lymph node cells from another group of mice were iv transferred to naive (C57BL x CBA)F1 mice (○). A few hours after transfer, the recipient mice were sc immunized with 1×10^7 BALB/c spleen cells. At all intervals a group of (C57BL x CBA)F1 mice was included, this group received spleen and lymph node cells from naive F1 mice (positive control). Also these mice were sc immunized a few hours after transfer. DTH reactivity was tested 6 days after sc immunization. Each experimental point respresents the arithmetic mean of the suppressive effect ± SE (n=6) calculated as a percentage of the positive control. The specific DTH response of this positive control ranged from 24-31%.

Transfer of iv induced suppression

Groups of (C57BL x CBA)F1 mice were iv immunized with an optimal dose of 5 x 10^7 irradiated BALB/c spleen cells. At different days after this suppressive immunization, one group of 6 mice was sc immunized with 1 x 10^7 BALB/c spleen cells, and from another group of 6 mice the spleen and lymph node cells were transferred into 6 naive syngeneic (i.e., (C57BL x CBA)F1) mice. A few hours later the recipients of 'suppressed' spleen and lymph node cells were also sc immunized with 1 x 10^7 BALB/c spleen cells. Both groups of mice were tested for DTH 6 days later. The date from these experiments, depicted in Fig. 2,

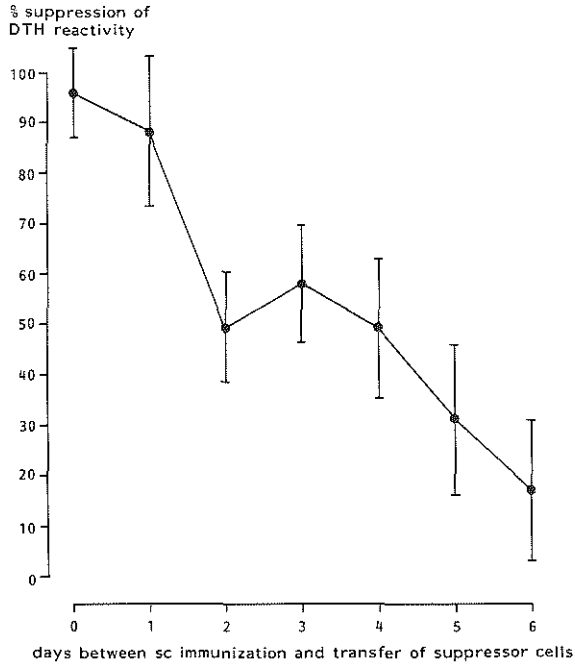

Fig. 3. Effect of infusion of activated suppressor cells at different intervals after sc immunization of the recipient mice. Spleen and lymph node cells from (C57BL x CBA)F1 mice, which were iv preimmunized with 5 x 10^7 irradiated BALB/c spleen cells, were systemically transferred at the same day or 1, 2, 3, 4, 5 or 6 days after sc immunization of the recipient mice with 1 x 10^7 BALB/c spleen cells. As positive controls, groups of (C57BL x CBA)F1 mice received spleen and lymph node cells from F1 mice at the same day or 1, 2, 3, 4, 5 or 6 days after sc immunization with BALB/c spleen cells. DTH reactivity was tested 6 days after sc immunization. Each experimental point represents the arithmetic mean of the suppressive effect ± SE (n=6), calculated as a percentage of the positive control.

show that transfer of the suppressive effect was possible only during a short period, and optimal on day 4 after the iv preimmunization. However, in the iv preimmunized mice themselves, the state of suppression persisted for at least 70 days.

Subsequently, the suppression was investigated after transfer of 'suppressed' spleen and lymph node cells at different intervals after the sc immunization of the recipient mice. (C57BL x CBA)F1 recipient mice received 'suppressed' spleen and lymph node cells from syngeneic donor mice on the same day as the sc immunization or 1 to 6 days later. The donor mice were always iv suppressed with 5×10^7 irradiated BALB/c spleen cells 4 days before transfer. Transfer of the suppressive effect was found to be maximal when it was performed on the same day as the sc immunization of the recipient mice (Fig. 3). The ability to transfer the suppression was found to be inversely propartional to the interval between sc immunization and transfer. Transfer of 'suppressed' spleen and lymph node cells on day 6, when the mice were challenged for DTH reactivity, hardly influenced the response.

The role of the spleen in the development of suppression after iv immunization

Splenectomized and sham-splenectomized (C57BL x CBA)F1 mice were iv immunized with 5×10^7 irradiated H-2 and non-H-2 incompatible BALB/c spleen cells or the same number of irradiated syngeneic spleen cells. Seven days later all mice were sc immunized with 1×10^7 BALB/c spleen cells and tested for DTH another 6 days later. The iv preimmunization appeared to induce suppression in both the splenectomized and the sham-splenectomized mice (Fig. 4A). The same results were obtained with splenectomized and sham-splenectomized B10.BR responder mice that were immunized with H-2 incompatible B10.D2 spleen cells, and with splenectomized and sham-splenectomized BALB/c responder mice that were immunized with non-H-2 incompatible B10.D2 spleen cells (Fig. 4A).

Subsequently, we investigated whether the spleen of suppressed mice is the principal site of residence of the suppressor cells. Therefore we transferred 1×10^8 spleen cells, 1×10^8 lymph node cell or 2×10^8 of a mixture of both cells types from suppressed (C57BL x CBA)F1 donor mice to naive recipients. A few hours later the recipient mice were sc immunized with 1×10^7 H-2 and non-H2 incompatible DBA/2 spleen cells and tested for DTH reactivity 6 days later. The lymph node cells appeared to be at least as capable as spleen cells of transferring suppression. Even lymph node cells from mice that were splenectomized before the iv preimmunization, could transfer suppression to naive recipients (Fig. 4B). Similar data were obtained with spleen and lymph node cells from BALB/c mice suppressed to non-H-2 incompatible B10.D2 spleen cells (Fig. 4B).

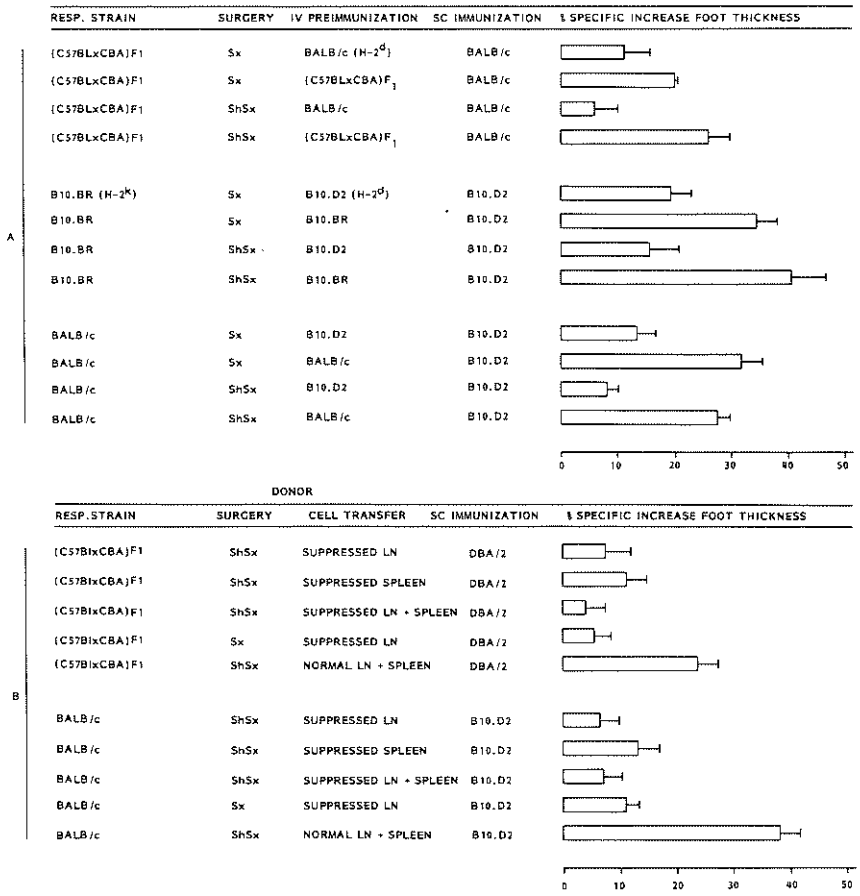

Fig. 4. (A) Effect of splenectomy on the iv induction of suppression. Responder mice were splenectomized (Sx) or sham-splenectomized (ShSx) four weeks before iv preimmunization with 5 x 10^7 irradiated allogeneic or syngeneic spleen cells. Seven days later all mice were sc immunized with 1 x 10^7 allogeneic spleen cells. Challenge for DTH was performed on day 6 after sc immunization. Each column represents the mean response ± SE (n=6). (B) Effect of splenectomy on the ability of lymph node cells to transfer the iv induced state of suppression. Donor mice were splenectomized (Sx) or sham-splenectomized (ShSx) 4 weeks before iv preimmunization with 5 x 10^7 irradiated allogeneic spleen cells. Four days after iv preimmunization, 1 x 10^8 spleen and/or 1 x 10^8 lymph node cells from the ShSx mice and 1 x 10^8 lymph node cells from the Sx mice were systemically transferred to syngeneic recipient mice. As a positive control, another group of recipient mice received spleen and lymph node cells from non-suppressed donors. One hour after transfer all mice were sc immunized with 1 x 10^7 allogeneic spleen cells and tested for DTH another 6 days later. Each column represents the mean response ± SE (n=6). The percentages specific suppression are shown in parentheses.

The role of T1 and T2 cells in the development of suppression after iv immunization

The development of suppression was investigated in ATx and ShTx DBA/2 mice, which were iv injected with either 5 x 10^7 irradiated non-H-2 incompatible BALB/c spleen cells or the same number of irradiated syngeneic spleen cells. Seven days later all mice were sc immunized with 1 x 10^7 BALB/c spleen cells and tested for DTH reactivity another 5 days later. The results show that depletion of T1 cells due to ATx did not interfere with the induction of suppression (Fig. 5A). The same results were obtained with ATx and ShTx

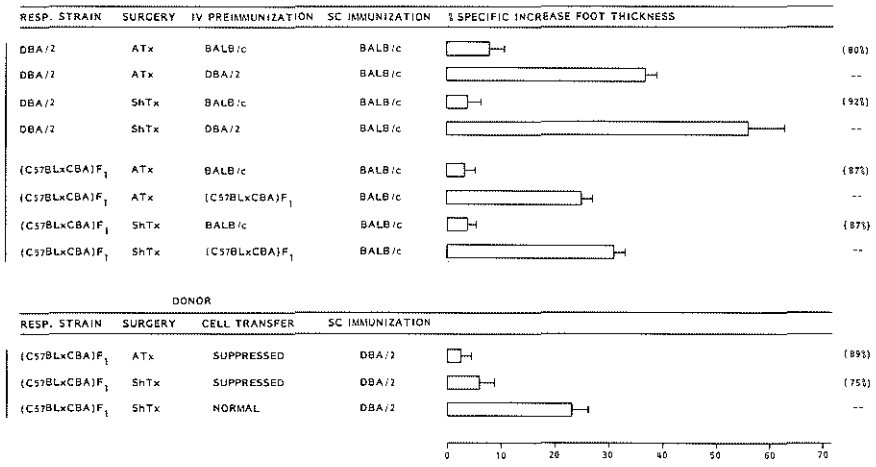

Fig. 5. (A) Effect of adult thymectomy on the iv induction of suppression. Responder mice were thymectomized (ATx) or sham-thymectomized (ShTx) ten weeks before iv preimmunization with 5 x 10^7 irradiated allogeneic or syngeneic spleen cells. Seven days later all mice were sc immunized with 1 x 10^7 allogeneic spleen cells. Challenge for DTH was performed on day 6 after sc immunization. Each column represents the mean response ± SE (n=6). (B) Effect of thymectomy on the ability to transfer the iv induced state of suppression. Donor mice were thymectomized (ATx) or sham-thymectomized (ShTx) ten weeks before iv preimmunization with 5 x 10^7 irradiated allogeneic spleen cells. Four days after iv preimmunization, the spleen and lymph node cells from these ATx and ShTx donor mice were systemically transferred to syngeneic recipient mice. Another group of recipient mice received normal spleen and lymph node cells from nonsuppressed donors (positive control). A few hours after transfer all mice were sc immunized with 1 x 10^7 allogeneic spleen cells. Challenge for DTH was performed on day 6 after sc immunization. Each column represents the mean response ± SE (n=6). The percentages specific suppression are shown in parentheses.

(C57BL x CBA)F1 responder mice immunized with H-2 and non-H-2 incompatible BALB/c spleen cells. The possibility to transfer suppression with spleen and lymph node cells from ATx suppressed mice was also investigated. Therefore, ATx and ShTx (C57BL x CBA)F1 donor mice were iv preimmunized with 5×10^7 irradiated H-2 and non-H-2 incompatible DBA/2 cells. Their spleen and lymph node cells were transferred into naive, syngeneic recipients 4 days later. A control group which received spleen and lymph node cells from naive ShTx mice was included. A few hours after transfer all recipient mice were sc immunized with 1×10^7 DBA/2 spleen cells and tested for DTH another 6 days later. The results show that T1 cell depletion also did not affect the capacity to transfer suppression (Fig. 5B).

The influence of T2 cell depletion was studied in (C57BL x CBA)F1 mice treated with ATS or NRS before iv injection of 5×10^7 irradiated H-2 and non-H-2 incompatible BALB/c spleen cells. Four days after iv suppression their spleen and lymph node cells were transferred into naive syngeneic recipients. A control group received spleen and lymph node cells from naive donor mice. A few hours later, all recipient mice were sc immunized and tested for DTH against BALB/c spleen cells. The data show that transfer of suppression was impossible after ATS treatment of the donor mice. The same result was obtained in a similar protocol with DBA/2 responder mice and H-2 and non-H-2 incompatible (C57BL x CBA)F1 spleen cells (Fig. 6).

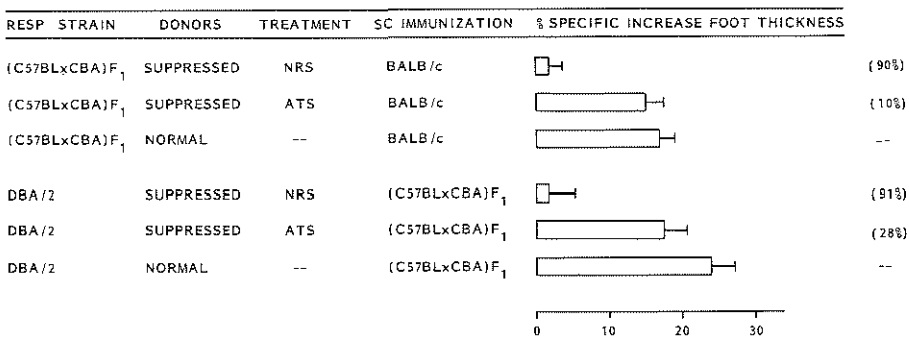

Fig. 6. Effect of anti-thymocyte serum (ATS) treatment on the transfer of suppression. Donor mice were treated with ATS or NRS on day -5 and day -2 and iv preimmunized with 5×10^7 allogeneic spleen cells on day 0. Four days later the spleen and lymph node cells were systemically transferred to syngeneic recipient mice. One hour after transfer all mice were sc immunized with 1×10^7 allogeneic spleen cells and tested for DTH another 6 days later. Each column represents the mean response ± SE (n=6). The percentages specific suppression are shown in parentheses.

Surface markers of the suppressor cells

The effect of ATS treatment shows that iv induced suppression of DTH to alloantigens is dependent on T cells. The Thy-1, Lyt-1 and Lyt-2 surface markers of these T cells were investigated. Thus, the spleen and lymph node cells from C3H/Tif mice suppressed to (C57BL x CBA)F1 alloantigens were depleted for T cells by anti-Thy-1.2 and complement, or for Lyt-1^+ cells by anti-Lyt-1.1 and complement or for Lyt-2^+ cells by anti-Lyt-2 and complement. After depletion, the residual cells were transferred into naive syngeneic recipients. As positive control, one group of recipient mice received normal spleen and lymph node cells. A few hours after transfer all mice were sc immunized with 1 x 10^7 allo-

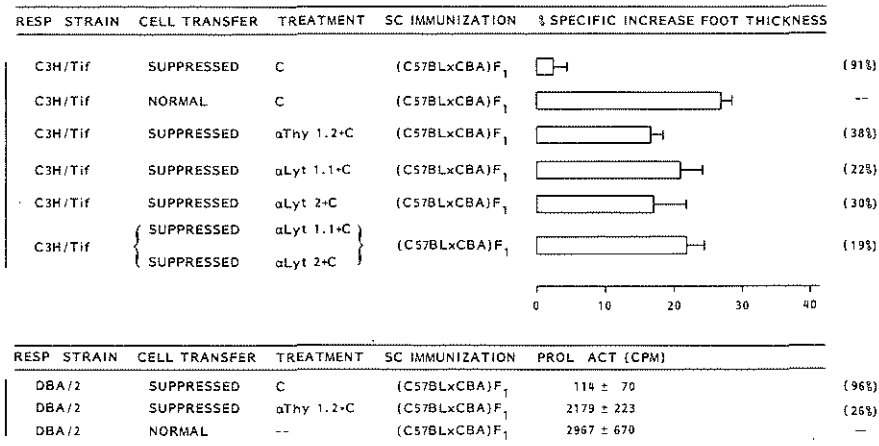

Fig. 7. (A) Surface markers of the suppressor cells involved in suppression of DTH to histocompatibility antigens. By means of systemic transfer, groups of responder mice received spleen and lymph node cells from normal, syngeneic mice or from syngeneic mice that had been iv injected with 5 x 10^7 irradiated allogeneic spleen cells 4 days previously ('suppressed cells'). Before transfer the suppressed cells were treated with the indicated monoclonal antibodies and complement (C). A few hours after transfer, all mice were sc immunized with 1 x 10^7 allogeneic spleen cells and tested for DTH another 6 days later. Each column represents the mean response ± SE (n=6). The percentages specific suppression are shown in parentheses. (B) Effect of depletion of suppressor T cells on the proliferative activity in the draining lymph nodes of sc immunized mice. Groups of responder mice received spleen and lymph node cells from normal, syngeneic mice or from syngeneic mice which had been iv injected with 5 x 10^7 irradiated allogeneic spleen cells 4 days previously ('suppressed cells'). On the day of transfer all mice were sc immunized with 1 x 10^7 allogeneic spleen cells and the proliferative activity in the draining lymph nodes was measured 5 days later.

geneic spleen cells and tested for DTH reactivity another 6 days later. The results (Fig. 7A) show that depletion of either Thy-1^+, Lyt-1^+ or Lyt-2^+ cells from the transferred suppressor cell population abrogates the suppression. Transfer of a combination of two suppressor population equivalents treated with either anti-Lyt-1.1 and complement or anti-Lyt-2 and complement, respectively, did not reestablish the suppressive effect. Moreover, the same results were obtained in a combination of DBA/2 responder mice and H-2 and non-H-2 incompatible (C57BL x CBA)F1 spleen cells (data not shown). Thus, the suppression induced by iv preimmunization with allogeneic spleen cells is dependent on suppressor T cells expressing Lyt-1 as well as Lyt-2 antigens.

