CELL-MEDIATED IMMUNITY TO HISTOCOMPATIBILITY ANTIGENS

Controlling factors, with emphasis on Graft-versus-Host reactions in mice

CELLULAIRE IMMUNITEIT TEGEN WEEFSELANTIGENEN.
CONTROLERENDE FACTOREN, MET NADRUK OP
TRANSPLANTAAT-ANTI-GASTHEER REACTIES BIJ MUIZEN

PROEFSCHRIFT

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VOORWOORD

Op één van de eerste bladzijden van dit proefschrift wil ik graag enkele woorden wijden aan de voorplaat. Het schilderij dat hier is weergegeven heeft als titel 'Artistoteles peinzend bij het borstbeeld van Homerus' en is door Rembrandt in 1653 geschilderd. Naar mijn idee zijn in Rembrandt, Aristoteles en Homerus drie essentiële eigenschappen voor een wetenschapper weergegeven: Aristoteles, de filosoof, was een briljant denker; Homerus, de dichter, was een begenadigd verteller en tenslotte Rembrandt, de schilder, was een begaafd interpretator, compositeur en 'uitvoerder'.
Echter, deze kenmerken zijn nog niet genoeg om optimale prestaties te leveren: er bestaat ook nog zo iets als 'sfeer'.
De jaren waarin ik mijn onderzoek heb verricht, kunnen het beste worden gekarakteriseerd door de openingszinnen van het boek 'A tale of two cities' van Charles Dickens: 'It was the best of times, it was the worst of times, it was the age of wisdom, it was the age of foolishness, it was the epoch of believe, it was the epoch of incredulity, it was the season of light, it was the season of darkness, it was the spring of hope, it was the winter of despair.....'.

CONTENTS

APPENDIX

ABBREVIATIONS

ATS	Anti-thymocyte serum
ATx	Thymectomy of adult mice
B cell	Bone marrow-derived lymphocyte
BMT	Bone marrow transplantation
CML	Cell-mediated lympholysis
CTL	Cytolytic T lymphocyte
dATP	Deoxyadenosine triphosphate
dGTP	Deoxyguanosine triphosphate
dGuo	2'-deoxyguanosine
DTH	Delayed-type hypersensitivity
GvH	Graft-versus-Host
H-antigen	Histocompatibility antigen
H-2 complex	Major histocompatibility complex of the mouse
H-2D locus	Marker locus of the D-region of the H-2 complex
H-2I-A locus	Gene locus in the I-A subregion of the H-2 complex
H-2I-E locus	Gene locus in the I-E subregion of the H-2 complex
H-2I-J locus	Gene locus in the I-J subregion of the H-2 complex
H-2K locus	Marker locus of the K-region of the H-2 complex
HLA	Human leukocyte antigen
HvG	Host-versus-Graft
H-Y antigen	Male specific histocompatibility antigen
Ia antigen	Serologically detectable I-region coded antigen
ILT reactivity	Immune lymphocyte transfer reactivity
ITL	Initiator T lymphocytes

iv	Intravenous
LD antigen	Leukocyte-defined antigen
LNS eq.	Lymph node seeking equivalent
MHC	Major histocompatibility complex
MLC	Mixed lymphocyte culture
MLR	Mixed lymphocyte reaction
Mls-locus	Minor lymphocyte stimulating locus
NLT	Normal lymphocyte transfer
NRS	Normal rabbit serum
PLN assay	Popliteal lymph node assay
RTL	Recruited T lymphocytes
sc	Subcutaneous
SD antigen	Serologically defined antigen
SRBC	Sheep red blood cells
T cell	Thymus-derived lymphocyte
T1 cells	Sessile T lymphocytes which are short-lived after ATx
T2 cells	Recirculating, long-lived T lymphocytes which are sensitive to ATS *in vivo*
TBMC	Tetraparental bone marrow chimera
TDL	Thoracic duct lymphocytes

AIM OF THE STUDY

Vertebrates possess a surveillance mechanism, called the immune system, that protects them from diseases caused by micro-organisms, such as parasites, bacteria and viruses. Furthermore, the immune system plays a central role in the rejection of foreign organ and tissue transplants. The immune system recognizes foreign invaders specifically and eliminates them selectively. Each entity that induces an immune reaction is called an antigen. Immune responsiveness involves two major effector mechanisms: humoral antibodies and cell-mediated reactions. All the cells that are involved in immune responses are a part of the lymphohemopoietic system, and arise from pluripotent hemopoietic cells in the bone marrow. The cells that are able to recognize antigens specificlly are called lymphocytes.

Two major types of lymphocytes can be recognized: T lymphocytes, or T cells, which undergo an essential differentiation phase within the thymus and B lymphocytes, or B cells, which largely mature within the bone marrow itself. In these organs the lymphocytes acquire characteristic cell surface markers, antigen-specific cell surface receptors and immunocompetence. Furthermore, T lymphocytes learn to discriminate between the components of the own body ('self') and external antigens ('non-self'). Following this maturation stage, the T and B lymphocytes migrate to peripheral lymphoid organs, such as lymph nodes and spleen, in which they are located in specific areas.

Upon encounter with the antigen for which they are specifically programmed, the lymphocytes enlarge and become active cells: T cells become involved in cellular immune reactions (e.g., delayed-type hypersensitivity reactions of which the Mantoux-reaction is a classical example; killing of virusinfected cells; rejection of organ transplants; resistance to certain tumors), or perform regulatory functions (i.e., help or suppression of other T and/or B cells). Antigen-activated B cells, on the other hand, transform into plasma cells, which produce proteins called antibodies. These antibodies bind specifically to the antigen that induced their production and are able to initiate several effector mechanisms for eliminating that particular antigen.

In diseases such as aplastic anemia, leukemia and immunodeficiency the patients frequently suffer from the consequences of an impaired immune system (e.g., recurrent infections with bacteria, viruses, parasites, etc.). One of the therapeutic modalities for the latter patients is the donation of a new immune system by transplantation of bone marrow cells. However, one of the major problems which complicate this treatment is Graft-versus-Host (GvH) disease, which occurs when immunologically competent cells (or their precursors) are transplanted into recipients who are immunologically crippled because of their underlying disease or their (chemo)therapy. The immunocompetent T cells derived from the donor marrow transplant recognize the recipient's tissue as foreign and react against them.

Although during the last 10 years a lot of progress has been made in the prevention and treatment of GvH disease after clinical bone marrow transplantation, there is still a lack of insight into the underlying mechanisms. The present state of knowledge in this field is reviewed in the next chapter, which puts emphasis on the cellular immunological aspects and the genetic incompatibilities that are required between the donor and the recipient.

It was the aim of the studies described in this thesis to investigate the cellular and genetic requirements for GvH reactions in a preclinical model, using mice as experimental animals, and especially to find ways to minimize or prevent the anti-host immune reactivity. After having established a procedure for the induction of suppressor T cells that could inhibit the anti-host immune response during GvH reactions, we characterized these T cells and their precursors. These studies are described in the Appendix publications.

GRAFT-VERSUS-HOST DISEASE: MECHANISMS AND CONTEMPORARY THEORIES

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I INTRODUCTION

For both the basic scientist and the clinical transplanter, Graft-versus-Host (GvH) reactions are still unresolved problems. It is now firmly established that GvH reactions represent a complex series of events, apt for studying the cellular dynamics that proceed from the specific stimulation of antigen-reactive lymphocytes in vivo. Interest has been focussed on the cellular and genetic requirements that underly GvH reactions and the various clinical symptoms of GvH disease.