In the combination of DBA/2 responder mice and immunizing (C57BL x CBA)F1 spleen cells we also studied the effect of suppressor cell transfer on the proliferative activity in the draining lymph nodes of the responder mice. Transfer of suppressor cells decreased the proliferative activity in the draining lymph nodes, and treatment of the suppressor cell population with anti-Thy-1.2 and complement abrogated the suppression of the proliferation (Fig. 7B). These results reinforce the conclusion from the transfer experiments shown in Fig. 3, which is that the suppressor T cells influenced the induction phase of the DTH response.

DISCUSSION

The data presented here show that iv immunization of mice with irradiated or non-irradiated allogeneic spleen cells can induce a state of DTH reactivity (Fig. 1A) as well as a state of T cell dependent suppression (Fig. 1B). The suppressive effect caused by the iv immunization becomes manifest only after secondary sc immunization (cf. (3) and Fig. 1B). When mice are *sc* preimmunized 1 to 7 days before secondary sc immunization, the subsequent DTH response is hardly affected (3), but at longer intervals and especially in the case of immunization with minor H antigens, secondary type responses can occur (30). So, the iv route of preimmunization seems to be obligatory for induction of suppression of DTH to H antigens.

In previous studies (3, 19, 20, 22) we always used irradiated allogeneic cells for the iv preimmunization in order to avoid anti-host reactivity by the injected allogeneic cells, which might confuse studies of the suppressor mechanism. Here we show that DTH reactivity can also be effectively suppressed by iv preimmunization with non-irradiated spleen cells (Fig. 1B). In the latter case, maximal suppression was already induced by a dose of 1×10^6 allogeneic spleen cells, which is about 50 times lower than in the case of irradiated allogeneic spleen cells (3). At higher doses, a smaller suppressive effect was found. The different dose response relationship of iv induced suppression by irradiated and non-

irradiated allogeneic spleen cells may be due to allogeneic effects and longer persistence of the antigen in the case of non-irradiated allogeneic cells.

It is remarkable that iv immunization with irradiated allogeneic spleen cells simultanously induces DTH reactive T cells and suppresssor T cells. Apparently, the suppressor T cells are unable to suppress the induction and functional activity of simultaneously activated DTH reactive T cells, but they do suppress the generation of DTH effector T cells after secondary sc immunization (Fig. 1B) and after transfer into secondary recipients (Fig. 7B). This is in harmony with studies of others showing that for suppression of the expression phase of DTH to haptens an additional suppressor T cell population is needed, which only occurs after sc immunization of the iv preimmunized animals (6, 8, 9, 31). However, as yet we have no real evidence that such an additional suppressor T cell subset is also involved in our model of suppression of DTH to H-antigens.

The suppressive effect induced by iv preimmunization with irradiated allogeneic spleen cells can be systemically transferred by spleen and lymph node cells only during the first few days after the iv induction (Fig. 2). However, when iv preimmunized mice themselves are sc immunized, a clear suppression of DTH reactivity is found up to 70 days after iv preimmunization. Similar results have been obtained in studies of suppression of DTH against haptens (32) and heterologous erythrocytes (33, 34) and in studies of CS (10). The authors of these studies suggest that this type of unresponsiveness is based on clone inactivation, as well as on active suppression. The clone inactivation was found to be a long-lived phenomenon and could not be transferred, while the transferable suppressor T cells only occurred during a short period after induction.

The inability to transfer suppression with spleen and lymph node cells at longer intervals after iv preimmunization might be due to the short life span of of activated suppressor T cells. Liew (35) has shown in a, to some extent, comparable system, that after booster immunization secondary type suppressor T cells appear in spleen and lymph nodes, suggesting the occurrence of suppressor memory T cells. The existence of alloantigen-specific suppressor memory T cells is further substantiated by our own studies showing that thoracic duct lymphocytes can adoptively transfer suppression during a substantial period following iv preimmunization (data not shown).

Suppressor T cells have been described for the afferent (10, 11, 32) as well as for the efferent limb (5, 8, 9, 13) of the immune response. Transfer of suppressor cells to recipient mice at different times *after* sc induction of DTH reactivity shows that maximal suppression occurs when the activated suppressor T cells are infused shortly after the sc immunization of the recipient mice (Fig. 3). Similar results have been found in systems employing hapten induced suppressor T cells by transferring so-called afferent phase suppressor T cells (10, 32). So-called efferent phase suppressor T cells, on the other hand, could suppress the DTH

response of recipient mice even when transferred on the day of challenge (5, 13). In our system of iv induced suppression of DTH against H-antigens we found that the proliferative response in the draining lymph nodes due to sc immunization can be suppressed by systemic transfer of suppressor T cells from recently iv preimmunized donor mice (Fig. 7B). This brings us to the conclusion that systemic transfer of iv induced suppressor T cells mainly affects the afferent limb of the DTH response against H-antigens.

It is often suggested that overwhelming of the spleen with a high dose of iv administered antigen bypasses the presentation of antigen by antigen-presenting cells, which would favor suppression instead of activation (2, 5, 36, 37). The requirement of the spleen for the induction of suppression was shown by Lagrange *et al.* (38). They were unable to induce suppression of DTH against SRBC by iv preimmunization of splenectomized mice. Sy *et al.* (39), on the other hand, showed that splenectomy did not prevent suppression of CS against 2,4-dinitro-1-fluorobenzene, but they could not transfer the suppression by lymph node cells from splenectomized mice. They concluded that the iv suppressive injection caused clone inactivation but did not induce transferable suppressor T cells. However, other studies showed that induction of afferent phase (5) and efferent phase (40) suppressor T cells was insensitive to splenectomy. The experiments presented in Fig. 4A show that suppression of DTH against H-antigens can be induced in mice splenectomized 4 weeks before iv preimmunization. Suppression could also be transferred with lymph node cells from mice splenectomized before the iv suppressive injection (Fig. 4B). So, we did not find the spleen to be essential in the induction of suppression of DTH against H-antigens.

From the typing experiments it appeared that the suppressor T cells essential for transfer of suppression are Lyt-1^+2^+ (Fig. 7A). In the literature it has been shown that especially the population of Lyt-1^+2^+ T cells is sensitive for ATx (41, 42), suggesting that this population constitutes mainly of T1 cells. Our data, showing the induction of suppressor T cells several months after ATx, indicate that the Lyt-1^+2^+ T cell population must be heterogeneous with regard to life-span and consists of ATx-resistant T2 cells as well as short-lived T1 cells (43). Recent flow cytofluorometry studies by Scollay (44) showed that ATx did not change the ratios between the different Lyt-subsets in spleen and lymph nodes, which also indicates the occurrence of long-lived Lyt-1^+2^+ T cells.

Although the suppressor T cells described in other assays were found to belong mostly to the Lyt-1^+2^- (8, 33, 35, 45) and Lyt-1^-2^+ (8, 33, 46) T cell subsets, also Lyt-1^+2^+ suppressor T cells have been described (35, 47). Thus, Liew described a Lyt-1^+2^+ suppressor T cell acting in suppression of DTH against H antigens (35). This cell was activated by a suppressive booster immunization, but the suppressor T cell induced by a single suppressive injection

appeared to be Lyt-1^+2^-. In our system, primary iv immunization activated a Lyt-1^+2^+ suppressor T cell (Fig. 7B), which is essential for transfer of the suppressive effect. However, we cannot exclude that other Lyt-1^+2^- or Lyt-1^-2^+ suppressor populations are involved as well. The occurence of a cascade of several phenotypically different, T cell subsets in suppression of DTH to haptenated syngeneic spleen cells and CS to certain haptens has been extensively documented by the group of Benacerraf (2, 8).

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CHAPTER IX

SUPPRESSION OF ANTIGRAFT IMMUNITY BY PREIMMUNIZATION III. CHARACTERIZATION OF SUPPRESSOR T CELLS INVOLVED IN SUPPRESSION OF THE EFFERENT PHASE OF DELAYED TYPE HYPERSENSITIVITY AGAINST ALLOANTIGENS

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ABSTRACT

Suppressor T (Ts) cells that can suppress delayed type hypersensitivity (DTH) against histocompatibility (H) antigens can be isolated from spleen and lymph nodes a few days after intravenous (iv) immunization of mice with irradiated allogeneic spleen cells. In the present paper we investigated the suppression of the efferent phase of DTH in order to characterize the Ts cells involved, and to compare them with the afferent phase Ts cells that have been characterized in a previous paper of this series. The DTH against third party alloantigens, which were not used for the iv suppressive immunization, could be suppressed by presenting the third party alloantigens together with the original alloantigens in the challenge inoculum for eliciting the DTH reaction. Thus, the ultimate suppressive effect by the Ts cells, which are active during the efferent phase of DTH, is nonspecific. This nonspecific suppression of DTH to alloantigens has previously been found for the afferent phase Ts cells as well. The essential population of 'efferent phase' Ts cells appeared to be Lyt 1^+2^+, just like the Ts cells involved in suppression of the afferent phase of DTH against alloantigens. We did not find any evidence for a H-2 or Igh restricted activation and function of the Ts cells active during both limbs of the DTH response to H antigens. In view of these similarities between afferent phase and efferent phase Ts cells we conclude that there are no arguments as yet to suppose that there is more than one type of T cells involved in the suppression of the afferent and efferent limb of DTH against H antigens.

INTRODUCTION

The *in vivo* cellular immune response by delayed type hypersensitivity (DTH) reactive T cells is regulated by a complex system of ao. amplifier (1), helper (2, 3) and suppressor T (Ts) cells (4–6). The previous studies of this series of papers deal with the regulation of DTH reactivity of mice against histocmpatibility (H) antigens (7, 8).

Subcutaneous (sc) immunization of mice with allogeneic spleen cells induces a state of host-vs-graft DTH reactivity to both major and minor H antigens (9, 10). The induction of DTH reactivity can be prevented by iv preimmunization of the responder mice with similar irradiated allogeneic spleen cells (7, 11). The iv route of preimmunization is obligatory for the induction of suppression, since sc preimmunization hardly reduces the DTH response (7). In contrary, at intervals longer than one month between primary and secondary sc immunization, especially in the case that the immunizing inoculum consists of minor H antigens only, secondary type responses can occur (9, 12).

Iv induced suppression is a dose dependent and long-lasting phenomenon (7). However, *transfer* of the suppressive effect with spleen and lymph node cells from suppressed mice is only effective during the first few days after iv injection of allogeneic irradiated spleen cells (8). The Ts cells involved were found to be Lyt-1^+2^+ (8). This systemic transfer of Ts cells to syngeneic recipients mainly affects the afferent phase of the host-vs-graft DTH response (8). The iv induced Ts cells are also capable of suppressing the antihost immune reactivity during acute and delayed graft-vs-host reactions (13). Precursors of the Ts cells belong to the pool of recirculating T lymphocytes, are insensitive to adult thymectomy and can be depleted by anti-thymocyte serum treatment *in vivo*.

Specificity studies have shown that the activation of Ts cells by alloantigens is antigen specific (7). However, their ultimate suppressive effect was found to be nonspecific (14, 15). These specificity studies have shown that afferent phase of the DTH against alloantigens is extremely susceptible to the regulatory activity of Ts cells. A similar conclusion has been drawn by several other investigators, who studied suppression of the induction and expression of contact sensitivity (CS) and DTH against haptens (4–6, 16, 17).

In contrast to our previous studies, most of the latter studies are focussed on the genetic and cellular requirements and the antigen specificity of the expression of the suppressive effect during the efferent phase of CS and DTH to haptens (4–6, 17). In this report we have studied the requirements for suppression of the efferent phase of DTH against alloantigens, the characterization of the cells involved in this type of suppression and the genetic requirements for the systemic transfer of the suppression by the Ts cells involved.

MATERIALS AND METHODS

Mice

Female BALB/c (H-2^d, Igh^a) and (C57BL/Rij x CBA/Rij)F_1 (H-$2^{b/q}$) mice were purchased from the Laboratory Animals Centre of the Erasmus University, Rotterdam, The Netherlands. Female DBA/2 (H-2^d), C3H/Tif (H-2^k) and C57BL/6J (H-2^b) mice were purchased from Bomholtgard, Ry, Denmark. Female BALB.B (H-2^b), BALB.K (H-2^k), (BALB/c x BALB.K)F_1 (H-$2^{d/k}$), B10.BR (H-2^k), B10.D2 (H-2^d), B10.G (H-2^q), B10.ScSn (H-2^b), (B10.D2 x B10.ScSn)F_1 (H-$2^{d/b}$) and DBA/1 (H-2^q) were puchased from OLAC Ltd., Bicester, U.K. Female C.B-20 (H-2^d, Igh^b) were obtained from the Institut für Biologisch-Medizinische Forschung A.G., Füllinsdorf, Switzerland. Female (BALB/c x C3H/Tif)F_1 (H-$2^{d/k}$) mice were bred at our own department.

Preparation of cell suspensions

Mice were killed by exposition to carbondioxide vapour. Spleens or lymph nodes were removed, placed in balanced salt solution (BSS) and squeezed through a nylon gauze filter to provide a single cell suspension. Nucelated cells were counted with a Coulter counter model BZI (Coulter Electronics, Harpenden, U.K.). The viability of the cell suspensions obtained was determined in the trypan blue exclusion test and was found to be at least 90%.

Immunization

Suppression was induced by iv immunization with 5 x 10^7 allogeneic spleen cells. Within 3 hours before iv injection the cells had been irradiated *in vitro* with 20 Gy, generated in a Philips Müller MG 300 X-ray machine as described in detail previously (18). DTH was induced by sc immunization in the inguinal area with 1 x 10^7 nonirradiated allogeneic spleen cells.

Systemic transfer of DTH reactivity

For systemic transfer of DTH, T cells were activated under graft-vs-host (GvH) conditions (19) by iv injection of 2 x 10^7 nucleated spleen cells into lethally irradiated allogeneic recipients within 4 hours after irradiation. Peak numbers of DTH reactive T cells occurred in the spleen and lymph nodes of the recipient mice at 4 to 6 days after irradiation (19). This could be demonstrated by transferring the spleen and lymph node cells to secondary recipients which were syngeneic to the original spleen cells donors. The secondary recipient mice were challenged for induction of a DTH response within 1 hour after transfer.

Systemic transfer of suppression

The state of suppression was transferred to recipient mice by iv injection of

spleen and lymph node cells from mice which had been iv suppressed 4 days previously. A few hours after transfer, the recipients were sc immunized for induction of DTH reactivity. Another 6 days later the recipients were challenged for induction of a DTH response.

Assay for immune lymphocyte transfer reactivity

The immune lymphocyte transfer (ILT) reactivity of lymph node cells from immunized donors, directed against H antigens of a particular recipient strain, was determined by sc injection of 5×10^6 of these lymph node cells into the dorsum of the right hind foot of mice of the relevant recipient strain. In the left hind foot the recipients received a sc injection of 5×10^6 lymph node cells from nonimmune mice, syngeneic with the immunized donor mice. The response induced by the latter injection is called a normal lymphocyte transfer (NLT) reaction. For ILT and NLT the cells were injected in a volume of 50 μl. The specific ILT reactivity was calculated as ILT – NLT and was expressed in 10^{-3} cm.

Selective elimination of Thy-1.2, Lyt-1.1 and Lyt-2 positive cells

Monoclonal IgM anti-Thy-1.2 antibodies (clone F7D5) were purchased from OLAC Ltd., Bicester, U.K. Monoclonal IgG2a anti-Lyt-1.1 (clone 7-20.6/3) was purchased from Cedar Lane Laboratories Ltd., Hornby, Ontario, Canada. Monoclonal IgM anti-Lyt-2 was obtained by *in vitro* culture of an IgM anti-Lyt-2 producing hybridoma, which was kindly provided by Dr. F.W. Fitch, Department of Pathology, University of Chicago, U.S.A. The procedure for the selective depletion of the lymphocyte subsets has been described in detail previously (8).

Assay for DTH

DTH reactions were determined by measuring the difference in thickness of the hind feet 24 hours after sc injection of a challenge dose of 2×10^7 of the appropriate allogeneic spleen cells into the dorsum of the right hind foot. As a control for background DTH reactivity, naive syngeneic mice were used which only received the challenge dose. The specific DTH response was calculated as the relative increase in foot thickness of the immune mice minus the relative increase in foot thickness of the control mice. The swelling of the control mice ranged between 15 and 25%.

RESULTS

Effect of iv induced suppressor cells on the efferent phase of DTH

We have previously shown that Ts cells induced by iv immunization with irradiated allogeneic spleen cells suppress the afferent limb of the DTH response (8). The effect of iv induced suppressor cells on the efferent phase of DTH

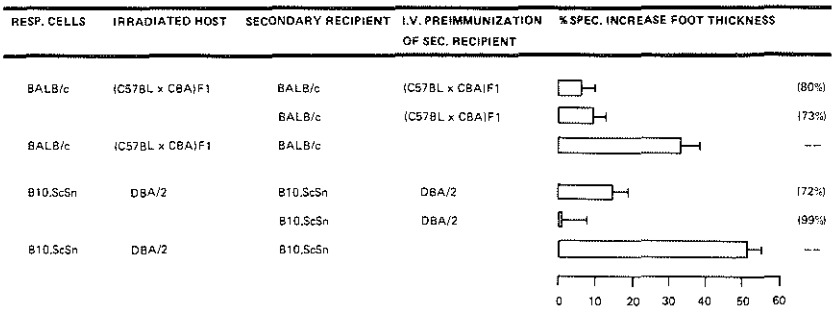

Fig. 1. Transfer of DTH effector T cells to iv suppressed recipient mice. DTH reactive T cells specific for (C57BL x CBA)F_1 alloantigens were generated by iv injection of 2 x 10^7 BALB/c spleen cells into lethally irradiated (C57BL x CBA)F_1 mice. Five days later spleen and lymph node cells of these recipients were iv transferred to a group of naive BALB/c recipient mice and to a group of BALB/c recipient mice which had been iv suppressed with 5 x 10^7 irradiated (C57BL x CBA)F_1 spleen cells 4 days earlier. A control group of BALB/c recipient mice, which were only suppressed, was included. One hr after transfer of the DTH effector T cells, all groups of BALB/c recipient mice were challenged with (C57BL x CBA)F_1 spleen cells. According to the same schedule, naive B10.ScSn recipient mice and B10. ScSn recipient mice which had been iv suppressed with irradiated 5 x 10^7 DBA/2 spleen cells, were iv infused with B10.ScSn DTH effector T cells activated against DBA/2 alloantigens. A control group of suppressed B10.ScSn mice was included. One hr after transfer of the DTH effector T cells all groups of B10.ScSn recipient mice were challenged for anti-DBA/2 DTH. In both experiments DTH responses were measured 24 hr after challenge. Each column respresents the mean response ± SE (n = 6). The percentage specific suppression is shown in parentheses. In both experiments significant suppression of DTH reactivity ($p < 0.01$) was found in all groups of mice which were suppressed by iv preimmunization with irradiated allogeneic cells.

against alloantigens has not been studied yet. Therefore the effect of these suppressor cells upon activated DTH effector T cells was investigated by iv infusion of such DTH effector T cells in previously iv immunized ('suppressed') mice. To this end, DTH effector T cells were generated in a GvH reaction.

DTH reactive T cells of BALB/c origin, activated under GvH conditions against (C57BL x CBA)F_1 alloantigens, were transferred to syngeneic BALB/c secondary recipients. The secondary recipient mice had been iv preimmunized with 5 x 10^7 irradiated (C57BL x CBA)F_1 spleen cells 4 days earlier. Control groups of BALB/c mice either received anti-F1 activated syngeneic lymphoid cells only or were iv preimmunized with 5 x 10^7 irradiated F_1 spleen cells only. The DTH reactivity of all groups of mice was evaluated by challenge with F_1 spleen cells immediately after transfer of the DTH effector T cells. The data from these experiments, depicted in Fig. 1, show that transfer of DTH reactivity was only succesful in naive secondary recipients and not in recipient mice which had been

iv preimmunized before. Similar data were obtained with B10.ScSn recipient mice which had been iv preimmunized with 5 x 10^7 irradiated DBA/2 spleen cells and were injected with syngeneic anti-DBA/2 reactive DTH effector T cells (Fig. 1).

Surface markers of iv induced suppressor cells

The surface markers of the suppressor cells that can suppress the efferent phase of DTH were determined in the immune lymphocyte transfer (ILT-NLT) assay. Therefore, suppressor cells were induced in C3H mice by iv injection of 5 x 10^7 irradiated (C57BL x CBA)F_1 spleen cells. Four days later, spleen cells were used as a source of suppressor cells. Another group of C3H mice was sc immunized with 1 x 10^7 F_1 spleen cells. Five days later the draining lymph nodes of these mice were used as source of DTH effector T cells. Subsequently, 5 x 10^6 immune lymph node cells were transferred together with either 1 x 10^7 naive spleen cells or 1 x 10^7 'suppressed' spleen cells into the dorsum of a hind

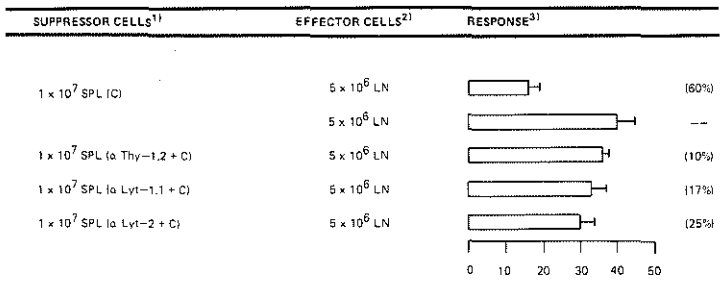

Fig. 2. Surface markers of iv induced suppressor cells active during the efferent phase of DTH.

1. C3H suppressor cells were induced by iv immunization of C3H mice with 5 x 10^7 irradiated (C57BL x CBA)F_1 spleen cells. Four days later the suppressed cells were treated with the indicated monoclonal antibodies and/or complement. After treatment, 1 x 10^7 of these cells were transferred into the dorsum of the right foot of (C57BL x CBA)F_1 recipient mice.
2. C3H DTH effector T cells were induced by sc immunization with 1 x 10^7 (C57BL x CBA)F_1 spleen cells. Five days later the draining lymph nodes were dissected and a cell suspension was made. Subsequently, 5 x 10^6 of these cells were transferred into the dorsum of the right hind foot of (C57BL x CBA)F_1 recipient mice. Suppressor and DTH effector cells were stimultaneously transferred. As a control, a similar number of unactivated lymphoid cells was injected into the dorsum of the left hind foot.
3. DTH in the (C57BL x CBA)F_1 recipient mice was measured as the difference in thickness (in 10^{-3} cm) between the right and left hind foot ± SE (n = 6) 24 hr after local transfer. The percentage specific suppression is shown in parentheses. Only the group of mice which received suppressor cells that were treated with complement only, showed a significant suppression of DTH.

foot of naive F_1 recipient mice, which were syngeneic with the spleen cells used for iv and sc immunization. Fig. 2, lines 1 and 2, show that the ILT response by the C3H DTH effector T cells was suppressed by the addition of C3H spleen cells from suppressed mice. Depletion of Thy-1.2 positive, Lyt-1 positive or Lyt-2 positive cells from the inoculum of DBA/2 suppressor cells, by using the appropriate monoclonal antibodies and complement, showed that the suppression of the efferent phase of the DTH response is dependent on Ts cells expressing Thy-1.2 and Lyt-1 as well as Lyt-2 surface antigens (Fig. 2, lines 3-5).

Suppression of DTH to third party bystander alloantigens during the efferent phase of DTH

We have shown earlier that iv induced Ts cells are antigen specific with regard to their antigen recognition (7). However, after restimulation of these Ts cells by the specific antigen(s), the DTH response to simultaneously administered third party alloantigens becomes suppressed as well (14, 15). The data presented above show that iv preimmunization also induces Ts cells which can suppress the efferent phase of DTH.

In the experiments described below, we investigated whether the simultaneous presentation of the specific and third party alloantigens during the efferent phase of DTH (in the challenge) only can also prevent the DTH response to the third party alloantigens. Therefore, B10.ScSn ($H\text{-}2^b$) mice were iv injected with irradiated H-2 compatible, but non-H-2 incompatible BALB.B ($H\text{-}2^b$) spleen cells. Seven days later these mice were sc immunized with H-2 incompatible B10.D2 ($H\text{-}2^d$) spleen cells. Another 6 days later the mice were challenged with either H-2 and non-H-2 incompatible BALB/c ($H\text{-}2^d$) spleen cells or a mixture of BALB.B and B10.D2 spleen cells.

A similar experiment was done with DBA/2 ($H\text{-}2^d$) responder mice, which were iv suppressed with non-H-2 incompatible B10.D2 ($H\text{-}2^d$) spleen cells, while DTH reactivity was induced with H-2 incompatible DBA/1 ($H\text{-}2^q$) spleen cells. Subsequently, DTH responses were elicited by challenge with either H-2 and non-H-2 incompatible B10.G ($H\text{-}2^q$) spleen cells or a mixture of B10.D2 and DBA/1 spleen cells.

It appeared in both combinations that the simultaneous presentation of the specific alloantigens used for iv preimmunization and the third party alloantigens in the challenge dose only, was suficient for the suppression of the DTH response to the third party alloantigens. Suppression was also found when the specific and the third party alloantigens were presented on separate cells (Fig. 3, expts. A and B). Similar data were obtained in a combination of mouse strains with a B10 background that were H-2 incompatible with the B10.BR responder mice (Fig. 3, expt. C).

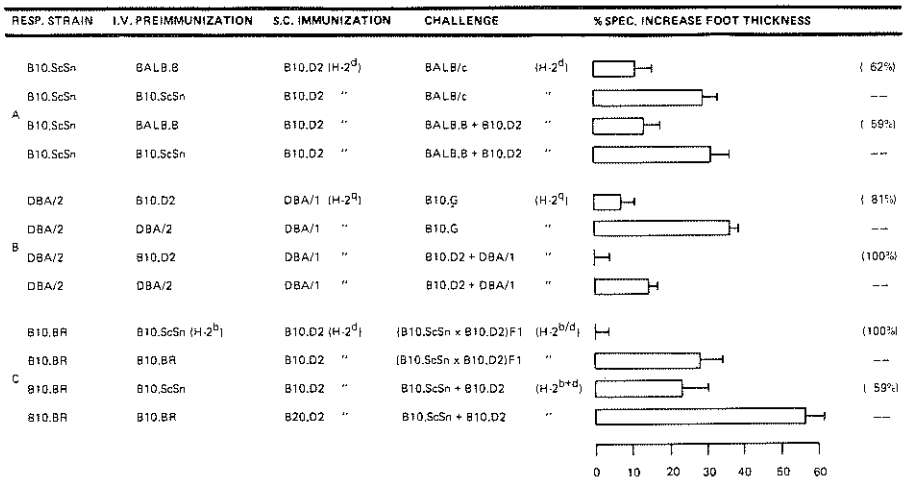

Fig. 3. Suppression of DTH to third party bystander alloantigens during the efferent phase of DTH. Responder mice were iv injected with 5 x 10^7 irradiated allogeneic spleen cells (the 'specific alloantigens') or syngeneic cells, and sc immunized with 1 x 10^7 allogeneic spleen cells (the 'third party alloantigens') 7 days later. Challenge for DTH was performed on day 6 after sc immunization. Each column represents the mean response ± SE (n = 6). In all experiments significant suppression of DTH ($p < 0.01$) against the third party bystander antigens was found when the specific and third party alloantigens were presented together in the challenge only (line 1). Significant suppression ($p < 0.01$) was also found when both types of alloantigens were presented on separate cells in a mixture (line 3). The columns of line 2 and 4 represent the positive controls. The percentage specific suppression and the H-2 haplotypes are shown in parentheses.

Transfer of non-H-2 specific Ts cells, which are induced by iv injection of H-2 and non-H-2 incompatible spleen cells

It has been shown in other studies (16) that iv injected hapten-modified H-2 incompatible lymphoid cells induce Ts cells that exert their suppressive effect only after transfer to the mouse strain from which the hapten-modified allogeneic cells were derived, and not after transfer to recipients which are syngeneic with the donors of the Ts cells. We investigated whether non-H-2 specific Ts cells induced by iv injection of H-2 *and* non-H-2 incompatible spleen cells are able to transfer their suppressive effect to recipients syngeneic with the donors of the Ts cells. Therefore, groups of BALB/c (H-2^d) responder mice were iv injected with either irradiated non-H-2 incompatible B10.D2 (H-2^d) spleen cells, irradiated non-H-2 and H-2 incompatible B10.ScSn (H-2^b) spleen cells, or irradiated syngeneic BALB/c spleen cells. Four days after the iv immunization, spleen and

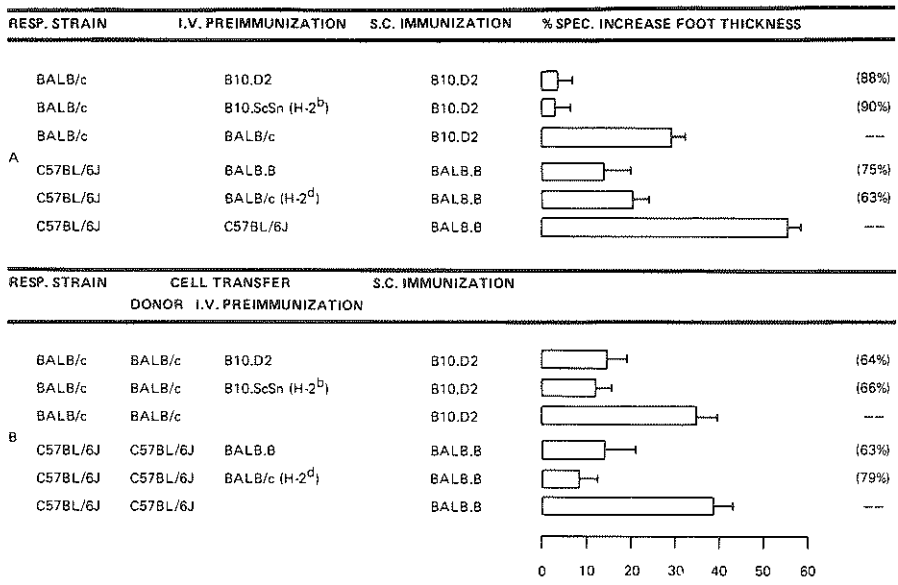

Fig. 4. (A). Suppression of DTH against non-H-2 alloantigens induced by iv injection of H-2 and non-H-2 incompatible spleen cells. Responder mice were iv injected with 5×10^7 irradiated H-2 compatible, but non-H-2 incompatible spleen cells, H-2 and non-H-2 incompatible spleen cells or syngeneic spleen cells. Seven days after iv preimmunization all groups of mice were sc immunized with 1×10^7 non-H-2 incompatible spleen cells. Challenge for non-H-2 specific DTH was done on day 6 after sc immunization.

(B.) Transfer of suppression by non-H-2 specific suppressor T cells induced by iv injection of H-2 and non-H-2 incompatible spleen cells. Donor mice were iv injected with 5×10^7 irradiated H-2 compatible, but non-H-2 incompatible spleen cells or H-2 and non-H-2 incompatible spleen cells. Four days after iv immunization, the spleen and lymph node cells from these mice were iv transferred to syngeneic recipient mice. Another group of recipient mice received spleen and lymph node cells from nonsuppressed donors (positive control). A few hours after transfer, all mice were sc immunized with 1×10^7 allogeneic spleen cells. Challenge for DTH was performed on day 6 after sc immunization. Each column represents the mean response ± SE (n = 6): The percentage specific suppression and the H-2 haplotypes are shown in parentheses. Significant suppression of DTH reactivity ($p < 0.01$) was found in all groups of responder mice which were suppressed by iv preimmunization with irradiated allogeneic spleen cells or received lymphoid cells from iv suppressed donor mice.

lymph node cells from some mice of these three groups were transferred to syngeneic recipeint mice. Immediately after this transfer, the recipient BALB/c mice were sc immunized with H-2 compatible and non-H-2 incompatible B10.D2 spleen cells and tested six days later for anti-B10.D2 DTH. The other mice of the three goups were sc immunized with B10.D2 spleen cells and six days later

tested for anti-B10.D2 DTH. It appeared that iv injection of H-2 and non-H-2 incompatible spleen cells was as effective as H-2 compatible spleen cells for induction of non-H-2 specific suppression. Futhermore, spleen and lymph node cells from the iv immunized donor mice did not reveal any difference in the capacity to transfer the suppressive effect to syngeneic or H-2 incompatible recipients (Fig. 4).

Similar data were found in C57BL/6J (H-2^b) responder mice iv injected with irradiated H-2 compatible BALB.B (H-2^b) and H-2 incompatible BALB/c (H-2^d) spleen cells (Fig. 4).

Lack of H-2 restriction in the suppression of the afferent phase of DTH by iv induced Ts cells

We investigated whether Ts cells that are induced by non-H-2 alloantigens recognize these antigens and suppress the induction of DTH effector T cells during the afferent phase of the DTH response in a H-2 restricted fashion. Therefore, BALB/c (H-2^d) donor mice were iv suppressed with irradiated H-2 compatible but non-H-2 incompatible B10.D2 (H-2^d) spleen cells, while BALB.K (H-2^k) mice were iv suppressed with irradiated H-2 compatible B10.BR (H-2^k) spleen cells. Four days later, spleen and lymph node cells from the suppressed BALB/c and BALB.K mice were transferred to naive BALB/c recipient mice. As a control, spleen and lymph node cells from non-suppressed BALB/c and BALB.K mice were transferred to separate groups of naive BALB/c recipients. Immediately after transfer, all recipients were sc immunized with H-2 compatible, but non-H-2 incompatible B10.D2 spleen cells. Six days later all mice were challenged with similar B10.D2 spleen cells. Suppression of the subsequent DTH response was found in the recipients of syngeneic BALB/c suppressor cells as well as in the recipients of H-2 incompatible BALB.K suppressor cells (Fig. 5 A).

Similar data were obtained with regard to the suppression of DTH against B10.D2 (H-2^d) coded non-H-2 alloantigens in BALB/c (H-2^d) responder mice infused with non-H-2 specific BALB/c (H-2^d) or BALB.B (H-2^b) suppressor cells (Fig. 5 B). Since systemic transfer of Ts cells mainly affects the afferent phase of DTH (8), these results strongly suggest that there is no special need for H-2 restricted recognition of the non-H-2 alloantigens by the Ts cells or for a H-2 restricted interaction between the Ts cells and the DTH reactive T cells during the induction of the DTH reactivity.

Lack of H-2 restriction in the suppression of the efferent phase of DTH by activated Ts cells

The requirement for H-2 compatibility between activated Ts cells and DTH effector T cells for suppression of the efferent phase of DTH was investigated.

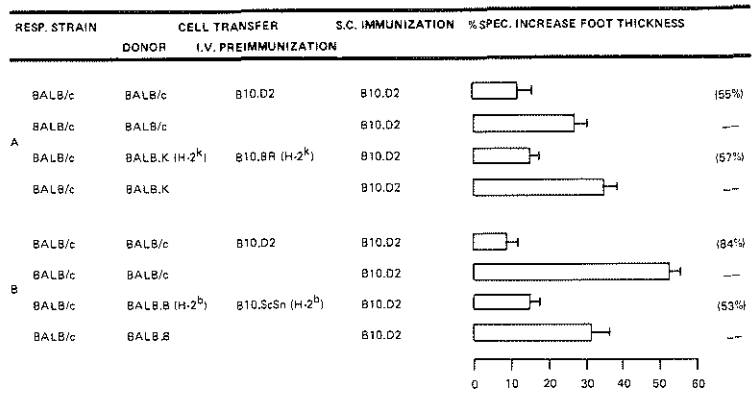

Fig. 5. Lack of H-2 restriction in the suppression of the afferent phase of DTH by Ts cells. BALB/c (H-2^d), BALB.K (H-2^k) and BALB.B (H-2^b) donor mice were iv injected with 5 x 10^7 irradiated B10.D2 (H-2^d), B10.BR (H-2^k) and B10.ScSn (H-2^b) spleen cells, respectively. Four days after iv immunization, spleen and lymph node cells from these mice were iv transferred to BALB/c responder mice. As positive controls, other groups of BALB/c responder mice received naive BALB/c, BALB.K or BALB.B spleen and lymph node cells. One hour after transfer, all mice were sc immunized with 1 x 10^7 non-H-2 incompatible B10.D2 spleen cells and tested for DTH antoher six days later. Each column represents the mean response ± SE (n = 6). The percentage specific suppression and the H-2 haplotypes are shown in parentheses. Significant suppression of DTH reactivity ($p < 0.01$) was found in all groups of responder mice which received spleen and lymph node cells from iv suppressed BALB/c, BALB.K and BALB.B donor mice.