The first formal description of GvH disease by Billingham and others appeared almost 30 years ago (1-5). According to Billingham (6) a GvH reaction can emerge when immunocompetent lymphocytes are introduced into a host that confronts the graft with a large degree of histo-incompatibility, and by itself is unable to mount a comparable immunologic attack against the intrusive donor lymphoid cells. Early experiments identified the 'immunologically competent cell' predicted by Medawar as the cell responsible for the initiation of GvH disease (7). Gowans demonstrated that small lymphocytes play a cardinal role in evoking GvH reactions (8). Since then many data have been gathered on the pathology of GvH, the cell-types involved in the primary interaction with antigen, the antigen recognition by these cells, the ensuing differentiation events and, last but not least, the expression of immunocompetence of the host, cell-mediated as well as antibody-mediated. Most of these results were obtained from studies with experimental animals

since studies in humans are necessarily restricted due to ethical aspects and the lack of appropriate controls.

In this paper we review GvH reactions with emphasis on animal studies, in particular those ensuing from reconstitution of irradiated hosts. Furthermore, we shall try to reconcile the different models and discuss the mechanisms that may underly GvH disease. Finally, we try to evaluate the relationship between GvH reactivity and autoimmunity and between GvH and the development of lymphoma.

II. GRAFT-VERSUS-HOST REACTIONS

1. Modes of induction

Among the most widely acknowledged forms of GvH reactions are: runt disease, in which an immunologically immature animal is exposed to alloreactive lymphoid cells from an adult donor; so-called 'secondary disease' in which the recipient initially is protected from the lethal effects of ionizing irradiation, i.e. primary disease, by engraftment of hemopoietic stem cells from an allogeneic donor, but subsequently suffers from the attack by mature alloreactive donor cells; parabiosis intoxication, which can occur in case of chronic cross-circulation of peripheral blood between two immunologically competent, but histoincompatible individuals; and F1 hybrid disease, which occurs following the injection of parental strain lymphoid cells into an F1 hybrid animal derived from two histoincompatible inbred strains (9). Of these forms of GvH reactions parabiosis intoxication does not fulfill the criteria set by Billingham.

An interesting form of GvH reaction can occur when maternal lymphocytes gain access to a histo-incompatible fetus via the placenta. Beer and Billingham (10,11) have shown that transfer of lymphocytes from donor rats, sensitized to allogeneic histocompatibility antigens can cause fatal runt disease in a large proportion of the progeny, provided the female rats had been mated with male rats from the corresponding allogeneic strain. The incidence of runt disease in these experiments was greater for sensitized lymphoid

cells than for non-sensitized cells.

Maternally induced runt disease has been described not only in rats. In mice, hamsters, guinea pigs, rabbits and humans the same phenomenon has been described (11,12). In all animal species, the timing of the immunization of the prospective mothers in relation to conception was found to be important. These experiments strongly suggest that runt disease is caused by immigrant lymphocytes from the maternal circulation. In support of this concept, it has been shown by Tuffrey et al. (13,14) that, in mice, maternal-fetal leukocyte traffic occurs. CBA female mice, homozygous for the T6 chromosome marker, were mated with sterile CBA (T6T6) males. After 2.5 days, fertilized eggs from the unrelated CFW mice, which lack the T6 marker, were placed into their uterine horns. The progeny was killed 40-60 days after birth and bone marrow and lymphoid tissues were cytogenetically examined: indeed, 3-30% of alien (T6T6) labeled cells were present in these tissues.

2. Kinetics

In an arbitrary way two types of GvH reactions can be distinguished after irradiation and reconstitution, namely, the acute and the delayed type. The acute or early type GvH reaction is caused by grafts containing a high number of T lymphocytes (e.g., mouse spleen, monkey and human bone marrow; see Table I) and causes mortality of the host within about 30 days after transplantation. Diminuition of T lymphocytes from the graft prior to transplantation results in a delay or abolition of the GvH reaction.

This can be achieved by physical methods (e.g., density gradient centrifugation) or biological approaches (i.e., incubation with drugs, plant lectins, host antigens, host cells plus ^{3}H-thymidine, or anti-lymphocyte serum with or without complement (reviewed in 9 and 16)). Acute GvH reaction is correlated with a defined pathology (see Section II.3).

The delayed type GvH reaction occurs in case of transplants which contain relatively low numbers of T lymphocytes (e.g., mouse bone marrow and fetal liver cells, human purified bone marrow cells; see Table I). Some investigators claim that delayed GvH reactions can also be provoked by immunocompetent cells which are not present in the graft at the time of transplantation, but develop in the host from a subpopulation of the transplanted cells. They might descend from pluripotent hemopoietic stem cells or, alternatively, from immature cells of the T lymphocytic series (17). The terms 'delayed' and 'chronic' GvH are often used indiscriminantly. Delayed GvH may eventually result in a 100% mortality of the irradiated recipients. This mortality might be attributed to infection or to 'suppressive' pathological symptoms resulting in incomplete reconstitution (see Section IV-1 and V-5). Chronic GvH usually presents itself with features of collagen vascular disease, such as a systemic lupus erythematosus-like syndrome (see Section II.3).

TABLE I. GvH-inducing potential of cells from various lymphoid organs

	Rodents	Dog	Monkey	Human
bone marrow	low	intermediate	high	high
spleen	high	?	?	?
lymph nodes	high	high	?	?
peripheral blood	high	high	high	high
thymus	low	?	?	intermediate
fetal liver	low	?	low	low
fetal thymus	low	?	?	low

From ref. 15.

3. Pathology

The pathology of radiation chimeras has been studied in detail in mice (18-21), man (22-24), monkeys (25), dogs (26), chickens (27,28) and rats (29). These investigations revealed more or less similar data.

The effect of the conditioning regimen (total body irradiation) is profound. Clinically, this effect is characterized by skin rash, which develops after irradiation and lasts for a few days, nausea and vomiting. When no bone marrow transplantation is attempted or the bone marrow graft fails to 'take', a pancytopenia ensues and the recipient dies within 1 to 3 weeks. Among the causes of death are bacterial infections, hemorrhages and anemia. Histologically, the bone marrow is aplastic, i.e. the cellularity is less than 5%, which is 10-15 times less than in normal healthy controls. Lymph nodes are also markedly hypoplastic, containing only a few small lymphocytes. In addition, lymph node sinuses are uniformly distended by erythrophagocytic histiocytes. In the skin, round dyskeratotic cells with pyknotic nuclei can be seen, as well as nonspecific foci of acute inflammation (30).

Although some variations exist in all species studied, GvH reactions cause cutaneous, hepatic and intestinal lesions. Despite the use of sibling donors matched at the major histocompatibility complex (MHC) and despite post-grafting immunosuppression, GvH disease occurs in approximately 70 percent of the patients with succesful marrow grafts.

The initial target organ of GvH reactions in almost all cases is the skin. Skin lesions are characterized by rash, bullae and desquamation. Hepatic and intestinal involvement usually appears several days after the rash. Intestinal involvement is mainly in the form of diarrhea, nausea and vomiting, but may progress towards abdominal pain and ileus. Liver disease is manifested by rises in serum bilirubin levels (mainly conjugated). Serum glutamic oxalacetic transaminase is usually in the range of 150 to 750 IU. Fever, wasting and a decreased performance status are also regularly seen with severe involvement. Although fever may be a manifestation of GvH disease per se, it is most commonly due to an associated infection caused by bacteriae (mostly gram-negative rods), viruses (cytomegaly virus and varicella-zoster virus) or fungi (e.g., candida albicans) (31-33).