Therefore, B10.BR (H-2^k) mice were iv suppressed with irradiated H-2 compatible but non-H-2 incompatible BALB.K (H-2^k) spleen cells, while B10.D2 (H-2^d) mice were iv suppressed with irradiated H-2 compatible but non-H-2 incompatible BALB/c (H-2^d) spleen cells. Suppressor cells from both groups of mice were tested for suppression of the efferent limb of the DTH response in the ILT-NLT assay. Therefore, the suppressor cells were transferred into the dorsum of a hind foot of (BALB/c x C3H)F_1 (H-$2^{d/k}$) mice in combination with B10.D2 DTH effector T cells against the BALB/c coded non-H-2 alloantigens. As a positive control another group of F_1 mice received B10.D2 DTH effector T cells only. The data presented in Fig. 6 A show that H-2 compatibility between the Ts cells and the DTH effector T cells was not needed for suppression of the efferent phase of DTH.

Subsequently we investigated whether recognition of the non-H-2 alloantigens by the Ts cells during the suppression of the efferent phase of DTH is subject to H-2 restriction. Therefore, Ts cells were induced by iv injection of B10.ScSn

(H-2^b) mice with irradiated H-2 compatible but non-H-2 incompatible BALB.B (H-2^b) spleen cells. In parallel, DTH effector T cells were induced by sc immunization of (B10.ScSn x B10.D2)F_1 (H-$2^{b/d}$) mice with non-H-2 incompatible BALB/c (H-2^d) spleen cells. Thereafter, (B10.ScSn x B10.D2)F_1 DTH effector T cells against BALB/c non-H-2 alloantigens were transferred alone or in combination with B10.ScSn anti-BALB.B Ts cells into the dorsum of a hind food of BALB/c mice. This experiment (Fig. 6 B) did not reveal any need for restricted recognition of the non-H-2 alloantigens by activated Ts cells for suppression of the efferent phase of DTH.

Lack of Igh restriction in the suppression of the afferent and efferent phase of DTH by Ts cells

Finally we investigated whether the Ts cells that are induced by non-H-2 alloantigens suppress the induction and expression of DTH effector T cells in an Igh restricted fashion.

For the afferent phase, BALB/c (Igh^a) and C.B-20 (Igh^b) donor mice were iv suppressed with irradiated H-2 compatible but non-H-2 incompatible DBA/2 spleen cells. Four days later spleen cells from suppressed BALB/c and C.B-20 mice were transferred to naive BALB/c recipient mice. As a control, spleen and lymph node cells from nonsuppressed BALB/c and C.B-20 mice were transferred to separate groups of BALB/c recipients. Immediately after transfer all recipients were sc immunized with DBA/2 spleen cells. Six days later all mice were challenged with similar DBA/2 spleen cells. The results show that Ts cells from BALB.K and C.B-20 mice were equally effective in suppressing the afferent phase of DTH (Fig. 7 A). Therefore, the afferent phase Ts cells for non-H-2 alloantigens are not Igh restricted.

For the efferent phase, BALB/c (Igh^a) and C.B-20 (Igh^b) mice were iv suppressed for DBA/2 non-H-2 alloantigens. The Ts cells from these BALB/c and C.B-20 mice were tested in the ILT-NLT assay for suppression of the efferent phase of DTH. Therefore, these suppressor T cells and BALB/c anti-DBA/2 DTH effector T cells were sc injected in the dorsum of a hind foot of DBA/2 mice. The data presented in Fig. 7 B show that Ts cells from BALB/c and C.B-20 mice were equally effective in suppressing the efferent phase of DTH. Therefore, Igh compatibility between Ts and DTH effector T cells is not needed for suppression of the efferent phase of DTH to non-H-2 alloantigens.

DISCUSSION

The experiments described here establish that iv injection of mice with irradiated allogeneic spleen cells induces Ts cells which affect the efferent limb

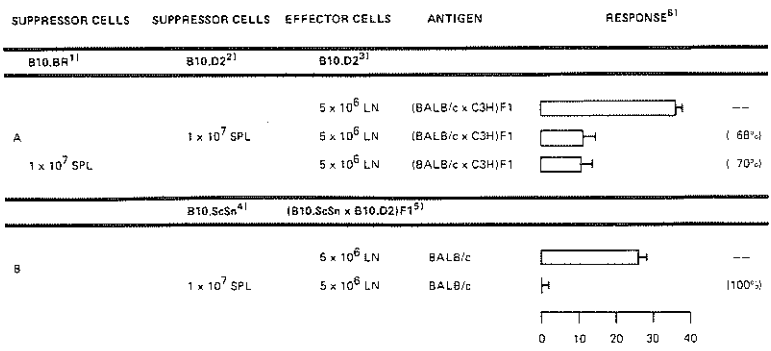

Fig. 6. (A). Lack of H-2 restriction in the interaction between Ts cells and DTH effector T cells in the efferent phase of DTH.

1. Ts cells were induced by iv immunization of B10.BR ($H\text{-}2^k$) mice with 5×10^7 BALB.K ($H\text{-}2^k$) spleen cells. Four days later, 1×10^7 spleen cells from these mice were transferred into the dorsum of the right hind foot of (BALB/c x C3H)F_1 ($H\text{-}2^{d/k}$) recipient mice.
2. Ts cells were induced by iv immunization of B10.D2 ($H\text{-}2^d$) mice with 5×10^7 irradiated BALB/c ($H\text{-}2^d$) spleen cells. Four days later, 1×10^7 spleen cells from these mice were transferred into the dorsum of the right hind foot of (BALB/c x C3H)F_1 ($H\text{-}2^{d/k}$) recipient mice.
3. B10.D2 ($H\text{-}2^d$) DTH effector T cells were induced by sc immunization with 1×10^7 BALB/c ($H\text{-}2^d$) spleen cells. Five days later, 5×10^6 cells from the draining lymph nodes were transferred into the instep of the right hind foot of (BALB/c x C3H)F_1 ($H\text{-}2^{d/k}$) recipient mice.

(B.) Lack of H-2 restriction in the interaction between Ts cells and antigen in the efferent phase of DTH.

4. Ts cells were induced by iv immunization of B10.ScSn ($H\text{-}2^b$) mice with 5×10^7 irradiated BALB.B ($H\text{-}2^b$) spleen cells. Four days later 1×10^7 spleen cells from these mice were transferred into the dorsum of the right hind foot of BALB/c ($H\text{-}2^d$) recipient mice.
5. (B10.ScSn x B10.D2)F_1 ($H\text{-}2^{b/d}$) DTH effector T cells were induced by sc immunization with 1×10^7 BALB/c ($H\text{-}2^d$) spleen cells. Five days later, 5×10^6 cells from the draining lymph nodes were transferred into the dorsum of the right hind foot of BALB/c ($H\text{-}2^d$) recipient mice.
6. Ts cells and DTH effector T cells were transferred simultaneously. As a control, the left hind foot of the recipient mice was injected with a similar number of naive lymphoid cells from the appropriate donor strains.

   DTH responses in the (BALB/c x C3H)F_1 and BALB/c recipient mice were measured 24 hr after local transfer and are expressed in 10^{-3} cm ± SE (n = 6). The percentage specific suppression is shown in parentheses. All groups of recipient mice which received Ts cells and DTH effector T cells showed a significant suppression of DTH ($p < 0.01$).

('challenge phase') of DTH to alloantigens. We have shown previously by studying the proliferative activity in the draining lymph nodes of mice after sc immunization with allogeneic spleen cells (7, 8) that iv preimmunization also induces Ts cells which suppress the afferent limb of DTH against alloantigens.

In contrast to Miller *et al.* (17, 20) who were able to transfer suppression of the efferent limb of CS against 2,4-dinitro-1-fluorobenzene (DNFB) by iv injection of activated Ts cells, we could not transfer efferent phase suppression intravenously, at least not with lymphoid cells isolated from donor mice 4 to 7 days after the iv suppressive injection.

Dietz *et al.* (21) demonstrated that Ts cells, which were isolated 7 days after iv induction of azobenzenearsonate (ABA) specific suppression, could only suppress the DTH response of the recipients when administered at the time of immunization. Transfer one or more days after immunization was ineffective. They showed that the afferent phase Ts cells induced second order Ts cells that suppressed the efferent phase.

In our experiments, the iv suppressed mice themselves displayed efferent phase suppression of DTH against alloantigens on day 6 after the secondary sc immunization, when the mice were challenged for DTH. Therefore we investigated whether lymphoid cells from iv suppressed and subsequently sc immunized mice were more potent in systemic transfer of efferent phase suppression. However, also under these conditions no suppressive effect could be demonstrated in mice which received these cells a few hours before challenge (data not shown). On the other hand, the experiments involving transfer of DTH effector T cells to iv suppressed recipients (Fig. 1) or cotransfer of DTH effector T cells and lymphoid cells from iv suppressed mice in an ILT-NLT assay (Fig. 2) show that iv injection of irradiated allogeneic spleen cells does induce Ts cells active during the efferent phase of DTH against alloantigens. These efferent phase Ts cells appeared to be Lyt-1^+2^+ (Fig. 2).

The occurence of 'bystander' suppression of DTH against third party alloantigens (Fig. 3) also demonstrates that iv immunization with irradiated allogeneic spleen cells induces 'efferent phase' Ts cells. Thus afferent phase Ts cells (8) as well as efferent phase Ts cells that can regulate DTH against alloantigens occur in spleen and lymph nodes 4 to 7 days after the iv suppressive injection.

The inability to transfer efferent phase suppression by iv injection of Ts cells in the period just before challenge may depend on an inefficient or inappropriate migration of the systemically transferred Ts cells to the hind foot, where the activity of DTH effector T cells has to be suppressed.

Although we know from previous experiments (7) that activation of alloantigen induced Ts cells is antigen specific, suppression of the efferent phase of DTH to third party bystander alloantigens (Fig. 3) shows that, after activation

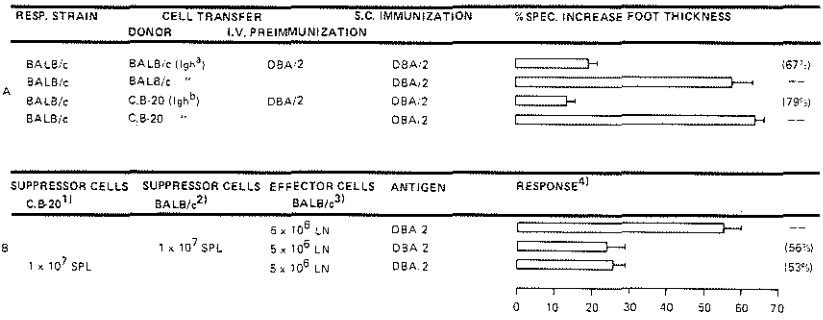

Fig. 7. (A.) Lack of Igh restriction in the suppression of the afferent phase of DTH by Ts cells. BALB/c (Igh^a) and CB-20 (Igh^b) donor mice were iv injected with 5×10^7 irradiated DBA/2 spleen cells. Four days after iv immunization spleen cells from these mice were iv transferred to BALB/c responder mice. As positive controls, other groups of BALB/c responder mice received naive BALB/c or C.B-20 spleen and lymph node cells. One hour after transfer, all mice were sc immunized with 1×10^7 non-H-2 incompatible DBA/2 spleen cells and tested for DTH another six days later. Each column represents the mean response ± SE (n = 6). The percentage specific suppression and the Igh allotype are shown in parentheses. Significant suppression of DTH reactivity ($p < 0.01$) was found in all groups of responder mice which received spleen and lymph node cells from iv suppressed BALB/c or C.B-20 donor mice.

(B.) Lack of Igh restriction in the interaction between Ts cells and antigen in the efferent phase of DTH.

1. Ts cells were induced by iv immunization of C.B-20 (Igh^b) mice with 5×10^7 DBA/2 spleen cells. Four days later, 1×10^7 spleen cells from these mice were transferred into the dorsum of the right hind foot of DBA/2 recipient mice.
2. Ts cells were induced by iv immunization of BALB/c (Igh^a) mice with 5×10^7 DBA/2 spleen cells. Four days later, 1×10^7 spleen cells from these mice were transferred into the dorsum of the right hind foot of DBA/2 recipient mice.
3. BALB/c (Igh^a) DTH effector T cells were induced by sc immunization with 1×10^7 DBA/2 spleen cells. Five days later, 5×10^6 cells from the draining lymph nodes were transferred into the dorsum of the right hind foot of DBA/2 recipient mice.
4. Ts cells and DTH effector T cells were transferred simultaneously. As a control, the left hind foot of the recipient mice was injected with a similar number of naive lymphoid cells from the appropriate donor strains. DTH responses in the DBA/2 recipient mice were measured 24 hr after local transfer and are expressed in 10^{-3} cm ± SE (n = 6). The percentage specific suppression is shown in parentheses. All groups of recipient mice which received Ts cells and DTH effector T cells showed significant suppression of DTH ($p < 0.01$).

of the Ts cells with the specific alloantigens, the ultimate suppressive effect by the efferent phase Ts cells is nonspecific. Suppression of DTH to third party bystander alloantigens even occured when the specific and third party antigens occurred on separate cells, suggesting that associative recognition of the alloantigens involved is not required for the Ts cells in order to display their non-

specific suppressive effect. Similar data on nonspecific bystander suppression of DTH against third party antigens *in vivo* have been described by ourselves for the afferent limb of DTH to alloantigens (14, 15), by Zembala *et al.* for suppression of the efferent limb of DTH against picrylchloride and oxazolone (22) and by Ramshaw *et al.* for DTH to protein antigens (23). Other studies dealing with suppression of CS and DTH to haptenized cells indicate that the suppressive effect of afferent (5, 16) and efferent phase (5, 17, 24, 25) Ts cells is antigen specific. However, these reports do not describe experiments which are appropriate to demonstrate nonspecific bystander suppressive effects.

Restriction requirements have been described in several Ts cell systems: for the activation of Ts cells (17), for the interaction of Ts cells and responder T cells in MLR (26, 27), for the interaction between different types of Ts cells and Ts cell factors in a suppressor cell cascade (5, 21, 25, 28, 29) and for the recognition of antigen by Ts cells (6, 30). The results depicted in Fig. 4 show that non-H-2 specific Ts cells can be induced by iv preimmunization with spleen cells that are H-2 as well as non-H-2 incompatible with the responder. No restriction requirements were found for these Ts cells in order to transfer suppression of the afferent phase of DTH to naive recipients. These results are at variance with those of Miller *et al.* (16, 17). They showed that hapten speicific Ts cells, induced by H-2 compatible haptenized cells, act during the efferent phase of the DTH response only and could be transferred to H-2 compatible as well as to H-2 incompatible recipient mice, while Ts cells induced with H-2 incompatible haptenized cells were afferent phase Ts cells and could only be transferred to recipients with the same H-2 haplotype as the donors of the haptenized allogeneic cells. The lack of H-2 restriction requirements for the efferent phase Ts cells found by Miller, *et al.* (17) is compatible with our data concerning suppression of the efferent phase of DTH against alloantigens by Ts cells (Fig. 6). Furthermore, our data show that systemic transfer of afferent phase Ts cells is not Igh restricted (Fig. 7 A). This is compatible with the afferent phase Ts1 cells acting in hapten specific Ts cells cascades (5, 2, 8). The Ts2 and Ts3 from these Ts cell cascades and their suppressor factors TsF2 and TsF3 have shown to be H-2 (I-J) and Igh restricted in the suppression of the efferent phase of DTH (5, 21, 31). Our data do not show any H-2 (Fig. 6) or Igh (Fig. 7 B) restriction requirement for Ts cells during suppression of the efferent phase of DTH against alloantigens.

To summarize, the data presented do not reveal any difference between the Ts cells active during the afferent (8) and efferent limb of DTH against alloantigens. Thus, despite of all well documented examples of different subsets of hapten specific Ts cells for suppression of the afferent and efferent phase of DTH and CS to haptens, there is no reason as yet to suppose that the afferent and efferent limb of DTH to alloantigens is regulated by different subsets of Ts cells.

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CHAPTER X

ALLOANTIGEN-SPECIFIC SUPPRESSOR T CELLS CAN ALSO SUPPRESS THE IN VIVO IMMUNE RESPONSE TO UNRELATED ALLOANTIGENS

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Delayed-type hypersensitivity (DTH) to both major histocompatibility complex (H-2) and non-H-2-coded antigens can be induced by subcutaneous immunization with allogeneic lymphoid cells in the mouse. While subcutaneous immunization with allogeneic cells preferentially induces DTH reactivity, intravenous immunization, especially with irradiated allogeneic cells, induces a state of suppression. Suppression is manifest both in direct host-versus-graft (HvG) (1) assays and under graft-versus-host (GvH) conditions (2), where spleen cells of suppressed mice are used to reconstitute irradiated allogeneic hosts. The suppression is mediated by T cells (2, 3). We have now studied the specificity of the suppressive effect by subcutaneous immunization of 'suppressed' mice with a combination of alloantigens comprising the antigen(s) used to induce the suppressor T cells as well as unrelated alloantigens. We report here that reaction against the third party alloantigens was effectively suppressed, provided these antigens were presented in combination with the antigen(s) that had induced the suppressor T cells. Both sets of alloantigens do not need to be physically associated.

Mice, suppressed to H-2 histocompatibility antigens, do not display a DTH response against these antigens, but respond normally against third party antigens (Table 1, groups A and E). The suppression is H-2 subregion-specific: mice, suppressed for either H-2I or H-2D antigens, display normal DTH reactivity after subcutaneous immunization with other H-2 subregion antigens (Table 1, groups B and C). Table 1, group D, shows that suppression of the HvG reactivity to H-2 subregions is haplotype-specific. We have also observed that intravenous injection of cells incompatible for minor histocompatibility antigens could not suppress DTH reactivity to subcutaneously administered H-2 alloantigens and vice versa. Thus suppressor T cells require restimulation with the original immunizing antigen: they are strictly antigen-specific as far as their activation is concerned.

In GvH reactions H-2I and MIs antigens activate DTH-reactive T cells (4). This activity is assayed in secondary recipients, after passive transfer of lymphoid cells from animals undergoing the GvH reaction (5, 6). The suppression found in HvG reactions is also found in GvH reactions (Table 1, group E).

The specificity of the suppressive effect was studied by subcutaneous immunization of 'suppressed' mice with a combination of alloantigens comprising the antigen(s) used to induce the suppressor T cells as well as unrelated alloantigens. Reaction against the third party alloantigens was effectively suppressed, provided these antigens were presented in combination with the antigen(s) that had induced the suppressor T cells. This holds also for GvH reactions (Table 2). The phenomenon was observed for complete H-2 differences (groups A and C) as well as for H-2 subregion differences (groups B and D). Intravenous preimmunization with an alloantigen suppressed the DTH response to a challenge by

Table 1 Specificity of antigen recognition by suppressor T cells

system	exp	resp. strain	i.v. preimmunization	s.c. immunization	% specific increase foot thickness
HvG	A	BALB/c ($H\text{-}2^d$)	BALB.B ($H\text{-}2^b$)	BALB.B ($H\text{-}2^b$)	
		BALB/c	BALB/c	BALB.B ($H\text{-}2^b$)	
		BALB/c	BALB.K ($H\text{-}2^k$)	BALB.B ($H\text{-}2^b$)	
	B	B10.AQR	B10.A (K)	B10.A (K)	
		B10.AQR	B10.AQR	B10.A (K)	
		B10.AQR	B10.T(6R)(I)	B10.A (K)	
	C	A.TH	A.TL (I)	A.TL (I)	
		A.TH	A.TH	A.TL (I)	
		A.TH	A.SW (D)	A.TL (I)	
	D	B10.Br (D^k)	B10.AKM (D^q)	B10.AKM (D^q)	
		B10.Br	B10.Br	B10.AKM (D^q)	
		B10.Br	B10.A(2R)(D^b)	B10.AKM (D^q)	
		B10.Br	B10.A(2R)(D^b)	B10.A(2R)(D^b)	
		B10.Br	B10.Br	B10.A(2R)(D^b)	
		B10.Br	B10.AKM (D^q)	B10.A(2R)(D^b)	

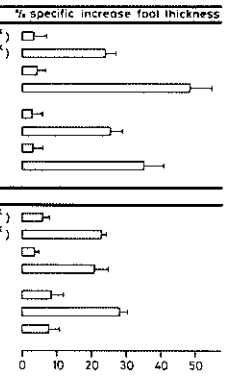
system	exp.	resp. cells	i.v. preimmunization	irr. host	
GvH	E	BALB/c ($H\text{-}2^d$)	BALB.B ($H\text{-}2^b$)	BALB.B ($H\text{-}2^b$)	
		BALB/c	BALB/c	BALB.B ($H\text{-}2^b$)	
		BALB/c	BALB.K ($H\text{-}2^k$)	BALB.B ($H\text{-}2^b$)	

0 10 20 30 40

Suppressor T cells were induced by intravenous (i.v.) preimmunization of groups of six mice with 5×10^7 X-irradiated (2,000 rad) allogeneic spleen cells. HvG reactivity was stimulated 7 days later by subcutaneous (s.c.) injection of 1×10^7 allogeneic spleen cells, distributed over the inguinal area. Six days later the mice were tested for DTH reactivity by injection of 2×10^7 of the same allogeneic spleen cells into the dorsum of the right hind foot. Foot swelling was measured 24 h after challenge. Untreated control mice received only the challenge dose. The swelling in these control mice varied between 12 and 20%. DTH responses are expressed as % specific increase in foot thickness, and corrected for nonspecific swelling in the control mice. The histograms represent the arithmetic mean ± s.e. The H-2 haplotype (group A), the H-2 subregion differences (group B and C), and the haplotype of the H-2D locus (group D) are shown in parentheses. GvH reactions were elicited by i.v. injection of 1×10^7 spleen cells ('responder cells') into X-irradiated (700 rad) allogeneic recipient mice. Five days after reconstitution the total cell yield of spleen, inguinal, axillary and mesenteric lymph nodes of a recipient mouse was injected i.v. into an untreated secondary recipient syngeneic with the spleen cell donor. DTH reactivity was measured as in HvG reactions. The H-2 haplotype of the mice and cells used (group E) are shown in parentheses. The origin of the H-2 subregions (K, I-A, I-J, I-E, D): A.SW s sss s, A.TH s sss d, A.TL s kkk d, BALB.B b bbb b, BALB/c d ddd d, BALB.K k kkk k, B10.BR k kkk k, B10.A k kkk d, B10.AKM k kkk q, B10.A(2R) q kkk b, B10.AQR q kkk d, B10.T(6R) q qqq d.

Table 2 Specificity of the suppressive effect mediated by suppressor T cells

system	exp.	resp. strain	i.v. preimmunization	s.c. immunization	challenge	% specific increase foot thickness
HvG	A	BALB.B ($H\text{-}2^b$)	BALB/c ($H\text{-}2^d$)	BALB/c x.K F_1 ($H\text{-}2^{d/k}$)	BALB/c x.K F_1 ($H\text{-}2^{d/k}$)	
		BALB.B	BALB.B	BALB/c x.K F_1 ($H\text{-}2^{d/k}$)	BALB/c x.K F_1 ($H\text{-}2^{d/k}$)	
		BALB.B	BALB/c ($H\text{-}2^d$)	BALB/c x.K F_1 ($H\text{-}2^{d/k}$)	BALB.K ($H\text{-}2^k$)	
		BALB.B	BALB.B ($H\text{-}2^b$)	BALB/c x.K F_1 ($H\text{-}2^{d/k}$)	BALB.K ($H\text{-}2^k$)	
	B	B10.A	B10.AQR(K)	B10.T(6R)(KI)	B10.T(6R)(KI)	
		B10.A	B10.A	B10.T(6R)(KI)	B10.T(6R)(KI)	
		B10.AQR	B10.A (K)	B10.A x.T(6R)F_1(KI)	B10.T(6R)(I)	
		B10.AQR	B10.AQR	B10.A x.T(6R)F_1(KI)	B10.T(6R)(I)	
system	**exp.**	**resp. cells**	**i.v. preimmunization**	**irr. host**	**challenge**	
GvH	C	BALB.B ($H\text{-}2^b$)	BALB/c ($H\text{-}2^d$)	BALB/c x.K F_1 ($H\text{-}2^{d/k}$)	BALB/c x K F_1 ($H\text{-}2^{d/k}$)	
		BALB.B	BALB.B	BALB/c x.K F_1 ($H\text{-}2^{d/k}$)	BALB/c x K F_1 ($H\text{-}2^{d/k}$)	
		BALB.B	BALB/c ($H\text{-}2^d$)	BALB/c x.K F_1 ($H\text{-}2^{d/k}$)	BALB.K ($H\text{-}2^k$)	
		BALB.B	BALB.B ($H\text{-}2^b$)	BALB/c x.K F_1 ($H\text{-}2^{d/k}$)	BALB.K ($H\text{-}2^k$)	
	D	A.AL	A.TH (KI)	A.TH (KI)	A.TH (KI)	
		A.AL	A.AL	A.TH (KI)	A.TH (KI)	
		A.AL	A.TL (K)	A.TH (KI)	A.TH (KI)	



The experimental details are the same as in Table 1. The H-2 haplotypes (groups A and C), or the H-2 subregion differences (groups B and D) are shown in parentheses. BALB/c x. KF1 means F1 hybrid of BALB/c x BALB.K, B10.A x T(6R)F1 means F1 hybrid of B10.A x B10.T(6R). The origin of the H-2 subregions of the A.AL strain is k kkk d. The other strains are described in the legend to Table 1.

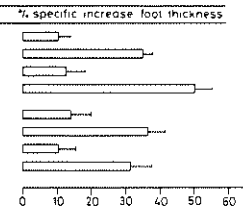
completely different alloantigens, as long as the preimmunizing antigen was also present in the inducing inoculum (Table 2, groups A-C). The same result was obtained after suppression with non-H-2 alloantigens, subsequent immunization with a mixture of the same non-H-2 alloantigens plus unrelated 'bystander' H-2 antigens, and eventual testing for DTH reactivity against the H-2 antigens only (Table 3, groups A and B). These experiments clearly show that the 'bystander' antigens and the antigens used to induce the suppressor T cells do not have to be presented by the same cells.

Nonspecific suppressive effects have been demonstrated previously *in vivo* (7, 8) and *in vitro* (9-13). Contact sensitizing agents (14, 15) as well as hapten-derivatized syngeneic (16, 17) and allogeneic (18) cells readily induce suppressor T cell activity. The generation of this activity is dependent on complex interactions of three sets of suppressor T cells in a regulatory circuit under control of IgH and H-2 genes (19, 20). Fresno *et al.* (11-13) have isolated an antigen-specific suppressor factor from continuously growing suppressor T cell clones. This factor was found to be a protein which, after interaction with antigen, breaks down into two peptides of 45,000 and 24,000 molecular weight. The former subunit suppresses both antigen-specific and other Lyt 1^+ T cells.

Intravenous preimmunization with X-irradiated allogeneic cells also induces suppressor T cell activity. The type of the suppressor T cell activated depends on

Table 3 Recognition of specific and 'bystander' antigens on separate cells

system	exp	resp strain	i.v. preimmunization	s.c. immunization	challenge	% specific increase foot thickness
	A	B10.D2 ($H\text{-}2^d$)	BALB/c	BALB.B ($H\text{-}2^b$)	B10.ScSn ($H\text{-}2^b$)	
		B10.D2	B10.D2	BALB.B ($H\text{-}2^b$)	B10.ScSn ($H\text{-}2^b$)	
		B10.D2	BALB/c	BALB/c + B10.ScSn ($H\text{-}2^b$)	B10.ScSn ($H\text{-}2^b$)	
		B10.D2	B10.D2	BALB/c + B10.ScSn ($H\text{-}2^b$)	B10.ScSn ($H\text{-}2^b$)	
	B	DBA/2 ($H\text{-}2^d$)	B10.D2	B10.G ($H\text{-}2^q$)	DBA/1 ($H\text{-}2^q$)	
		DBA/2	DBA/2	B10.G ($H\text{-}2^q$)	DBA/1 ($H\text{-}2^q$)	
		DBA/2	B10.D2	B10.D2 + DBA/1 ($H\text{-}2^q$)	DBA/1 ($H\text{-}2^q$)	
		DBA/2	DBA/2	B10.D2 + DBA/1 ($H\text{-}2^q$)	DBA/1 ($H\text{-}2^q$)	



Suppressor T cells against minor histocompatibility antigens were induced as described in the legend to Table 1, which also gives the details of the assays. The H-2 haplotype differences are shown in parentheses.

the dose of antigen and on treatment of the mice with cyclophosphamide (21). Liew (7), using high antigen doses and cyclophosphamide to induce suppressor T cell activity, has shown that suppression of DTH to H-2 subregion products can be induced if, and only if, the H-2 incompatibility includes the I-J subregion. The suppressor T cells are then antigen-specific, recognize the allo-I-J molecules and suppress also the DTH response to other H-2 subregion gene products if these are presented on the same cells as the allo-I-J determinants (7). The suppressor T cells activated by our protocol (which differs from Liew's by using a lower dose of antigen and no cyclophosphamide) do not need to recognize allo-I-J determinants (Table 1) and will nonspecifically suppress the response to 'bystander' antigens, irrespective of whether the specific and 'bystander' antigens are physically associated.

Various mechanisms have been proposed to date for the beneficial effect of blood transfusion on kidney transplant survival (22, 23). The data of Fresno *et al.* (11-13), combined with our finding of nonspecific suppression of the *in vivo* immune response to 'bystander' alloantigens, even if they are not physically associated with the specific antigens, may well explain the blood transfusion effect. Sharing histocompatibility antigens by the transfused blood cells and the transplanted kidney and/or the passenger blood cells might reactivate antigen-specific suppressor T cells after transplantation, and that might supress the anti-graft reactions in a nonspecific way.

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CHAPTER XI

SUPPRESSION OF DELAYED TYPE HYPERSENSITIVITY TO THIRD PARTY 'BYSTANDER' ALLOANTIGENS BY ANTIGEN-SPECIFIC SUPPRESSOR T CELLS

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SUMMARY

Delayed type hypersensitivity (DTH) against alloantigens can be induced by sc immunization with allogeneic cells. The induction of DTH can be suppressed by iv preimmunization of the mice with similar allogeneic spleen cells, provided the cells are irradiated before injection. This supression is mediated by T cells. The suppressor activity can be induced not only by H-2 and non-H-2 coded antigens, but also by H-2 subregion coded antigens. Suppression induced by K, I or D subregion coded antigens is specific for that particular subregion as well as for its haplotype. I-J coded alloantigens were found to be not necessary for the induction of antigenspecific suppressor T cells. After restimulation of suppressor T cells by the 'specific' alloantigens, the DTH to simultaneously administered third party alloantigens becomes suppressed as well. This nonspecific suppression of DTH to third party 'bystander' alloantigens also occurs when the 'specific' and the third party antigens are presented on separate cells, provided that both cell types are administered together at the same site. The simultaneous presentation of both sets of alloantigens during the induction phase of DTH only is sufficient to prevent the normal development of DTH to the third party antigens.

INTRODUCTION

Suppression of cellular immunity in mice by intravenous (iv) preimmunization is a well-known phenomenon. Most studies deal with suppression of contact sensitivity (1-3) or delayed type hypersensitivity (DTH) to haptens (4, 5) after iv preimmunization with hapten-modified syngeneic (1-5) or allogeneic lymphoid cells (2). Some of them have shown that the development of suppressor activity depends upon complex interactions of three sets of suppressor T cells in a regulatory circuit under control of IgH and H-2 genes (3, 6).

Studies concerning suppression of cellular immunity against alloantigens are less numerous. Suppression of DTH to alloantigens (7-10) and suppression of skin (11, 12) and heart allograft rejection (13) can be induced by iv preimmunization with different types of donor cells. Most investigators have reported that, in addition to iv preimmunization with donor antigen, pretreatment with antilymphocyte serum (12, 14) or cyclophosphamide (7, 8, 13) is necessary to obtain the suppressive effect. Our own studies have shown that the use of immunosuppressive drugs or antisera is superfluous in order to induce suppression of DTH to alloantigens by iv preimmunization (9, 10). Furthermore, in our hands, the antigen dose needed for optimal suppression is rather small as compared with the other studies (7, 8). This iv induced suppression persists for a long period (9) and can be transferred by T lymphocytes only (10). We have shown by *in vivo* [^{3}H]TdR-incorporation studies that the iv induced suppressor T cells suppress the induction of DTH by subcutaneously administered H-2 and non-H-2 alloantigens (9). The *activation* of T cells responsible for suppression of DTH to H-2 alloantigens was found to be specific for the H-2 haplotype of the cells used for iv preimmunization. Furthermore, iv injection of cells incompatible for non-H-2 alloantigens could not suppress DTH to H-2 alloantigens or vice versa (9).

Our previous studies, however, did not deal with the ability of H-2 subregion coded antigens to induce suppressor T cells by iv preimmunization. This is of particular interest, since it has been reported that I-J alloantigens are required in order to induce the suppressive activity (7). The present paper pays attention to this particular aspect and, in addition, shows that the *suppressive effect* mediated by the antigen-specific suppressor T cells *in vivo* is nonspecific.

MATERIALS AND METHODS

Mice

A.SW (H-2^s), BALB/c (H-2^d), B10.A (H-2^a), and C3H/HeJ (H-2^k) mice were purchased from the Laboratory Animals Centre of the Erasmus University,

Rotterdam. DBA/2 mice were purchased from the Radiobiological Institute TNO, Rijswijk, The Netherlands. A.TL (H-2^{tl}), A.TH (H-2^{t2}), BALB.B (H-2^{b}), BALB.K (H-2^{k}), (BALB/c x BALB.K)F1 (H-$2^{d/k}$), B10.ScSn (H-2^{b}), B10.D2 (H-2^{d}), B10.BR (H-2^{k}), B10.G (H-2^{q}), (B10.ScSn x B10.D2)F1 (H-$2^{b/d}$), B10.AQR (H-2^{yl}), B10.T(6R) (H-2^{y2}), B10.AKM (H-2^{m}), B10.A(2R) (H-2^{h2}), and DBA/1 (H-2^{q}) mice were purchased from OLAC Ltd., Bicester, United Kingdom. (B10.T(6R) x B10.A)F1 (H-$2^{y2/a}$), and B10.MBR (H-2^{bq1}) were bred at our own department. The age of the responder mice varied between 10 and 24 weeks. Only female mice were used.

Preparation of cell suspensions

Mice were killed by carbon dioxide. Spleens were removed, placed in 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. The viability of the cell suspensions obtained was at least 90%.

Immunization

Suppression was induced by iv preimmunization with 5 x 10^7 allogeneic spleen cells. Within 3 hours before iv injection the cells had been irradiated *in vitro* with 20 Gy, generated in a Philips Müller MG 300 X-ray machine as described in detail previously (15). DTH was induced by sc immunization with 1 x 10^7 nonirradiated allogeneic spleen cells. When mixtures of spleen cells derived from two different mouse strains were used for sc immunization, the cells were treated with mitomycin C. The interval between iv induction of suppression and sc immunization for DTH was 7 days. Six days after the sc immunization the mice were tested for DTH reactivity by injection of a challenge dose into the dorsum of the right hind foot.

Mitomycin C treatment

Treatment of spleen cells with mitomycin C (Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan) was carried out as described by Blomgren and Svedmyr (16). A number of, 2 to 10 x 10^8, spleen cells was incubated for 30 min at 37°C in 40 ml BSS containing 25μg mitomycin C per ml. After treatment with mitomycin C, the cells were washed with BSS three times.

Assay for DTH

DTH reactions were determined by measuring the difference in thickness of the hind feet 24 hr after sc injection of a challenge dose of 2 x 10^7 of the appropriate allogeneic spleen cells into the dorsum of the right hind foot. As a control for background DTH reactivity, naive syngeneic mice were used which

only received the challenge dose. The specific DTH response was calculated as the relative increase in foot thickness of the immune mice minus the relative increase in foot thickness of the control mice. The swelling of the control mice ranged between 15 and 25%.

RESULTS

Induction of suppressor T cells by H-2 subregion-coded alloantigens and their specificity

To investigate whether H-2 subregion-coded antigens can induce a state of suppression by iv immunization, and to study the specificity of the suppressor T cells (10) involved, we employed several combinations of H-2K, H-2I and H-2D incompatible mouse strains. For H-2K, B10.AQR mice were iv injected with either 5×10^7 irradiated H-2K incompatible B10.A spleen cells, H-2I incompatible B10.T(6R) spleen cells, or syngeneic B10.AQR spleen cells. Seven days later the mice were sc immunized with 1×10^7 B10.A or B10.T(6R) spleen cells and challenged with similar spleen cells another six days later. It appeared that the iv preimmunization of B10.AQR mice with H-2K incompatible spleen cells did suppress the anti H-2K DTH reactivity, but not the anti H-2I DTH reactivity (Fig. 1, exp. A and B). Similarly, iv preimmunization with H-2I or H-2D incompatible spleen cells led to a H-2I of H-2D subregion specific suppression of the DTH reactivity, respectively (Fig. 1, exp. B-D).

Subsequently we investigated whether the iv induced suppression to H-2 subregion coded antigens is specific for the haplotype of the cells used for immunization. Thus, B10.BR ($H\text{-}2D^k$) mice were iv injected with either irradiated H-2D incompatible B10.AKM ($H\text{-}2D^q$) spleen cells, B10.A(2R) ($H\text{-}2D^b$) spleen cells, or syngeneic B10.BR spleen cells. Seven days later the mice were sc immunized with B10.AKM or B10.A(2R) spleen cells and challenged with similar cells another six days later. It appeared that the suppression of the DTH reactivity to H-2D subregion coded alloantigens was haplotype specific (Fig. 1, exp. E). The same result was obtained with a combination of B10.A ($H\text{-}2K^k$), B10.MBR ($H\text{-}2K^b$), and B10.AQR ($H\text{-}2K^q$) mice, which have different haplotypes for the H-2K subregion (Fig. 1, exp. F).