Histologically, the mildest change observed in skin is focal or diffuse vacuolar degeneration of epidermal basal cells and of acanthocytes. In severe cases, focal or diffuse spongiosis (separation and intercellular edema of basal cells and acanthocytes) and dyskeratosis or eosinophilic degeneration of epidermal cells is observed. In more severe cases, clefts and spaces (acantholysis, epidermolysis) occur after necrosis of basal cells and acanthocytes in the basal and more superficial layers, resulting in separation of the dermal-epidermal junction. In the worst cases, there is frank loss of the epidermis. Similar changes can be found in hair-follicle epithelium in synchrony with the epidermal lesions. The collagen of the papillary dermis displays moderate severe necrosis in severe GvH reactions.

Inflammatory changes consist primarily of mononuclear infiltration of epidermis and papillary dermis (34). In the oral and esophageal mucous membranes, hyperbasophilic lymphocytes accumulate in large numbers around superficial small vessels and then massively infiltrate the overlaying basement membrane into a spongiotic epithelium. Once intraepithelial, the individual lymphocyte acquires a characteristic 'halo' (30).

Two types of liver injury can be distinguished, depending on the type of acute GvH disease. Whereas the patients with early-onset acute GvH disease have predominantly hepatocellular necrosis, those with late onset acute GvH disease have predominantly bile duct injury (early onset acute GvH occurring at day 4-18 after transplant and late onset acute GvH occurring at day 16-56 after transplant). The hepatocellular necrosis is characterized by periportal acidophilic cells and hepatocellular dropout as well as small foci of acidophilic cells in the midzone region. The necrotic foci are associated with a mild to moderate infiltrate of lymphoid mononuclear cells (35).

The bile duct injury of late-onset acute GvH disease is evidenced by epithelial dysplasia with nuclear and cellular polymorphism, hyperchromasia, angulation of the nuclear membrane, and frequently by pyknotic nuclei. Cellular debris is often evident in the ductal lumen. Lymphocytes occur both epiductally and as infiltrations of duct walls (36).

The gastro-intestinal tract of individuals suffering from acute GvH disease displays lesions of both the small and large intestines, which are most severe in the distal ileum.

The mildest histopathologic alteration is focal dilatation and degeneration of the mucosal glands with a concomitant infiltration by lymphocytes. Degeneration appears to take place in a piecemeal fashion. Affected crypt cells contain bare nuclei. Later on cellular fragmentation occurs and eventual disintegration of entire crypts. In the severest cases diffuse mucosal denudation occurs. With increasing severity, mucosal and submucosal edema, flattening of villi, epithelial cell atypia and necrosis, mucosal and submucosal mononuclear cell infiltrate and bacterial and/or fungal colonization can be found (34).

Bone marrow specimens from lethally irradiated animals examined very early after bone marrow allografting, exhibit a hypocellularity ranging from 5 to 10 percent of normal. Degenerated megakaryocytes are common, but by day 10 nonvacuolated, normal forms predominate. Marrow regeneration is noted earlier than in recipients of autologous marrow, appearing as foci of proliferating stem cells. Ribbons of normoblasts disposed among dense collections of erythroblasts, blast forms of the myeloid series and mature granulocytes become the dominant microscopic feature. In the lymph nodes atrophy develops after an early proliferative phase; total numbers of lymphoid cells decrease and finally the lymphoid cells almost completely disappear. During this process foci of lymphocytokaryorrhexis can be observed (30).

Chronic GvH disease may present itself in various forms: it may develop as a progressive disease subsequent to continuously active GvH disease, as quiescent onset subsequent to the resolution of acute disease, or as a de novo entity. In man, it generally

develops more than 100 days after transplantation (37).

Chronic GvH disease is characterized by progression of the skin lesions to scleroderma-like phenomena. A sicca syndrome may accompany the skin lesions. Patients with chronic GvH suffer from infections caused by bacteriae (e.g., staphylococci, pneumococci and streptococci) and viruses (e.g., herpes zoster) (38,39).

Histologically in the skin progressive basal cell vacuolar degeneration can be shown together with epidermal thinning. The dermis is often fibrotic and edematous. Immunofluorescence staining shows IgG surrounding dermal collagen bundles (39). The liver shows some portal fibrosis, but is otherwise relatively free of pathological changes. Biopsies of the gastro-intestinal tract shows a plasmacytoid-lymphocytic submucosal infiltrate in the small intestine; extensive mucosal and submucosal esophageal fibrosis may also occur similar to systemic sclerosis.

The lymphoid system shows a generalized lymphadenopathy. Microscopic examination reveals decreased numbers of lymphocytes with an increased number of plasmacytoid cells. Reticulohistiocytic cell proliferation is prominent in the medulla and in perifollicular areas.

In chronic GvH disease salivary glands and lacrimal glands display lymphatic infiltration similar to that observed in Sjögren's syndrome. Furthermore, a spectrum of immune abnormalities is observed including hypergammaglobulinemia, IgM paraproteinemia, immune-complex disposition, plasma cell hyperplasia, lymphocytotoxic antibodies and autoantibodies to autologous or donor lymphocytes. Taken together, chronic GvH disease is a syndrome of collagen

vascular disease associated with disordered immune regulation features, immunodeficiency and autoimmunity (38,39).

4. Grading systems

Grading systems for GvH disease have been devised to relate clinical signs and histological changes to survival times. Thomas et al. (31) attempted to classify the overall severity of clinical GvH disease in a grading system ranging from 0 to IV based upon clinical and histological staging systems (Tables II, III and IV). Analysis of survival among their patients suggested that patients can be placed into two groups: those without clinically evident GvH disease (Grade 0) and those with GvH disease involving skin only (Grade I) show a survival of 55%, whereas patients with Grades II to IV have a survival of only 15%. The grading system may become more meaningful as advances are made in the treatment of GvH disease and the accompanying infections. A comparable grading system is used by the American College of Surgeons (National Institutes of Health Organ Transplant Registry, 1975) (40).

GvH disease may be very difficult to diagnose. It can be confused by conditions affecting several organs, e.g., virus infections (cytomegalovirus), collagen vascular disease or irradiation. Likewise specific diseases of the liver (chronic aggressive hepatitis, biliary cirrhosis, drugs), of the skin (virus infections, drug eruptions, lupus erythematodes, allergic reactions) and of the gastrointestinal tract (bacterial, viral and protozoal infections) may cause histological changes which are very difficult

to differentiate from those caused by GvH disease (see for detailed descriptions the reports of Lerner et al. (34), Slavin and Santos (23), Beschorner et al. (29), Shulman et al. (39) and Sale et al. (41)).

Complications of chronic GvH disease may vary from autoimmune disorders to the formation of lymphomas (see the Chapters IX and X).

It is striking that the target organs of GvH disease are mainly restricted to skin, liver, gastrointestinal tract, oral mucosa, and lymphoid and hemopoietic organs. These tissues are directly or indirectly in contact with the milieu exterieur and with pathogenic microorganisms. One might speculate that these microorganisms and the changes they induce after ingestion by body cells lead to the expression of Ia antigens by these cells, which then function as targets for the ongoing GvH reaction (42,43). This concept is supported by the data of Van Bekkum and Knaan. These authors have shown that barrier-maintained mice with sterile intestines do not suffer from GvH disease after irradiation and allogeneic bone marrow transplantation (44). Also in the clinical situation elimination of the gastrointestinal microorganisms and patient isolation have been shown to prevent or reduce the GvH disease (45, 45a).