The specificity of the suppressive effect by antigenspecific suppressor T cells

Previous studies (9, 10) and those above show that suppressor T cells, induced by iv preimmunization with alloantigens, are antigenspecific. This conclusion brings us to the question concerning the specificity of the suppressive effect mediated by these suppressor T cells. What happens when 'suppressed' mice are

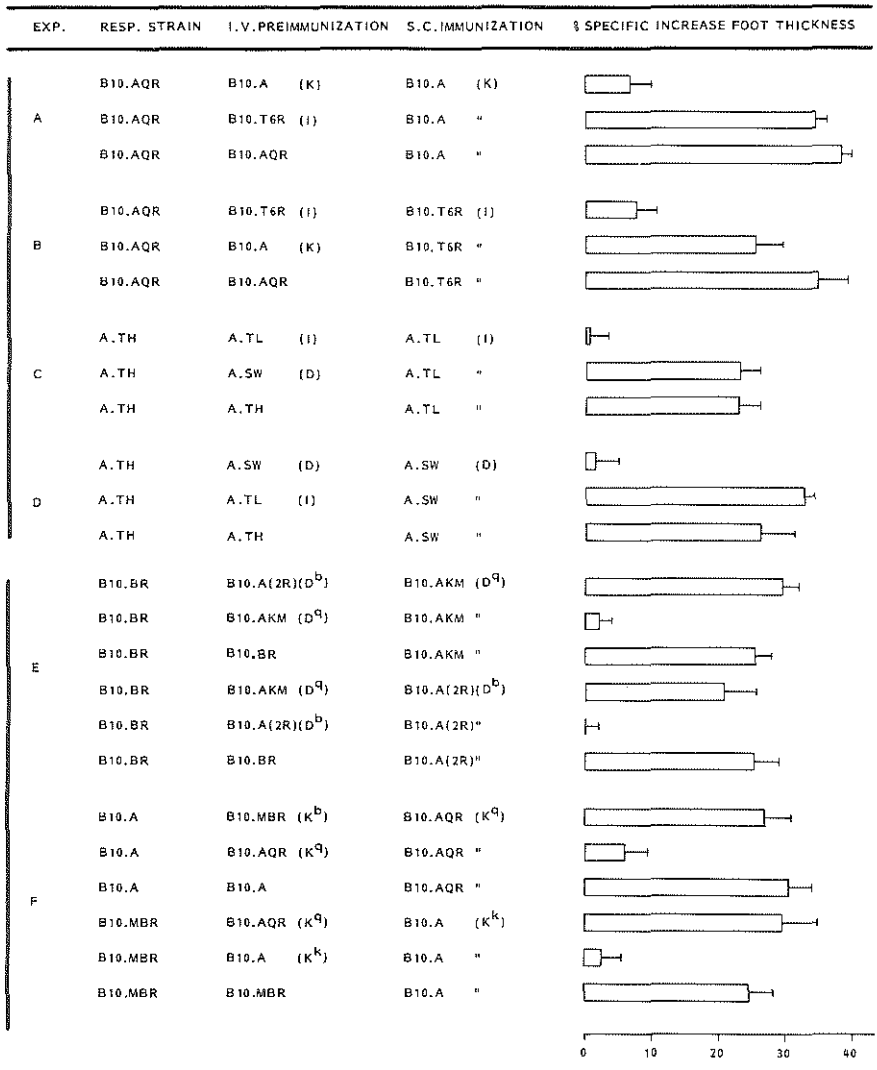

Fig. 1. Induction and specificity of suppressor T cells regulating DTH to H-2 subregion coded antigens. Responder mice were iv injected with 5×10^7 irradiated allogeneic or syngeneic spleen cells and sc immunized with 1×10^7 allogeneic spleen cells 7 days later. Challenge for DTH was performed on day 6 after sc immunization. Each column respresents the mean response ± SE (n=6). In all experiments significant suppression of DTH reactivity ($p < 0.01$) was only found in mice iv and sc immunized with the same H-2 subregion coded antigens. The H-2 subregion differences (exp. A-D), the haplotype of the H-2D locus (exp. E) and the haplotype of the H-2K locus (exp. F) are shown in parentheses.

sc immunized with third party alloantigens in combination with the alloantigens that had been used for induction of the antigenspecific suppressor T cells?

This was investigated in mouse strain combinations that were either fully H-2 incompatible, only H-2 subregion incompatible, non-H-2 incompatible, or H-2 and non-H-2 incompatible. Thus, BALB.B (H-2^b) mice were iv injected with irradiated H-2 incompatible BALB/c (H-2^d) or syngeneic BALB.B spleen cells. Seven days later these mice were sc immunized with H-2 incompatible BALB.K (H-2^k) or (BALB/c x BALB.K)F1 (H-$2^{d/k}$) spleen cells and challenged with similar spleen cells another six days later. A similar experiment was done with B10.BR (H-2^k) responder mice, which were iv suppressed with H-2 incompatible B10.ScSn (H-2^b) spleen cells, while DTH reactivity was induced and elicited with B10.D2 (H-2^d) or (B10.ScSn x B10.D2)F1 (H-$2^{b/d}$) spleen cells. It appeared that iv preimmunization with alloantigens indeed suppressed the DTH response to the third party alloantigens, but only when these third party antigens were presented together with the alloantigens that previously had induced the suppressor T cells (Fig. 2, exp. A and B).

The same data was obtained in combinations of mouse strains that were H-2K and/or H-2I incompatible and in combinations that were H-2 and/or non-H-2 incompatible. Thus, iv preimmunization with H-2K and H-2I coded alloantigens could suppress the anti H-2I and anti H-2K DTH response, respectively, when the subregion coded alloantigen used for iv preimmunization was also present in the induction and challenge-phase of DTH (Fig. 2, exp. C and D). Similarly, suppressor T cells induced by either H-2 or non-H-2 coded alloantigens also suppressed the anti non-H-2 and anti H-2 DTH response, respectively, provided the antigens used for iv suppression were also present in the inoculum used in the induction and challenge-phase of DTH (Fig. 2, exp. E-L).

These results show that alloantigen-specific suppressor T cells effectively suppress the reaction against third party alloantigens, provided these antigens are presented as 'bystanders' to the antigen(s) that had induced the suppressor T cells.

Effect of the mode of presentation of the specific and 'bystander' antigens

In the previous section it was shown that the ultimate suppressive effect by the antigen-specific suppressor T cells is nonspecific. However, it is unclear from these experiments whether the simultaneous presentation of the 'specific' and the 'bystander' antigens is required in the induction as well as the challenge-phase or that combined presentation in the induction phase is sufficient. Furthermore, the above experiments do not reveal whether or not associative recognition of the specific and 'bystander' antigens is involved. Therefore, subsequent experiments were devoted to these questions. Thus, BALB.B (H-2^b) responder mice were iv injected with irradiated H-2 incompatible BALB/c (H-2^d) or

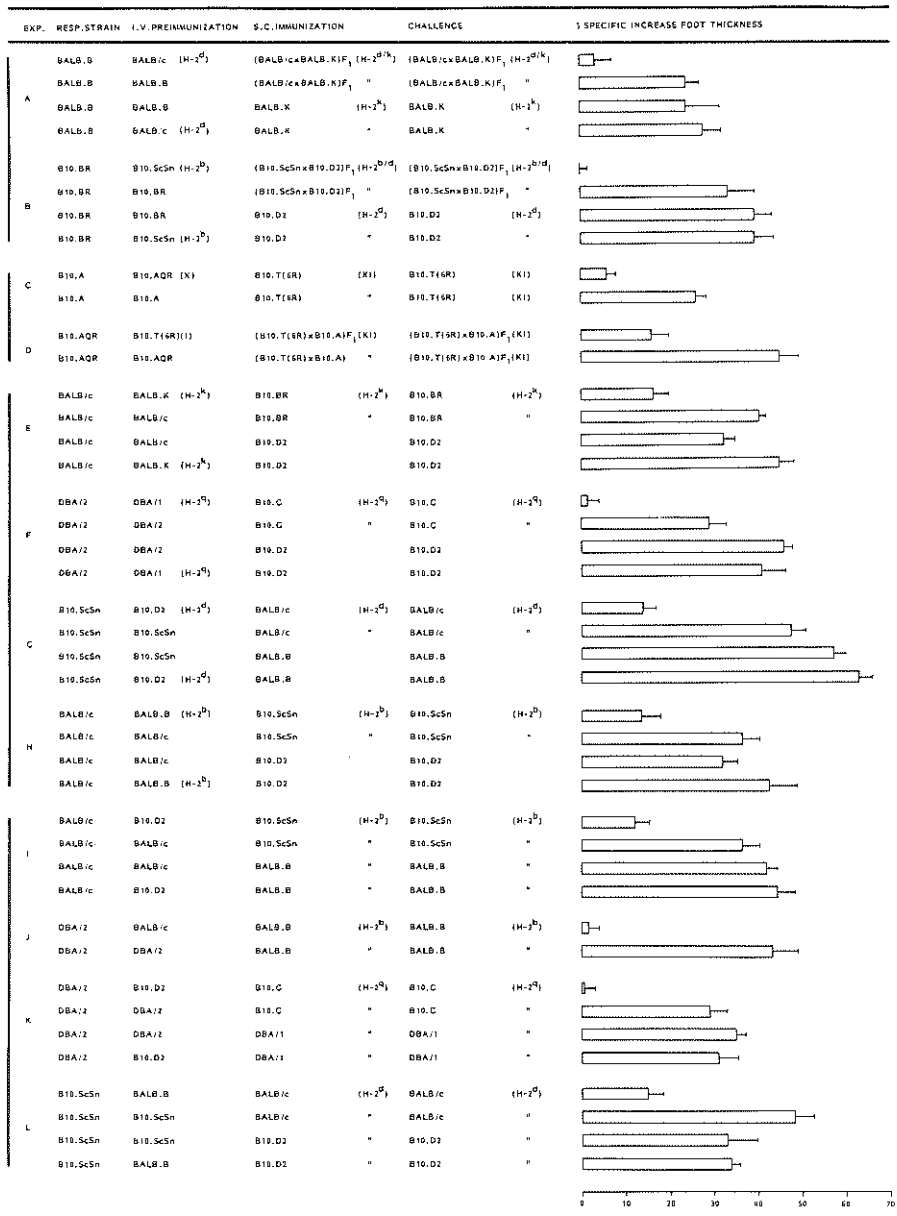

Fig. 2. Specificity of the suppressive effect upon DTH to alloantigens by alloantigen-specific suppressor T cells. Responder mice were iv injected with 5 x 10^7 irradiated allogeneic or syngeneic spleen cells and sc immunized with 1 x 10^7 allogeneic spleen cells 7 days later.

Challenge for DTH was performed on day 6 after sc immunization. Each column respresents the mean response ± SE (n=6). In all experiments significant suppression of DTH reactivity ($p < 0.01$) against third party antigens was found, when the third party antigens were presented as 'bystander' antigens in combination with the antigens used for the iv induction of antigen specific suppressor T cells, the 'specific' antigens. The suppressed combinations are shown in the first line of each experiment. The nonsuppressed DTH response against the combination of 'specific' and third party antigens is shown in the second line. The other histograms of each experiment represent a positive control and a specificity control. The H-2 haplotypes are shown in parentheses.

syngeneic BALB.B spleen cells. Seven days later the mice were sc immunized with H-2 incompatible (BALB/c x BALB.K)F1 ($H\text{-}2^{d/k}$) spleen cells or a mixture of BALB/c ($H\text{-}2^{d}$) and BALB.K ($H\text{-}2^{k}$) spleen cells. Another six days later all mice were challenged with BALB.K spleen cells (Fig. 3, exp. A). Similar experiments were done in combinations incompatible for the whole H-2 complex (Fig. 3, exp. B), or H-2K and H-2I (Fig. 3, exp. C and D), or H-2 and non-H-2 alloantigens (Fig. 3, exp. E and F). The results from all these experiments show that simultaneous presentation of the alloantigens used for iv preimmunization and the 'bystander' alloantigens, during the induction of DTH only, is sufficient to suppress the DTH reactivity to the 'bystander' alloantigens. Suppression was also found when the 'specific' and third party 'bystander' alloantigens were presented on separate cells, provided that the mix of both cell types was administered to the same site (Fig. 3). However, when the specific and third party 'bystander' alloantigens were administered to separate sites during the sc immunization, no suppression of DTH reactivity against the third party alloantigens was found (fig. 4, exp. A-C).

Suppression by partially overlapping sets of non-H-2 alloantigens

Finally we investigated whether the overlap between the non-H-2 coded alloantigens of two H-2 compatible, non-H-2 incompatible donor strains can be sufficient to activate enough iv induced antigen-specific suppressor T cells to suppress the DTH reactivity against the incompatible non-H-2 alloantigens. Thus, BALB/c ($H\text{-}2^{d}$) mice were iv injected with irradiated allogeneic B10.D2 ($H\text{-}2^{d}$) spleen cells, DBA/2 ($H\text{-}2^{d}$) spleen cells, or syngeneic BALB/c ($H\text{-}2^{d}$) spleen cells. Seven days later all mice were sc immunized with B10.D2 spleen cells. Another six days later they were tested for DTH by challenge with similar spleen cells (Fig. 5, exp. A). Similar experiments were done in three other combinations (Fig. 5, exp. B-D). The results of these experiments show that a partial overlap between the sets of non-H-2 alloantigens of the two donor strains used for iv and

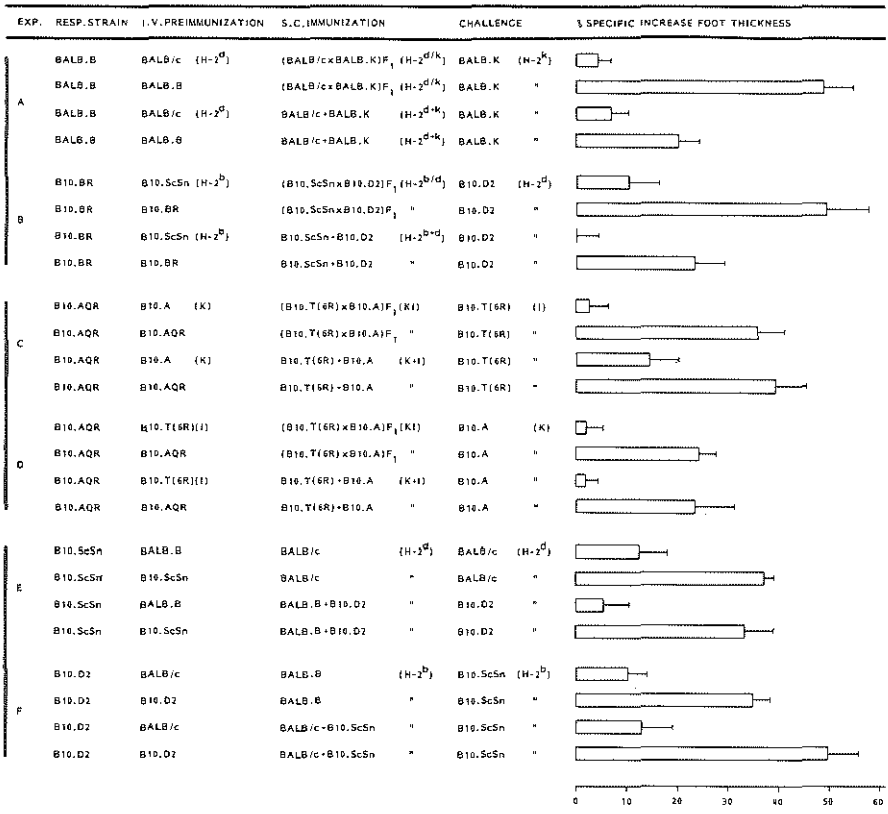

Fig. 3. Effect of the mode of presentation of 'specific' and third party 'bystander' alloantigens upon the suppression of DTH to alloantigens. Responder mice were iv injected with 5×10^7 irradiated allogeneic or syngeneic spleen cells and sc immunized with 1×10^7 allogeneic spleen cells 7 days later. Challenge for DTH was performed on day 6 after sc immunization. Each column represents the mean response ± SE (n=6). In all the experiments significant suppression of DTH reactivity ($p < 0.01$) against third party 'bystander' antigens was found, when the 'specific' and third party 'bystander' antigens were presented only during the sc induction of the DTH reactivity (line 1). Significant suppression ($p < 0.01$) was also found when both types of antigens were presented on separate cells, but simultaneously administered in a mix at the same site (line 3). The histograms on lines 2 and 4 represent the positive controls. The H-2 haplotypes are shown in parentheses.

sc immunization, respectively, is sufficient to obtain suppression of the same magnitude as in the case of using the same allogeneic donor strain for iv and sc immunization.

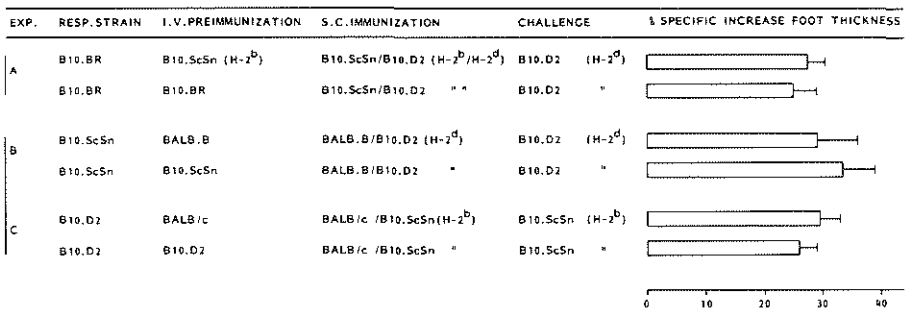

Fig. 4. Effect of administration of the 'specific' and the third party alloantigens to separate sites upon the induction of DTH against the third party alloantigens. Responder mice were iv injected with 5 x 10^7 irradiated allogeneic of syngeneic spleen cells. Seven days later all mice were sc immunized in the brachial area with 1 x 10^7 spleen cells presenting the 'specific' alloantigens. Simultaneously, they were sc immunized in the inguinal area with 1 x 10^7 spleen cells presenting the third party alloantigens. Challenge for DTH was performed on day 6 after sc immunization. Each column represents the mean response ± SE (n=6). In all experiments no significant difference was found in DTH reactivity between the responder mice, which were iv preimmunized with irradiated allogeneic (the 'specific' antigens) or with irradiated syngeneic cells. The H-2 haplotypes are shown in parentheses.

DISCUSSION

The data presented in this paper demonstrate that suppression of DTH to alloantigens can be induced not only by H-2 and non-H-2 coded antigens, but also by the H-2 subregion coded antigens K, I and D (Fig. 1). The suppression for a particular H-2 subregion is specific for that subregion as well as for its haplotype (Fig. 1). To express their suppressive effect, the suppressor T cells involved must be restimulated with the original antigen. Quite similar results have been obtained by others in the suppression of mixed lymphocyte reactions (MLR) (17, 18). They showed that suppression of MLR can be mediated by K, I or D specific suppressor T cells (18, 19).

Our results do not confirm the observation of Liew (7) that suppression of DTH to H-2 subregion coded antigens only occurs if an I-J incompatibility is included. The requirement of an I-J allogeneic effect has also been described in suppression of contact sensitivity when suboptimal conditions are choosen for suppression, while under optimal conditions no I-J allogeneic effect was found to be needed (20). The biochemical nature of the I-J antigens, however, is still uncertain. Studies in which monoclonal antibodies were used have shown that I-J is probably heterogeneous (21). Moreover, molecular and genetic studies

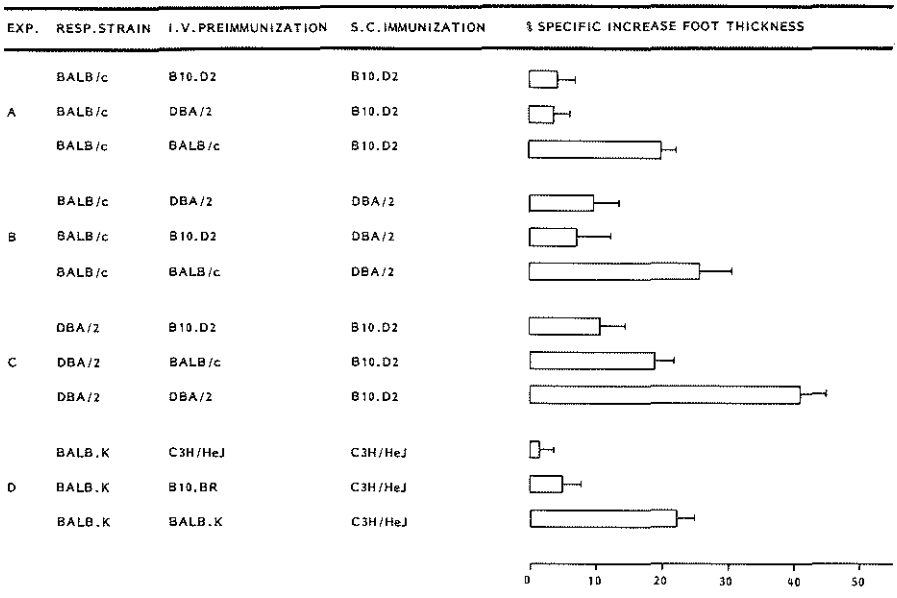

Fig. 5. Supression of DTH to non-H-2 alloantigens by partially overlapping sets of non-H-2 alloantigens. Responder mice were iv injected with 5×10^7 irradiated allogeneic or syngeneic spleen cells and sc immunized with 1×10^7 allogeneic spleen cells 7 days later. Challenge for DTH was performed on day 6 after sc immunization. Each column represents the mean response ± SE (n=6). In all experiments significant suppression of DTH reactivity ($p < 0.01$) was found after iv preimmunization with non-H-2 coded alloantigens, independent of whether the non-H-2 alloantigens used for iv suppression, for the sc induction of DTH reactivity, and for the challenge were derived from the same strain (line 1) or from different H-2 compatible mouse strains (line 2). Line 3 represents the positive control.

could not demonstrate the existence of an I-J locus between I-A and I-E (22).

In contrast to our experimental set up, Liew (7) made use of cyclophosphamide (CY) treated responder mice. Earlier investigations dealing with suppression of DTH to heterologous eryhrocytes have shown that the Lyt phenotype of the suppressor T cell that can transfer suppression depends on whether or not the responding mice are treated with CY. When CY was used, the suppressor T cells appeared to be Lyt-1^+2^- (23), while in the absence of CY Lyt-1^-2^+ suppressor T cells were found (24). Transfer experiments with suppressor T cells induced according to our protocol have shown that the suppression can be abolished by treatment with anti Lyt-1 as well as with anti Lyt-2 and complement (unpublished data). We are presently investigating whether the suppression in our system is

determined by one type of suppressor T cells or by several subsets of phenotypically different suppressor T cells. Taken together the above observations suggest that in the experiments described by Liew (7) and us different suppressor T cell systems or different parts of a more complicated suppressor T cell circuit are involved.

The most important aspect of the present studies is the finding that after restimulation of suppressor T cells by the 'specific' antigens, the induction of the *in vivo* immune response to simultaneously administered third party antigens is also significantly suppressed. In some experiments a residual response was found (Fig. 2). This response, however, was always less than half the value of the response against the third party and the 'specific' antigens together. The residual response cannot be explained by supposing that it represents the response against the third party antigens, since the DTH response against the third party antigens only was of the same magnitude as the DTH response against the third party antigens and the 'specific' antigens together (Fig. 2).

Most studies concerning the specificity of suppression of contact sensitivity (CS) and DTH reactivity against haptens deal with suppression during the effector phase of the response (3-5, 25, 26). By *in vivo* proliferation studies (9) and adoptive transfer experiments (unpublished data) we have shown that suppressor T cells induced by iv preimmunization with alloantigens suppress the induction as well as the expression phase of the DTH reaction. The present study shows that the simultaneous presentation of the 'specific' and third party antigens during the sc induction of DTH only is sufficient for suppression of the response to the latter.

In MLR it has been shown that antigen-specific suppressor T cells induced by H-2 subregion or non-H-2 coded antigens could suppress the response to third party alloantigens when the suppressor T cells were reactivated by the 'specific' antigens (27, 28). Similar results have been obtained in experiments on the suppression of *in vitro* antibody formation (29, 30). Nonspecific suppression of the *in vivo* immune response against 'bystander' antigens by antigen-specific suppressor T cells has been described for the effector phase of CS to picrylchloride and oxazolone (25. 26) and for DTH to protein antigens (31, 32) and H-2 subregion coded antigens (7). In the latter studies it was shown that suppression of DTH against the 'bystander' antigens only occurs when the 'specific' and 'bystander' antigens are physically associated.

In the suppression of CS to 4-hydroxy-3-nitrophenol no suppression of CS to 'bystander' antigens has been found, even not in animals that had been primed as well as challenged with both the 'specific' and 'bystander' antigens (33). However, this lack of nonspecific suppression might be due to the separate administration of both antigens. The experiments presented in this paper reveal that suppression

of DTH to third party alloantigens also occurs when the 'specific' and the 'bystander' antigens are present on separate cells, provided that both cell types are administered together at the same site (Fig. 3). When both cell types were injected at separate sites (e.g., the 'specific' antigens in the brachial area and the third party antigens in the inguinal area), no suppression of DTH to the third party antigens was found (Fig. 4). This suggests that the mediators accounting for the nonspecific suppressive effects are only effective in the close proximity of the site of production.

The nonspecific suppression of DTH to 'bystander' alloantigens by specifically reactivated suppressor T cells and the observation that the 'specific' and 'bystander' antigens do not need to be physically associated explain why allogeneic cells from different H-2 compatible mouse strains can induce suppression of DTH against each other (Fig. 5). As there is always an overlap between the non-H-2 alloantigens of two given H-2 compatible mouse strains (34), the shared antigens are able to reactivate a part of the iv induced antigen-specific suppressor T cells. The nonspecific suppressive effect mediated by these activated suppressor T cells apparently is sufficient to suppress the DTH reactivity against the non-shared antigens as well.

The nonspecific T cell dependent suppression of DTH against third party alloantigens can be explained by the recent data of Fresno *et al.* (35-37). They have isolated an antigen-specific suppressor factor from continuously growing T cell clones. This factor is a protein which, after interaction with the specific antigen, breaks down in two peptides of 45,000 and 24,000 MW. The former subunit suppresses antigenspecific and other Lyt-1^+ T cells. The data of Fresno *et al.*, combined with the present data about nonspecific suppression of the *in vivo* immune response to 'bystander' alloantigens, even if they are not physically associated, may well explain the beneficial blood transfusion effect upon kidney transplant survival (38). Sharing histocompatiblity antigens by the transfused blood cells and the transplanted kidney and/or the passenger blood cells might reactivate antigen-specific suppressor T cells after transplantation, which might suppress the anti-graft reactions in a nonspecific way.

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CHAPTER XII

GENERAL DISCUSSION

Several T cell subpopulations have been described with different functions within the immune system, for instance helper T cells (Th), amplifier T cells (Ta), DTH reactive T cells (Tdth), cytotoxic T cells (Tc) and suppressor T cells (Ts). The Lyt cell surface markers make it possible to discriminate between some of these subpopulations.

Th cells and most of the Tdth cells are Lyt-1^+2^-, whereas Tc and Ts cells are Lyt-1^-2^+ or Lyt-1^+2^+ (1). Recently a monoclonal antibody against the murine T4 cell surface marker has been described (2). The T4 marker discriminates better for Th cells and Tdth cells than the Lyt 1 marker.

The relationship between Th cells and T cells that mediate DTH has been studied by several investigators (3-5). They were not able to prove that B cell help and DTH reactivity can be mediated by one and the same T cell. Using Lyt-1^+2^- *cloned* Th cells we were able to demonstrate that Th cells can mediate DTH responses (chapter III). The clones used for these studies have been shown to release several biologically active mediators, the induction of which is strictly antigen-specific and H-2IA restricted (6). Weiss and Dennert (7), also demonstrated that uncloned and cloned alloantigen specific Lyt-1^+2^- T cell lines are able to elicit antigen specific DTH responses. Lin and Askonas (8) showed that an Lyt-1^-2^+ influenza A specific cytotoxic T cell clone was also able to elicit DTH responses after combination with the specific antigen. This is not amazing since some studies have shown that Lyt-1^-2^+ T cells may also mediate DTH (9).

The question is whether Th cells are more effective in eliciting DTH responses than Tc cells. When we compare the reactivity in DTH of the cloned Th cells used in our experiments with the reactivity of the cloned T cell line used by Weiss and Dennert (7) or with the reactivity of the Tc cells used by Lin and Askonas (8), it appears that Th cells are much more effective. Further research is needed to reveal whether this difference is due to the different culture conditions, or reflects an intrinsic difference between both T cell subpopulations.

Priming of mice with SRBC or minor H-antigens not only induces DTH reactive effector T cells but also long-lived memory T cells (10). These memory T cells are insensitive to treatment with antimitotic drugs and have to be reactivated before secondary type DTH responses can be elicited (10). Studies by others (11, 12) investigating as to whether long-lived memory cells recirculate

or mainly belong to a resident population suggest that memory T cells can have either nature dependent on the type of cellular immune response studied.

We investigated by means of thoracic duct cannulation whether the memory T cells involved in secondary DTH against SRBC and minor H-antigens recirculate (chapter IV). It was shown that thoracic duct lymphocytes (TDL) from mice which were immunized with SRBC or minor H-antigens several weeks previously, could adoptively transfer the ability of secondary DTH to these antigens. By passing such immune TDL through intermediate recipients we demonstrated that these SRBC or minor H-antigen specific memory T cells recirculate from blood to lymph. Thus, these memory T cells have the same characteristics as memory cells involved in secondary humoral immune responses (13-15).

While secondary DTH to SRBC and minor H-antigens can be readily induced, secondary DTH to fully H-2 incompatible allogeneic cells does not occur (chapter V, and ref. 10). Even adoptive transfer of TDL from mice immunized with H-2 incompatible spleen cells to irradiated recipients did not reveal a memory effect (chapter IV). This is in contrast with the results of Hall *et al.* (12). They have shown that in rats a dramatic and specific increase of allograft reactivity can be demonstrated after immunization with MHC coded antigens, provided an adoptive transfer system is used.

Subsequently we investigated whether class I and class II alloantigens separately are similarly incapable of eliciting secondary DTH (chapter V). It appeared that class I alloantigens, just like SRBC and minor H antigens, are able to induce secondary type DTH, whereas class II alloantigens and combinations of class II and class I alloantigens do not induce secondary type DTH responsiveness. This lack of secondary DTH against a set of H-antigens that includes class II alloantigens cannot be explained by the suppressive effect of enhancing antibodies (16) or by the lack of appropriate restriction molecules (chapter VI). The experiments show that priming with class II alloantigens has a dominant effect, which prevents secondary DTH reactivity to class I alloantigens. The final proof for the existence of a dominant suppressive mechanism can be obtained in transfer experiments. Therefore one should investigate whether cells or factors isolated from mice which are primed, or primed and boosted, with a set of alloantigens that include class II alloantigens, can suppress a secondary type anti-class I DTH response in mice that received multiple immunization with class I alloantigens only.

It is generally accepted that recognition of SRBC (chapter III) and minor H-antigens (17, 18) by DTH effector T cells is restricted by H-2 coded molecules. However, there is no general agreement about the existence of restricted recognition of H-2 coded alloantigens (17, 18). Smith and Miller (18) and Weiss and Dennert (7) have shown that *in vivo* activated H-2 specific DTH effector T cells and IA

specific T cell lines can express DTH reactivity after transfer to H-2 incompatible naive recipients. They concluded that H-2 specific DTH effector T cells are unrestricted. We have shown, on the other hand, that the anti-class I DTH response is restricted by class II molecules, whereas the anti-class II DTH response is restricted by class I molecules (chapter VI). Moreover, to our opinion the results of Smith and Miller and Weiss and Dennert do not exclude that their responses were actually H-2 restricted. To explain this we hypothesized the following (fig. 1). During the induction of DTH effector T cells to H-2 subregion coded alloantigens, these antigens are recognized by the reactive T cells in the context of the other H-2 molecules on the surface of the same allogeneic cells or, after processing by antigen-presenting cells (APC) in the context of the H-2 molecules of the APC. When the alloantigens are presented on cells from which the other H-2 molecules are syngeneic with the responder mouse, only synrestricted DTH effector T cells will be induced. On the other hand, when the particular H-2 alloantigens are presented on an allogeneic H-2 background, the DTH effector T cells induced by direct interaction with the allogeneic cells will be allorestricted, while the DTH effector T cells induced after alloantigen processing and presentation by APC will be synrestricted.

Van der Kwast (17) has shown in studies concerning H-2 restricted recognition of minor H-antigens in DTH, that macrophage processing of H-antigens is only important during induction of DTH and not during the expression phase. Therefore DTH effector T cells should recognize H-2 alloantigens on an allogeneic H-2 background only when the specific H-2 alloantigens are presented in

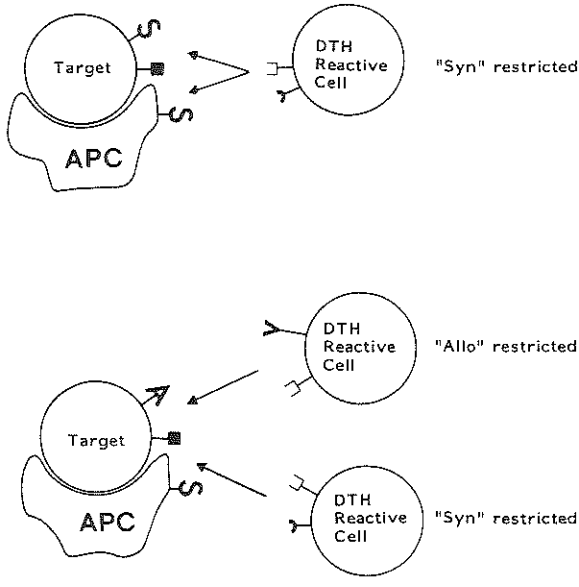

Fig. 1. Model for syngeneically and allogeneically H-2 restricted recognition of H-2 subregion coded alloantigens.

combination with the allogeneic H-2 restriction molecules during the activation of the DTH reactive T cells.

The studies which are discussed above (chapters IV – VI) show that the sc route is an adequate way to induce DTH against H-antigens. The effect of iv administration of different doses of allogeneic spleen cells has also been investigated. It appeared that iv immunization with allogeneic spleen cells can induce a state of DTH reactivity as well as a state of suppression (chapter VIII). The state of suppression due to the iv immunization becomes manifest after secondary sc immunization only and is not due to a shift in the moment of peak responsiveness (chapter VII). Two sc injections of the same antigen, on the other hand, do not lead to a lower DTH response than a single sc immunization. On the contrary, we have shown in situations where minor H- or class I coded H-antigens are used for primary and secondary sc immunization, that accelerated DTH responses evolve (chapter V). Thus the iv route of preimmunization seems to be obligatory for the induction of suppression of DTH against H-antigens. It was found that this state of suppression depends on antigen-specific Ts cells (chapters VII and VIII). From studies of DTH against heterologous erythrocytes (20, 21) and haptens (22 – 24), it is known that iv administration of high doses of such antigens also induce a state of dominant suppression which depends on Ts cells.

Suppression of DTH against H-antigens can be induced by iv injection of irradiated allogeneic spleen cells as well as by iv injection of non-irradiated allogeneic spleen cells. To avoid allogeneic effects and persistence of the injected allogeneic cells, we used irradiated allogeneic cells for the induction of suppression, usually.

The suppressive effect appeared to be long-lasting (chapter VII). Transfer of the suppressed state with T cells from iv immunized donor mice to naive recipients was, on the other hand, only effective during the first few days after the iv suppressive injection (chapter VIII). The systemic transfer of Ts cells mainly affected the afferent limb (induction) of DTH (chapter VIII). Transfer of suppression of the efferent phase of DTH could be demonstrated only when the Ts cells were mixed with DTH effector T cells *in vitro* and the subsequent DTH response was quantitated in the immune lymphocyte transfer assay (chapter IX).

The precursors of the Ts cells belong to the pool of recirculating T lymphocytes, which are insensitive to ATx and can be depleted by ATS treatment *in vivo* (chapter VIII). In contrast to other examples of DTH suppressor systems (25, 26), the presence of the spleen was not needed for induction of the antigen-specific Ts cells (chapter VIII).

Analyses of the Lyt-1 and Lyt-2 markers on the cell surface of the Ts cells, showed that for suppression of the afferent as well as the efferent phase of

DTH to alloantigens Lyt-1^+2^+ Ts cells are essential. Other authors described hapten specific Ts cells which affect either the afferent (27) or the efferent limb (22, 28) of DTH. Furthermore, cascades of different Ts cell subsets have been described, which affect both limbs of the DTH response (29). The Ts subsets which are involved in suppressor cell cascades could be distinguished from each other by different Lyt phenotypes and by different H-2 and Igh restriction requirements (29, 30). Our experiments could not demonstrate any need for restricted recognition of antigen and DTH reactive T cells by the afferent and efferent phase Ts cells (chapter IX). Thus until now there are no arguments to suppose that there is more than one type of Ts cell essential for both limbs of the DTH response against H-antigens.