TABLE II. Overall clinical grading of the severity of GvH disease

Grade	Degree of organ involvement
0	no clinically evident GvH disease
I	+ to ++ skin rash, no gut involvement, no liver involvement, no decrease in clinical performance
II	+ to +++ skin rash, + gut involvement or + liver involvement (or both), mild decrease in clinical performance
III	++ to +++ skin rash, ++ to +++ gut involvement, or ++ to +++ liver involvement (or both), marked decrease in clinical performance
IV	similar to Grade III with ++ to +++ organ involvement and extreme decrease in clinical performance

From ref. 31.

TABLE III. Proposed clinical stage of GvH disease according to organ system

Stage	Skin	Liver	Intestinal tract
+	maculopapular rash <25% of body surface	bilirubin 2-3 mg/100 ml	>500 ml diarrhea per day
++	maculopapular rash 25-50% of body surface	bilirubin 3-6 mg/100 ml	>1000 ml diarrhea per day
+++	generalized erythroderma	bilirubin 6-51 mg/100 ml	>1500 ml diarrhea per day
++++	generalized erythroderma with bullae formation and desquamation	bilirubin >15 mg/100 ml	severe abdominal pain, with or without ileus

From ref. 31.

TABLE IV. Proposed histopathologic stage of GvH disease according to organ systems

Stage	Skin	Liver	Intestinal tract
+	basal vacuolar degeneration or necrosis (or both)	<25% abnormal small interlobular bile ducts	dilatation of glands, single cell necrosis of epithelial cells
++	+ plus spongiosis, dyskeratosis, and eosinophilic necrosis of epidermal cells	25-50%	+ plus necrosis plus dropout of entire glands
+++	++ plus focal microscopic epidermal-dermal separation	50-75%	++ plus focal microscopic mucosal denudation
++++	frank epidermal loss	>75%	diffuse microscopic mucosal denudation

From ref. 31.

III. ASSAY SYSTEMS

A variety of GvH assays has been developed. Some are often used (e.g., the mortality assay, splenomegaly assay and popliteal lymph node assay), others are rarely used (e.g., liver infiltration assay, splenic explant assay, Elkins' renal assay). We shall discuss the assays most frequently used; for the other assays we refer to the reviews by Elkins (46) and Grebe and Streilein (9).

1. Mortality assay

The course of GvH disease and its severity can be assayed by determining the body weight change, the incidence of clinically apparent disease and the mortality rate. These methods are simple to carry out and can provide a valid indication that a GvH reaction has been induced. From these the mortality rate is considered as the most reliable parameter. Mortality can be expressed in two ways, namely as mortality rate and as cumulative mortality. The former expresses mortality at a given time point, the latter as the total mortality at the end of the observation period.

It can hardly be overemphasized, however, that the results obtained by the mortality assay are substantially influenced by host-related and environmental factors, such as effects of irradiation, infection, hematologic and immunologic functions of the graft, etc. Very striking is the effect of the gastrointestinal microorganisms on the mortality in GvH. While in certain donor-recipient combinations of conventionally bred mice a 100%

mortality is observed, similar combinations involving germfree or decontaminated mice show 100% survival (44).

Hence, it is difficult to attribute mortality to the donor anti-host alloreaction only. Consequently, the experiments have to be performed under well-standardized conditions.

Generally, in mice the mortality is the highest after lymphoid cell transplantation into H-2 incompatible recipients (9). Lethal GvH disease can also occur after transplantation of bone marrow cells into lethally irradiated, H-2 identical but non-H-2 incompatible, allogeneic mice. Under such conditions the mortality mainly occurred between days 20 and 80 after irradiation (47,48).

2. Splenomegaly assay

In 1959, Simonsen and Jensen described a phenomenon which has grown out to one of the best known and widely used GvH assays (49). They observed that neonatal mice, rats and chickens injected with allogeneic lymphoid cells display splenomegaly. This prompted these investigators to use the splenomegaly as a measure of the GvH reaction. GvH splenomegaly peaks at 8 to 10 days after donor cell inoculation. The so-called splenic index is calculated as a quotient, i.e.,

$$\frac{\text{experimental spleen weight / host body weight}}{\text{control spleen weight / control body weight}}$$

A splenic index greater than 1.30 is indicative of significant GvH reactivity (49).

Over a certain range of donor cells the splenomegaly shows a logarithmically linear relationship to the number of lymphocytes injected. Splenomegaly is not only an expression of the specific anti-host immune reactivity but, secondarily, is influenced by a response of the host lymphoid and/or hemopoietic cells (50). Therefore, it is difficult to compare spleen indices of hosts of different genotype even when inoculated with lymphoid cells from identical donors. Moreover, the results may be confused by the accompanying runt disease (46). Lastly, the extent to which the spleen can enlarge is limited (see also the section about the popliteal lymph node assay below).

3. Popliteal lymph node assay

The popliteal lymph node assay is based on the observation of Levine (51) that the popliteal lymph nodes (PLN) may enlarge after injection of parental strain lymphoid cells into a foot of F1 hybrid rats. Within seven days after footpad injection, the draining PLN in rats may become 50 times as heavy as the original ones. This reaction was found to be a local manifestation of GvH reaction and most optimal after injection of lymphoid cells into semi-allogeneic hosts (51).

Ford et al. (52) have shown that in the PLN assay the mean lymph node weight was linearly related to the dose of cells injected, when plotted on a double log scale. The method was found to be 10 times more sensitive and also more convenient than several other assays, because fewer donor cells are needed to elicit GvH reactions, the contralateral PLN can be used as a control and lastly, there is no accompanying runt disease.

The degree of lymph node enlargement produced by a given dose of cells was influenced by the volume in which they were injected. The larger the volume, the smaller the PLN weight gain and vice versa. Moreover, the age of the recipients is important: the older the recipients, the less sensitive they are. The sex of the recipients, on the other hand, does not affect the PLN enlargement (52).

Application of the PLN assay was extended to mice by Hardt and Claësson (53). These authors determined the number of nucleated cells in the 'test' lymph node and the contralateral 'control' lymph node. The lymph node index was calculated as the ratio between these numbers. Although in mice the PLN assay is less sensitive than in rats (52,53), it is a very convenient and accurate assay in this species as well.

The PLN assay is able to detect MHC as well as non-MHC differences. The main stimulus seems to be the Class II alloantigens of the MHC (54). In MHC-identical strain combinations, about 100 times more cells are required to produce the same lymph node enlargement as in MHC incompatible combinations (52). In mice, only non-MHC alloantigens coded for by the Mls locus produce a significant response at relatively low cell doses (55).

The major advantage of the PLN assay above the splenomegaly assay in quantifying GvH reactivity of lymphoid cells is that the lymph nodes can enlarge many times more than the spleen in the splenomegaly assay. This holds for both the rat and the mouse (52,53).

Injection of F1 hybrid cells into the footpad of parental mice usually induces a Host-versus-Graft (HvG) reaction (56).

This HvG reaction peaks on day 4, in contrast to PLN GvH reactions which peak on day 7. Injection of alloreactive lymphoid cells into the footpad of fully MHC incompatible recipients results in combined GvH and HvG responses producing a smaller PLN enlargement than unidirectional GvH reactions. The HvG component in this reaction can be abrogated by sublethal irradiation of the recipients 24 hr before donor cell injection (9). Thereby the GvH reaction augments (9).

PLN hypertrophy is highly dependent on host radiosensitive cells which by themselves do not seem to provide immunogenic stimuli. These stimuli seem to be mainly provided by host radioresistant cells (9).

Two mechanisms may underly the lymph node enlargement in the PLN assay: proliferation in situ and trapping of recirculating cells (57). In the experiments of Piguet et al. (50) lymph node enlargement coincided with the kinetics of proliferation. Trapping of recirculating cells might be enhanced by the longer migration pathway in enlarged lymph nodes. Emeson and Thursh (58) demonstrated that ^{51}Cr-labeled F1 lymphoid cells, injected intravenously (iv) into syngeneic hosts 24 hr before sacrifying them to determine PLN enlargement, are trapped in the challenged lymph node and contribute considerably to the lymphadenopathy. However, it seems that the former possibility might well be true: Ford et al. (59) have shown that most of the cells proliferating in the enlarging popliteal lymph node originated from the F1 hybrid host.

4. Anti-host delayed-type hypersensitivity

Our laboratory has developed a delayed-type hypersensitivity (DTH) assay which is appropriate for measuring anti-host immune reactivity after transplantation of allogeneic lymphoid cells into lethally irradiated recipients. This assay is based on passive transfer of alloactivated donor T-cells to naive, i.e., unprimed, secondary recipients syngeneic with the donor (60,61). Subsequently, the non-irradiated secondary recipients are challenged in the dorsum of the right hind foot with spleen cells that are syngeneic with the irradiated recipients, i.e., the animals used for allo-activation of the passively transferred cells. The DTH response to the challenging cells is measured as the difference in thickness of the hind feet 24 hr later. The DTH response is calculated as the relative increase in foot thickness of the recipients of GvH-activated cells minus the relative increase in foot thickness of naive control mice which only received the challenge (see Fig. 1).

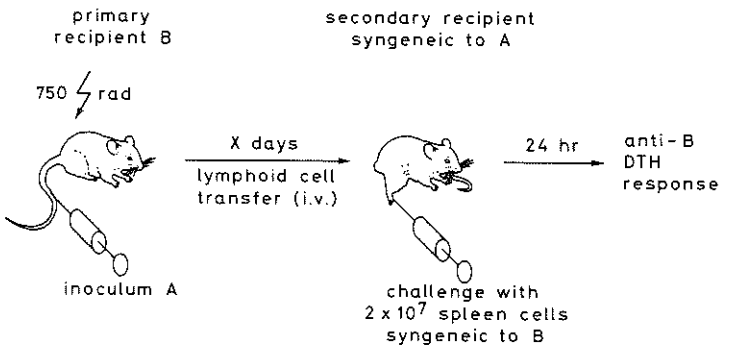

Fig. 1. Scheme of the experimental system used to demonstrate GvH-related DTH against host histocompatibility antigens.

Advantages of the assay are its reproducibility, the short interval whereafter the DTH alloreactivity can be measured and the direct measurement of the generation of anti-host directed T cell activities (60,61), which is independent of the microbiological status of the recipients. Disadvantages, on the other hand, are its laboriousness and the fact that it can only be used in donor recipient combinations involving H-2I and/or Mls differences. H-2K, H-2D and non-H-2 alloantigens other than those encoded for by the Mls locus are by themselves unable to elicit a GvH-related DTH reaction (62).

5. Inhibition of hemopoietic activity

Blomgren en Andersson (63) have described a GvH assay which is based on the principle that the ^{59}Fe uptake by the spleen of lethally irradiated mice infused with syngeneic bone marrow cells is a measure of the ongoing erythropoiesis. The latter can be inhibited by simultaneous injection of the mice with alloreactive T cells. The degree of inhibition is positively correlated to the number of alloreactive T cells injected. Using this method it is possible to estimate the relative immunological reactivity of sensitized and non-sensitized lymphoid cells against alloantigens. The authors have demonstrated that, using this assay, the GvH reactivity of only 6×10^4 lymph node cells or cortisone-resistant thymocytes can be measured. Lymph node cells which had been sensitized in irradiated allogeneic mice prior to injection into test mice of the same strain were 2-4 times more reactive than the non-sensitized control lymphocytes (63). The advantage of this assay is the elimination of host lymphoid cell influences. However, the major disadvantage

is the laboriousness of the test. Furthermore, the subset of donor T cells responsible for the suppression of erythropoiesis has not been defined.

6. Phagocytic index assay

The phagocytic index assay is based on the observation of Howard (64) that two weeks after injection of mouse parental lymphoid cells into F1 hybrids, iv injected colloidal carbon is cleared from the blood stream at an enhanced rate.
This rate of phagocytosis is measured in the phagocytic index assay. An advantage of this assay is that quantitative data can be obtained without the need of sacrifying the animal. However, there is a major disadvantage: alterations in the mononuclear phagocyte system resulting from the GvH reaction itself cannot be distinguished from complications due to the GvH reaction, such as infection (46).
This assay is hardly used any more.

7. Chorioallantoic membrane assay

The chorioallantoic membrane (CAM) assay has already been described by Murphy (65) in 1916, although he was unable to recognize its immunological basis and could not satisfactorily explain the phenomena observed. He placed chicken spleen and bone marrow fragments onto the chorioallantoic membrane of an allogeneic chick embryo. During culture the explanted fragments enlarged and developed into white nodules, which comprised mainly leucocytes (65). Later studies revealed that each nodule or 'pock' represents the site

were a single donor cell has lodged and reacted against the histocompatibility antigens of the host (66). The genesis of the 'pocks' represents donor cell proliferation. The CAM 'pock' count is the only method for the direct enumeration of cells capable of initiating GvH reactions. However, the visibility of the pocks may be subject to extraneous influences, such as infection (46).

8. In vitro assays

The afferent arm or initiation phase of the GvH reaction in vivo is generally thought to be represented by the mixed lymphocyte culture (MLC) assay. The in vitro MLC assay is based on blast transformation and proliferation of cocultured lymphocytes obtained from allogeneic individuals. Stimulation of cell division in the MLC is the outcome of recognition of histocompatibility antigen determinants by specific, immunologically competent T lymphocytes (67). When either population of lymphocytes can proliferate, the assay is called a 'two-way MLC'. When the lymphocytes from one individual cannot proliferate (e.g., due to irradiation or treatment with mitomycin C) the assay is called a 'one-way MLC'. The extent of proliferation is usually measured by tritiated thymidine (^{3}H-TdR) incorporation.

The MLC assay has been employed to answer several fundamental questions concerning the cellular and genetic requirements of alloreactivity. The data of Wilson, who did most of the exploring studies, can be summarized as follows: the responding cell in the MLC is a small lymphocyte; the addition of macrophages to the cultures does not significantly affect the proliferative response;

proliferative responses occur only when the cocultured lymphocyte subpopulations differ at the MHC; and proliferative responses in cultures of parental and F1 hybrid cells are unidirectional (68). Studies of Bach et al. (69-71) and Meo et al. (72) revealed that H-2I region coded alloantigens are most potent in inducing MLC reactions. H-2K and H-2D coded alloantigens in the absence of H-2I region differences induce relatively weak MLC reactions.
Similar observations were made in man by Eijsvoogel et al. (73).

Lymphocytes from donors made tolerant to allogeneic histocompatibility antigens at birth were specifically unreactive in MLC against cells bearing antigens of the tolerance inducing strain, but not to third party cells (68). The same phenomenon has been described for GvH reactions: Gowans et al. (74) demonstrated that lymphocytes from tolerant animals were unable to produce runt disease in the relevant F1 hosts. Lymphocytes from donors systemically presensitized at adult age exhibit a curtailed proliferative activity in mixed cultures with cells from donor strain animals to which they had been immunized (75). This phenomenon can be explained by the observations of Cerottini et al. (76) that during MLC's cytotoxic T cells are generated which can specifically lyse lymphoblasts derived from the original stimulator individual, thereby reducing the immunogenic stimulus. However, it is equally well possible that the preimmunization induced specific (77,78) or nonspecific (79) suppressor T cell activity.