The alloantigen specific Ts cells can be induced not only by H-2 and non H-2 coded alloantigens but also by H-2 subregion coded alloantigens (chapters VII, X & XI). Our studies do not confirm the observation of Liew (31) that suppression of DTH against H-2 subregion antigens only occurs when an I-J incompatibility is involved. An important aspect of our studies is the finding that after restimulation of the Ts cells by the specific antigens, the induction of the *in vivo* immune response to simultaneously administered third-party alloantigens is also significantly suppressed (chapters IX – XI). This suppressive effect on DTH to third party alloantigens is called 'bystander suppression'. Simultaneous presentation of the specific- and the third party-alloantigens either during the sc induction of DTH or during the challenge to elicit the DTH reaction, is sufficient for bystander suppression. This reinforces the conclusion from our transfer experiments (chapters VIII & IX) that the iv induced Ts cells can affect either limb of the alloantigen specific DTH response.

Bystander suppression also occurs when the specific and the third party-alloantigens occur on separate cells, provided that both cell types are administered together at the same site (chapters IX – XI). A summary of our data on suppression of DTH to specific and third party-alloantigens is given in table 1.

The next step in future research of alloantigen specific suppression should be the study of the nature of the putative suppressor factor(s). We have shown already that the ultimate suppressive effect by the Lyt-1^+2^+ Ts cells is non-specific (chapters IX – XI). From other studies (32, 33) it is known that most antigen specific Ts cells release antigen specific suppressor factors. These factors have to interact with the specific antigen (32) or with the specific antigen and another T cell (33) before a nonspecific suppressor factor is released. Most of the studies which describe the nature and working mechanism of suppressor factors are dealing with well-defined antigens like haptens (24, 29, 30, 33). In our studies (chapters VIII & IX) we are dealing with several different alloantigens. These alloantigens may have different requirements for the optimal induction of

Table 1. *Suppression of specific- and thirdparty alloantigens.* *

i.v. preimmunuzation	s.c. immunization	challenge for DTH	suppression of DTH response
A	A	A	yes
A	B	B	no
A	AB	AB	yes
A	AB	B	yes
A	A+B	B	yes
A	A/B	B	no
A	B	AB	yes
A	B	A+B	yes

* Summary of data presented in chapters IX - XI.

A and B indicate groups of non-overlapping alloantigens; A represents the 'specific alloantigens' and B the 'third party alloantigens'; AB indicates that both sets of alloantigens are expressed together on the same allogeneic cells; A+B indicates that the alloantigens A and B are presented together in a mixture of cells expressing either A or B; A/B indicates that the alloantigens A and B are presented simultaneously at separate sites.

the appropriate Ts cells. Isolation and screening of suppressor factors with a defined antigen specificity is more difficult when a large number of different alloantigens is involved. The use of strain combinations with smaller and more defined alloantigenic differences may facilitate the study of the suppressor factors involved in suppression of DTH to alloantigens. This indicates the use of recombinant mouse strains (chapter X – XI) or the C57BL/6 mutant strains (34). The C57BL/6 mutants have small mutations in the H-2 K or I region, which result in biochemically and serologically well-defined structural alterations in the K or I molecules. Preliminary experiments have shown that DTH responses and suppression of DTH can be achieved in combinations of C57BL/6 mice and C57BL/6 mutant mice with a mutation in the K or I region.

The data of nonspecific suppressor factors (32, 33) in combination with our own data of bystander suppression of DTH to H-antigens may offer an explanation for the above described (viz. 1.5) beneficial blood transfusion effect upon kidney transplant survival (35).

The blood transfusion donor and the kidney donor have both a unique pattern of compatible and incompatible H-antigens with the kidney transplant patient. In an outbred population, like the human population, there will be always an overlap between the H-antigens of the donor of the transfused blood and the kidney donor (fig. 2). After kidney transplantation, these shared alloantigens

might reactivate specific Ts cells, which are induced by the blood transfusion. Subsequently, these reactivated Ts cells might suppress the kidney graft rejection in a nonspecific way.

TRANSPLANT RECIPIENT | BLOOD DONOR | KIDNEY DONOR

Fig. 2. Illustration of the putative overlap between the H-antigens of the transplant recipient, the blood-donor and the kidney-donor. A-F refer to different H-antigens. An overlap between the H-antigens of the blood-donor and the kidney-donor might cause the beneficial effect of blood transfusion on kidney transplant rejection (viz. 1.5).

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SUMMARY

Cellular immunity *in vivo* was studied in mice by means of the delayed type hypersensitivity (DTH) assay with emphasis on the response to histocompatibility (H) antigens. The first part of the experimental work of this thesis (chapters III – VI) deals with the cellular and genetic requirements for induction and expression of DTH, while the second part (chapters VII – XI) deals with the requirements for suppression of DTH.

In most instances, T cells mediating DTH responses share the Lyt phenotype and H-2 restriction requirements with helper T cells. This raises the question as to whether helper activity and DTH can be mediated by the same activated T cells. In chapter III this was investigated by making use of clones of SRBC specific Th cells. DTH responses could be elicited after local transfer of cloned Th cells together with the specific antigen in the dorsum of a hind foot of syngeneic recipient mice. The C57BL/6J-derived cloned Th cells mediated DTH responses only in mice which were H-2 K and I-A compatible with C57BL/6J mice. With the available mouse strains we could not distinguish for K or I-A restriction. Transfer of cloned Th cells to T cell deficient syngeneic nu/nu mice showed that host T cells were not essential for the DTH response. As few as 1000 cloned Th cells injected together with the specific antigen were able to elicit a specific DTH response. In comparison with *in vivo* activated lymph node cells, the cloned Th cells were far more effective on a per cell basis. Thus, helper activity and DTH can be mediated by the same T cells.

Secondary type DTH responses can be induced against SRBC and minor H-antigens. We investigated whether memory T cells involved in secondary DTH against SRBC and minor H-antigens recirculate. This was done by thoracic duct cannulation (chapter IV). It was found that thoracic duct lymphocytes (TDL) from mice which had been immunized with the specific antigens several weeks previously could adoptively transfer the capacity of secondary DTH to the specific antigens. By passing these immune TDL through an intermediate recipient it was demonstrated that the memory T cells involved recirculate from blood to lymph.

Priming and booster immunization with fully H-2 incompatible cells did not induce secondary type DTH. We investigated whether class I and class II alloantigens separately are also incapable of eliciting secondary DTH (chapter V). It appeared that class I coded alloantigens (H-2 K and D), just like SRBC and minor H-antigens, could induce secondary type DTH, whereas class II coded alloantigens (H-2I) and combinations of class II and class I alloantigens failed to induce secondary type DTH. From these experiments we concluded

that priming with class II alloantigens has a dominant effect which prevents secondary DTH to class I alloantigens.

The question as to whether the elicitation of DTH reactivity against class I or class II coded alloantigens is restricted by other H-2 molecules, has been investigated in chapter VI. These experiments provided evidence that DTH reactive T cells which are activated against class I alloantigens, need to recognize these antigens in the context of syngeneic class II molecules to elicit a DTH response, while T lymphocytes activated against class II alloantigens need to recognize these antigens in the context of syngeneic class I molecules. Thus, with regard to the requirement of MHC restriction, DTH against class I alloantigens and class II alloantigens does not differ essentially from DTH against minor H-antigens and conventional antigens like heterologous erythrocytes.

In chapters IV – VI it is shown that subcutaneous (sc) immunization is appropriate for inducing DTH against H-antigens. Subcutaneous preimmunization does even induce a state of secondary type DTH reactivity when minor H-antigens or class I coded alloantigens are administered. Intravenous (iv) preimmunization, on the other hand, induces a state of suppression which becomes manifest after a subsequent secondary sc immunization for the induction of a DTH response (chapter VII).

The iv route was found to be obligatory for the suppressive effect and the extent of suppression appeared to depend on the dose of iv injected irradiated allogeneic spleen cells (chapter VII). It has been shown that the intravenous injection of H-antigens induces Ts cells by which the suppressive effect could be transferred to naive recipient mice (chapters VIII & IX).

Although the iv induced suppressive effect persisted in the suppressed mice for at least 70 days, systemic transfer of Ts cells was effective during the first few days after their induction only. The systemic transfer mainly affected the afferent limb of DTH. Efferent phase Ts cells could be demonstrated only by cotransfer of lymphoid cells from iv suppressed mice and DTH effector T cells in the immune lymphocyte transfer assay (chapters VIII & IX).

For suppression of the afferent as well as the efferent phase of alloantigen specific DTH Lyt-1^+2^+ Ts cells appeared to be essential. We did not find evidence for a H-2 or Igh restricted activation and function of the Ts cells active in both limbs of alloantigen specific DTH. Therefore we concluded that there are no arguments as yet to suppose that there is more than one type of Ts cells involved in the suppression of the afferent and efferent limb of DTH against H-antigens (chapters VIII & IX).

Specificity studies showed that the activation of the Ts cells by alloantigens is antigen-specific (chapters VII, X & XI). However, after restimulation of the Ts

cells by the specific antigens, the induction of the *in vivo* immune response to simultaneously administered third-party alloantigens was also significantly suppressed. This nonspecific suppression of DTH to third party 'bystander' alloantigens also occurred when the specific and the third-party alloantigens were presented on separate cells, provided that both cell types were administered together at the same site. Simultaneous presentation of both sets of alloantigens either during the sc induction of DTH or in the challenge to elicit the DTH reaction, was also sufficient for bystander suppression (chapters IX – XI).

For a detailed insight into the nature of the bystander suppressive effect, future research should be focussed on the isolation and characterization of the suppressor factor(s) which are released after stimulation of Ts cells with the specific antigen.

SAMENVATTING

Cellulaire immuniteit *in vivo* werd bestudeerd bij de muis met behulp van de vertraagd type overgevoeligheidsreaktie (DTH), waarbij de nadruk lag op het onderzoek naar de reaktie tegen transplantatie of histocompatibiliteits (H) antigenen.

Het eerste deel van het onderzoek, dat in dit proefschrift is beschreven (hoofdstuk III – VI), heeft betrekking op de cellulaire en genetische voorwaarden voor het optreden van een DTH reaktie, terwijl het tweede gedeelte (hoofdstuk VII – XI) betrekking heeft op de voorwaarden voor het onderdrukken van een DTH reaktie.

Meestal hebben de T lymphocyten die een DTH reaktie veroorzaken het zelfde Lyt fenotype en dezelfde voorwaarden voor H-2 restrictie als helper T (Th) cellen. Dit roept de vraag op of T cellen die in staat zijn om helper activiteit te geven ook in staat zijn tot een DTH reaktie. In hoofdstuk III werd dit nader bestudeerd, waarbij gebruik gemaakt is van klonen van Th cellen die specifiek waren voor het antigeen SRBC.

DTH reakties konden worden opgeroepen door lokale injektie van gekloneerde Th cellen, samen met het specifieke antigeen, in een achterpoot van syngene recipient muizen. De van C57BL/6J muizen afkomstige gekloneerde Th cellen gaven alleen DTH reakties in muizen die H-2 K en I-A compatibel waren met C57BL/6J muizen. Met de geteste muizenstammen kon geen onderscheid worden gemaakt tussen K of I-A restrictie. Transfer van gekloneerde Th cellen naar T cel deficiënte nu/nu muizen toonde aan dat T cellen van de gastheer niet noodzakelijk waren voor de reaktie. Reeds 1000 gekloneerde Th cellen, welke samen met het specifieke antigeen werden ingespoten, waren voldoende om een DTH reaktie op te roepen. Beoordeeld op het effekt per cel, bleken gekloneerde Th cellen veel effektiever dan *in vivo* geaktiveerde lymfkliercellen. De konklusie uit deze experimenten is, dat dezelfde T cellen die in staat zijn om helper aktiviteit te geven ook een DTH reaktie kunnen opwekken.

Sekundair type DTH reakties kunnen worden geinduceerd tegen SRBC en minor H-antigenen. Wij onderzochten of memory T cellen, die betrokken zijn bij de sekundaire DTH reaktie tegen SRBC of minor H-antigenen, recirculeren. Dit werd onderzocht met behulp van *ductus thoracicus* cannulatie (hoofdstuk IV). Gevonden werd dat *ductus thoracicus* lymfocyten (TDL), afkomstig van muizen die enige weken eerder waren geimmuniseerd, in staat waren om het vermogen tot sekundaire DTH reaktiviteit voor het specifieke antigeen over te dragen aan naïve muizen. Door deze immune TDL iv toe te dienen aan een intermediaire recipient en vervolgens te isoleren uit de *ductus thoracicus*, kon worden aange-

toond, dat de betrokken T memory cellen recirculeren van bloed naar lymfe.

Primaire en sekundaire immunisatie met cellen die volledig H-2 incompatibel zijn, induceert geen sekundair type DTH reaktie. We onderzochten of klasse I of klasse II alloantigenen afzonderlijk ook het vermogen missen om een sekundaire DTH reaktie op te wekken (hoofdstuk V). Het bleek dat klasse I gecodeerde alloantigenen (H-2 K en D), net als SRBC en minor-H antigenen, een sekundair type DTH reaktie konden induceren. Klasse II gecodeerde alloantigenen (H-2I) of combinaties van klasse II en klasse I alloantigenen waren hiertoe niet in staat. Uit deze experimenten konkludeerden wij dat primaire immunisatie met klasse II alloantigenen een dominant effekt heeft, dat er voor zorgt dat sekundair type DTH reakties tegen gelijktijdig aangeboden klasse I alloantigenen worden voorkomen.

De vraag of een DTH reaktie gericht tegen klasse I of klasse II alloantigenen wordt bepaald door andere H-2 moleculen, is onderzocht in hoofdstuk VI. Op grond van deze experimenten werden aanwijzingen verkregen dat DTH reaktieve T cellen, welke geaktiveerd zijn tegen klasse I alloantigenen, deze antigenen moeten herkennen in de kontekst van syngene klasse II moleculen. T lymfocyten die tegen klasse II alloantigenen zijn geaktiveerd, daarentegen, moeten deze antigenen herkennen in de kontekst van syngene klasse I moleculen. Wat betreft het optreden van MHC restrictie verschilt DTH tegen klasse I en klasse II alloantigenen dus niet wezenlijk van DTH tegen minor H-antigenen en conventionele antigenen, zoals heterologe erythrocyten.

Uit hoofdstuk IV – VI blijkt dat subcutane (sc) immunisatie een geschikte manier is om DTH tegen H-antigenen te induceren. Subcutane preimmunisatie met minor-H antigenen of klasse I alloantigenen induceert zelfs een staat van 'memory' voor sekundaire DTH reaktiviteit. Intraveneuze (iv) preimmunisatie, daarentegen, induceert een staat van suppressie welke tot uiting komt bij een daarop volgende sc immunisatie voor de induktie van DTH (hoofdstuk VII).

Voor het verkrijgen van het suppressief effekt bleek iv immunisatie een vereiste en de mate van suppressie bleek afhankelijk van de dosis iv geinjekteerde, bestraalde miltcellen (hoofdstuk VII). Door de intraveneuze injektie van H-antigenen werden Ts cellen geinduceerd, waarmee het suppressief kon worden overgedragen naar naïve muizen (hoofdstuk VIII & IX).

Hoewel in gesupprimeerde muizen het iv geinduceerde suppressieve effekt aanhield gedurende een periode van minstens 70 dagen, bleek iv toediening van Ts cellen slechts effektief gedurende de eerste paar dagen na induktie van suppressie. Iv toediening van Ts cellen beinvloedde voornamelijk de induktie van een staat van DTH. Het vóórkomen van Ts cellen die de expressie van DTH onderdrukken, kon alleen worden aangetoond door gelijktijdige iv toediening van

lymphoïde cellen van gesupprimeerde muizen en DTH effektor T cellen in de 'immune lymphocyte transfer assay' (hoofdstuk VIII & IX). Voor suppressie van zowel de afferente als de efferente fase van de alloantigeen specifieke DTH bleken Lyt-1^+2^+ Ts cellen essentieel. Wij vonden geen aanwijzing voor een H-2 of Igh 'restricted' activatie en functie van Ts cellen welke aktief zijn gedurende beide fasen van de alloantigeen specifieke DTH. Op grond van deze resultaten konkludeerden wij dat er geen argumenten zijn om te veronderstellen dat er meer dan één type Ts cel betrokken is bij de afferente en efferente fase van DTH tegen H-antigenen.

Specificiteitsstudies toonden aan dat de aktivatie van de Ts cellen door alloantigenen antigeenspecifiek is (hoofdstuk VII, X & XI). Echter, na restimulatie van de Ts cellen door de specifieke antigenen, werd de induktie van DTH tegen gelijktijdig aangeboden 'third party' alloantigenen ook onderdrukt. Deze niet-specifieke suppressie van DTH tegen 'third party bystander' alloantigenen, trad ook op wanneer de specifieke en de 'third party' alloantigenen werden aangeboden op afzonderlijke cellen. Een voorwaarde daarbij was dat beide celtypen gezamenlijk werden aangeboden op dezelfde plaats. De gezamenlijke aanbieding van *beide* sets alloantigenen, alleen gedurende de sc induktie van DTH reaktiviteit of alleen gedurende de 'challenge' om een DTH reaktie op te roepen, was ook voldoende om de DTH tegen 'bystander' alloantigenen te onderdrukken.

Voor een beter inzicht in de aard van het suppressief effekt op DTH tegen 'bystander' antigenen zou toekomstig onderzoek zich moeten richten op de isolatie en de karakterisering van suppressorfaktor(en), welke worden vrijgemaakt na stimulatie van Ts cellen met het specifieke antigeen.

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Lex Hussaarts bedank ik voor zijn ontwerp van het kaft van dit boekje.

CURRICULUM VITAE

In 1970 diploma HBS-B behaald aan het van der Waals Lyceum te Amsterdam. Studie biologie van 1970-1979 aan de Universiteit van Amsterdam. Doktoraal examen met als hoofdvak fytopathologie bij Prof. Dr. K. Verhoeff, Fytopathologisch Laboratorium te Baarn. Bijvakken: genetica bij Drs. H. Wiering, Genetisch Instituut, U.v.A. en cellulaire immunologie bij Prof. Dr. W. Leene, Laboratorium voor Celbiologie en Histologie, U.v.A. Onderwijsaantekening biologie.

Tijdens doktoraalstudie van april 1974 tot september 1979 voor de halve werktijd als assistent verbonden aan het Genetisch Instituut, U.v.A.

Van oktober 1979 tot april 1983 werkzaam op het Laboratorium voor Celbiologie en Genetica van de Erasmus Universiteit te Rotterdam, waar het in dit proefschrift beschreven onderzoek werd verricht onder leiding van Prof. Dr. R. Benner.

Vanaf 1983 verbonden aan de afdeling Immunologie van het Centraal Diergeneeskundig Instituut te Lelystad.