The MLC may be used in predicting the outcome of bone marrow transplantation in mice: Rodey et al. (80) demonstrated a correlation

between MLC reactivity and chronic GvH disease in animals immunosuppressed with cyclophosphamide and 4 Gy (400 rad) X-irradiation and subsequently transplanted with bone marrow cells from various allogeneic strains. In combinations where no or only weak MLC reactivity could be detected between donor and host cells, the GvH disease was very mild.

While the MLC is the *in vitro* equivalent of the afferent arm or initiation phase of the GvH reaction, cell-mediated lympholysis (CML) *in vitro* has been considered as representing the efferent arm or effector phase of the GvH reaction, namely the alloreaction causing the disease and ultimate death of the recipients (81). Already activated cytotoxic lymphocytes generated during an acute GvH reaction *in vivo* do not produce GvH reactivity in the splenomegaly assay (82). Moreover, cytotoxic donor T cells failed to cause lethal GvH disease (83). Furthermore, skin transplant rejection under Host-versus-Graft conditions has been shown to depend on Lyt-1$^+$ T cells (84) and the genetic requirements for skin graft rejection correlate with those of DTH reactive T cells and not with those of cytotoxic T cells (84). Therefore, it is questionable whether the CML assay only is a good representative for the immunologic events responsible for the ultimate death of the individuals suffering from GvH disease (see also Section V.4).

IV. GENETIC REQUIREMENTS

1. MHC alloantigens

In several *in vivo* and *in vitro* assays for GvH reactivity it has been shown that, generally, the most severe GvH reactions occur when the donor and the recipient differ at the MHC. This has been found in a variety of species, including mouse (85), rat (86), dog (26), monkey (25), man (87) and chicken (88). In the mouse, the MHC is located on chromosome 17 and composed of three major classes of loci: Class I loci residing in the K and D regions, Class II loci residing in the I region and Class III loci residing in the S region (89). Klein and Park have shown that the strongest GvH reaction, as measured in the splenomegaly assay, is induced by a H-2I region disparity (90). Disparity at the H-2K and H-2D regions caused only a borderline effect in this assay. Similar observations have been done in the PLN assay, by measuring the radioactive IUdR incorporation in spleen and lymph nodes (91,92) and in the MLC (69-71).

Until recently, mapping studies suggested the existence of several subregions within the H-2I region of the MHC, namely I-A, I-B, I-J, I-E and I-C. The functions of each of the subregions of H-2I have recently been extensively reviewed by Klein et al. (93). Studies using several GvH assays have shown that the main stimulus for proliferation is provided by the I-A subregion of the H-2I region of the MHC (94).

fluorescence technique, that during a GvH reaction donor cells acquire host cell-derived H-2K, H-2D and Ia alloantigens on their cell surface. Mapping studies indicated that next to recipient type H-2K and H-2D alloantigens, only recipient type I-A subregion products could be identified on the donor cells. Host I-E/C and I-J subregion products were not observed on the donor cells. It has also been shown by Prud'homme et al. that Lyt-1+2- cells pick up the host I-A alloantigens, whereas the K/D alloantigens are picked up by Lyt-1-2+ cells.

Klein (96) demonstrated by using the mortality assay that in a particular I-C disparate combination (i.e., B10.S(7R) versus B10.S(9R)) the reaction was so strong that almost all the irradiated F1 hybrids that had been injected with the allogeneic bone marrow cells died within 3 weeks after the inoculation. Such a severe reaction is usually only observed in combinations differing at the entire H-2 complex or at least the I-A subregion (97). Furthermore, the severity of GvH induced in I-C disparate mouse strain combinations varied as a function of the interallelic strain combination and was particularly influenced by properties of the recipient I-C determinants. Thus, I-C determinants of recipients bearing the s-haplotype led to strong GvH reactions, whereas $I\text{-}C^d$ determinants induced moderate GvH reactions, even when donor strains carrying different I-C alleles were used (98,99). However, in these studies the used mouse strain combinations were not incompatible for I-C alone. They therefore represent merely an indication for the GvH-inducing capacity of the right part of the H-2I subregion, from which the I-E subregion has proven GvH-inducing capacity (94).

Reviewing the functional aspects of the H-2 complex, Klein recently concluded that, at present, there is no good evidence any more for the existence of the I-B, I-J and I-C subregions (93). Furthermore, using the technique of gene transfer, Steinmetz and colleagues (100) correlated the various histocompatibility loci with their serologically defined gene products. These authors have shown that the genetic map of the I region confines the I-B and I-J subregion to a very small part of the DNA, thus indicating that there is little space for the genetic information for possible I-B and I-J subregions.

In summary, the various classes of H-2 coded products behave the same in the splenomegaly GvH assay, the PLN assay (101) and the MLC assay. Class I products (i.e., H-2K and H-2D coded alloantigens) cause weak or moderate proliferative GvH reactions, whereas Class II products (i.e., H-2I coded alloantigens) cause strong lymphoproliferative GvH reactions. Class III products (i.e., H-2S region coded products) do not participate at all in GvH reactions.

In contrast, the reaction of donor T cells against the various H-2 subregion coded antigens is different in assays measuring the effector phase of GvH reaction. Severe GvH disease with a 100% cumulative mortality within one month occurs only in donor-recipient combinations differing for the entire H-2 complex or for H-2K plus H-2I-A (97). It has been reported that much less severe GvH disease with a cumulative mortality of 50% or less and death of individual mice spread over the entire observation period is observed when the donor and host differ at either the K, I or D region alone. In these particular experiments with irradiated recipients the degree of GvH disease induced by these three regions, when taken singularly,

was about the same (97).

In non-irradiated recipients, on the other hand, differences at K/D or I cause completely different syndromes (102). The above results from Klein and Chiang (97) with the mortality assay are also not in line with data from the in vitro CML assay. In the latter, K and D region coded alloantigens induce substantial CML responses, while I region coded alloantigens induce only weak CML responses (103-105).

Significant GvH reactions as measured in the splenomegaly assay and the PLN assay also occur when donor and host differ from each other by mutations in the H-2K or H-2D region (106).
Such mutations represent substitutions of nucleotides in the DNA coding for the H-2K or H-2D molecule. These mutations lead to substitutions of amino acids in the polypeptide chain of the H-2K or H-2D molecule and thereby to a different steric configuration of this molecule (107). Amino acid substitutions in an H-2K or H-2D molecule can also lead to a significant MLC response (108). This suggests that such mutant H-2K and H-2D molecules have a stronger proliferation inducing capacity than their normal, non-mutated allogeneic counterparts.

In man, the MHC is called the HLA system. This system consists of Class I antigens, called HLA-A, B and C, Class II antigens, called HLA-D/DR, and Class III antigens coding for complement factors. The HLA of man and the H-2 of mice are homologous to a great extent. The murine H-2K, D and L molecules are homologous with HLA-A, B and C molecules and the H-2I region is homologous with the HLA-D/DR region. These structurally homologous gene products direct largely identical functions (109) in all vertebrate species. Thus, in man, in general, strongest GvH reactions are observed in donor-recipient combinations

including an incompatibility at HLA-D/DR (86,109).

2. Non-MHC alloantigens

Not only MHC-coded alloantigens give rise to strong proliferative responses, in the mouse also the products of the Mls locus can do so (110). This locus is located on chromosome number 1. Till now five codominant alleles have been described, designated Mls^a, Mls^b, Mls^c, Mls^d and Mls^e (110,111). These determinants develop late in neonatal life (112) and are present on B cells (113), macrophages (114), bone marrow stem cells (115) and tooth germ (116), but not on T cells (113).

Mls determinants have a widely differing stimulating capacity in MLC: Mls^a, Mls^d and Mls^e are strongly stimulatory, Mls^c is intermediate and Mls^b is not at all stimulatory (110,111). However, these results were obtained with particular strain combinations and it is possible that Mls determinants stimulate differently when associated with different H-2 haplotypes. This view is supported by the observation of Rychlikova et al. (117) that Mls^b cells responding to Mls^a antigens give a much weaker MLC response when both donor strains are $H\text{-}2^b$ than when both strains are $H\text{-}2^k$. Other non-MHC coded histocompatibility antigens than those encoded for by the Mls locus do hardly or not induce a MLC response (118).

Until recently, no cytotoxic effector cells against Mls determinants could be demonstrated (119). However, using the newly developed congenic mouse strain BALB.D2.MA, which is a recombinant strain of BALB/c bearing the Mls^a locus from DBA/2, strong anti-Mls^b cytotoxic T cell activity was detected after preimmunization of the

restimulation in vitro.

No cytotoxic activity could be detected in the reverse direction (i.e., Mls^b anti-Mls^a) although it is this direction that MLC reactivity occurs after cocultivation of BALB/c and DBA/2 lymphocytes (120).

GvH reactions can also be elicited across Mls differences. Rodey et al. (80) described that lymphoid cells from the C3H/He ($H\text{-}2^k$, Mls^c) strain caused a cumulative mortality of 87% at 100 days when tested in immunosuppressed AKR ($H\text{-}2^k$, Mls^a) recipients. This is in agreement with the observation that Mls^a determinants induce a strong MLC response. In the PLN assay, too, it has been demonstrated that Mls incompatibilities can elicit GvH reactions (55). The lack of correlation between the GvH disease inducing capacity and the CML inducing capacity of Mls locus coded antigens suggest that cytotoxic T lymphocytes are not necessarily required for mortality in H-2 compatible, Mls locus incompatible donor-recipient combinations.

Our own laboratory (118) has recently shown that in H-2 compatible, Mls incompatible donor-recipient combinations anti-host directed DTH effector T cells are generated. Other non-H-2 alloantigens do not induce this activity. The capacity of Mls-locus antigens to induce distinct anti-host DTH reactivity correlates with the capacity to induce a one-way MLC response. Mls^a and Mls^c determinants initiate both a positive MLC response as well as distinct GvH-related DTH reactivity. The Mls^b determinant, on the other hand, which is unable to induce in vitro proliferation (110) induces only marginal and short-lasting GvH-related DTH reactivity (118).

After transplantation of H-2 and Mls matched bone marrow cells into lethally irradiated mice, delayed GvH reactions may develop, indicating that GvH reactions can also be induced by minor histocompatibility antigens other than Mls locus coded antigens (47,48,121). The same observation was made in dog (122) and man (31). Korngold and Sprent (47) demonstrated that untreated bone marrow cells from B10.BR ($H\text{-}2^k$, Mls^b) mice caused a high incidence of lethal GvH disease when transferred to heavily irradiated CBA/J ($H\text{-}2^k$, Mls^d) recipients. They found 80% mortality at 80 days post transplantation. By comparing various H-2 compatible donor-recipient combinations they showed that at least three minor H antigen differences should exist between donor and host in order to elicit lethal GvH disease. However in neonatal mice tested in splenomegaly and mortality assays, minor H differences cause minimal reactions unless the lymphoid cell donors are presensitized (46,123).

A remarkable observation in human GvH disease has been reported by Rappaport et al. (124). These authors describe that patients who received bone marrow cells from identical twin donors (i.e., HLA plus non-HLA identical) occasionally developed GvH disease. The same was demonstrated in other animal species (124a). This indicates that, next to genetic factors, epigenetic factors may also elicit GvH disease. For instance, total body irradiation or viral infection may cause altered recipient self-antigens which are recognized by the donor lymphocytes.

3. Hybrid histocompatibility antigens

Fathman and colleagues (125,126) have demonstrated the expression of murine MLC stimulating determinants on F1 hybrid stimulator cells that are absent on cells from either parental strain. These hybrid molecules result from the interaction of maternal and paternal H-2I region products on the cell surface, thus creating new specificities not occurring on the cells from either parent (127,128). Furthermore, it has been shown by Sandrin et al. (129) that the I-A^k allele can cause the absence of several other MHC products on F1 hybrid cells. The reason for this 'low expression effect' is not clear.

We have shown in our GvH-related DTH assay that, in the mouse, FI hybrid lymphoid cells may respond to histocompatibility antigens of parental-type irradiated recipients. Thus, (C57BL x CBA)F1 spleen cells evoke an anti-host DTH response towards lethally irradiated (7.5Gy) CBA recipients, albeit of a smaller magnitude than in the opposite direction. Similar data were obtained in other F1 hybrid to parent combinations (Table V). This suggests that the F1 hybrid cells do not express all the determinants of the histocompatibility antigens that the parental cells do, so that they are able to respond to these determinants. As the GvH-related DTH assay only detects reactivity towards H-2I and Mls locus coded antigens (62) these data suggest that H-2I region coded products are susceptible to posttranslational modulation.

TABLE V. Hybrid histocompatibility antigens in GvH reaction

Spleen cell donor	Irr. recipient	DTH response
(C57BL x CBA)F1	C57BL	14.4 ± 2.7
(C57BL x CBA)F1	CBA	16.0 ± 2.8
(A.TH x A.TL)F1	A.TL	11.8 ± 3.9
(C3H x BALB/c)F1	BALB/c	8.2 ± 3.1
(C3H x BALB/c)F1	BALB.K	13.4 ± 1.9
C57BL	(C57BL x CBA)F1	30.0 ± 2.5
CBA	(C57BL x CBA)F1	25.0 ± 1.7

Anti-host DTH reactivity in lethally irradiated parental mice reconstituted with 5 x 10^7 F1 hybrid spleen cells. As a control, for 2 parent to F1 combinations (C57BL→(C57BL x CBA)F1 and CBA→(C57BL x CBA)F1) the anti-host DTH reactivity is also given. In such parent to F1 combinations the anti-host DTH responses are always higher. For details of the assay system: see Section III-4. DTH responses were measured at 24 h after challenge and expressed as the percentage specific increase of foot thickness ± 1 SEM (n=6).

V. CELLULAR REQUIREMENTS

1. Role of thymus-derived lymphocytes

From the studies of Gowans (8) it has become clear that small lymphocytes are the cells that induce GvH. He showed that GvH reactivity remained in a suspension of thoracic duct lymphocytes, which had been depleted of viable large lymphocytes by overnight incubation at 37°C. After this procedure the only morphologically identifiable cell type left was the small lymphocyte.

Cantor (130) and Golub (131) showed that treatment of parental spleen or lymph node cells with anti-Thy-1 serum plus complement abolished their capacity to induce a GvH reaction in neonatal F1 hosts, thus demonstrating that GvH reactions are mediated by T lymphocytes.

It was shown by Yoshida and Osmond (132), using the PLN assay in rats, that lymphocytes from different anatomical origins displayed different capacities to induce GvH reactions: blood lymphocytes were the most effective, lymphocytes from mesenteric lymph nodes and spleen were less effective and bone marrow and thymus cells were the least effective (see Table VI). Similar data have been presented by other authors using different assays (133,134).

McGregor demonstrated that the origin of the GvH reactive cells lies in the bone marrow. The bone marrow contains precursor cells which, during thymic passage, differentiate into GvH inducing T

cells (135). Umiel demonstrated that fetal liver cells could also give rise to GvH reactive cells after being subjected to thymic influence in vivo (136,137).

TABLE VI. Relative potential of mouse tissues for repopulation (hemopoietic stem cells) and for induction of acute GvH disease (T lymphocytes)

	CFU-s/10^5 nucleated cells	GvH potential
bone marrow	600	2-3
fetal liver	100	1
spleen	60	20-50
peripheral blood	6	20-50
lymph nodes	-	100
thymus	-	2-5

From ref. 15.

Bone marrow cells from aged mice (30 months old) were more efficient to produce mortality in irradiated F1 recipients than bone marrow cells from young mice (138). Spleen cells from aged parental strain mice, on the other hand, were less capable of producing splenomegaly in neonatal F1 hybrid recipients (139). These results are compatible with the decreasing incidence of immunocompetent T cells in the spleen and the increasing incidence in the bone marrow of aging mice (140).

Originally, the observation that thymocytes hardly cause detectable GvH and the fact that the presence of the thymus is essential for the development of immunologic competence seemed to create a paradox.

It was shown in mice (141,142) and rats (143) that thoracic duct lymphocytes (TDL) and lymphocytes from other lymphoid organs of neonatally thymectomized animals were unable to cause splenomegaly or runt disease. This capacity could be restored by grafting a thymus to these donors or by injecting them with thymic extracts (144,145).

Sprent and Basten (146) have shown that thymectomy of neonatal mice reduces the number of long-lived cells at adult age by 50 percent, while the number of short-lived lymphocytes remains constant This indicates that the long-lived cells consist mainly of T lymphocytes and the short-lived cells mainly of B lymphocytes (146). This suggests that the GvH reactive T cells predominantly are long-lived cells.

Although the thymus mainly consists of immature, short-lived cells which reside in the cortex, also a small population of more mature cells occur. The latter are located in the thymic medulla (147).

The immature thymocytes, which are incapable of mounting GvH reactions, and the mature thymocytes which are capable, differ from each other a.o. in their sensitivity to corticosteroid hormones (148). The immature thymocytes are corticosteroid-sensitive while the mature thymocytes are resistant. Since the latter population constitutes 4 to 5 percent of all thymocytes (149,150), it is not surprising that, as a whole, thymocytes have only a weak capacity to mount GvH reactions. The immature corticosteroid-sensitive thymocytes may acquire GvH reactivity after interaction with macrophages (151).

The notion that mature recirculating T lymphocytes are the principle mediators of GvH was reinforced by the observation that donor treatment with anti-thymocyte serum (ATS) resulted in an abolition of their spleen and lymph node cells to cause splenomegaly (152) or anti-host DTH reactivity (61). Also the occurrence of secondary disease in irradiated mice was reduced after engraftment with allogeneic bone marrow cells from ATS-treated donors. Under such conditions the weight loss and mortality of the recipients were delayed (153).

2. Role of proliferation of donor cells

Fox (154) described that after injection of T6-chromosome-marked CBA spleen cells into F1 mice, the spleen and lymph nodes of the recipients contained many mitotically active cells bearing the T6 chromosome marker. This suggests that the murine acute GvH reaction is associated with donor cell proliferation.

That donor cells need to proliferate in order to induce GvH was first shown by Meuwissen and Good (155) in the splenomegaly assay by treatment of donor cells with mitomycin C, a drug which, in appropriate concentrations, inhibits cell division, but leaves immunological function intact. Cheever et al. (156) found that elimination of the specifically activated, proliferating T cells by ^{3}H-TdR suicide *in vitro* greatly diminished the ability of the residual cells to induce GvH. Also the full development of specific anti-host DTH during acute GvH reactions is dependent on proliferation of the reactive T cells (157). Lafferty et al. (158), however, showed that in the chorioallantoic membrane assay non-dividing (either mitomycin C-treated or irradiated) donor cells could cause GvH reactions in recipient embryos, provided they were brought into contact with embryonic hematopoietic cells of recipient type origin immediately after treatment. This was done by mixing both cell populations *in vitro* before inoculation.

Similarly, Scollay et al. (159), using the splenic explant technique, demonstrated that parental lymphoid cells treated with mitomycin C at doses which completely prevent subsequent proliferation, were able to cause enlargement of newborn F1 spleen fragments. Moreover, these mitomycin C treated parental cells gave rise to splenomegaly in newborn F1 mice, provided they were mixed with newborn F1 spleen cells prior to injection. Hence, donor cells may produce GvH reactions without proliferation, but only under particular conditions. Maximal GvH reactions, however, require proliferation of the donor T cells.

Lymphoid cell proliferation in lymphoid as well as non-lymphoid organs does not necessarily correlate with mortality. Spach and Motta (160) have shown that the rate of ^{3}H-TdR incorporation in spleen, lymph nodes, liver and kidney increased 1, 3, 9 and 5-fold, respectively, after inoculation of B10.D2 lymphoid cells into the H-2 plus non-H-2 disparate (B10.BR x DBA/2)F1 mice as well after injection into non-H-2 disparate (B10.D2 x DBA/2)F1 mice. However, there was a great difference in mortality between both groups of F1 hybrids. The mice subjected to GvH across H-2 plus non-H-2 differences were all dead by day 21 while the mice subjected to GvH across non-H-2 differences alone survived more than 28 days.

3. Helper T cells

Cantor and Boyse (161-166) have shown that murine T lymphocytes can be subdivided into subpopulations bearing specific membrane antigens. These markers correlate with distinct functional activities. Three well-defined subpopulations exist: Lyt-1, Lyt-23 and Lyt-123 positive T cells. Lyt-1+ T cells develop helper activity after stimulation by antigen, enhance the generation of killer cells and activate macrophages in DTH reactions, whereas Lyt-23+ T cells express suppressor and/or cytotoxic activity (167). Lyt-123+ T cells can amplify helper T cells and suppressor T cells during antibody production (168). Observations of Scollay et al. (169) and Mathieson et al. (170) suggest that there are two separate lines of intrathymic differentiation, one leading to Lyt-1+ cells and the other to Lyt-123+ cells.

The latter cells might further differentiate into Lyt-23+ cells after their migration as Lyt-123+ cells to the periphery (171). Lyt-1+ T cells belong to the T2 subpopulation of long-lived, recirculating T cells. A portion of the Lyt-123+ subpopulation can be eliminated by adult thymectomy and thus corresponds to the T1 subpopulation of short-lived, sessile T cells. The remainder of the Lyt-123+ T cells probably belong to the T2 subpopulation of recirculating T cells (172,173).

Studies by Vallera et al. (174) have shown that Lyt-1+ T cells are involved in lethal GvH reactions: treatment of BALB/c bone marrow cells with monoclonal anti-Lyt-1 antibodies plus complement prior to injection into lethally irradiated C57BL/6 recipients resulted in more than 80% survival at 100 days. C57BL/6 recipients receiving untreated BALB/c bone marrow cells, on the other hand, were all dead by day 35. Furthermore, treatment of bone marrow cells with anti-Lyt-2 antibodies did not prevent GvH mortality. However, in these studies the bone marrow cells were treated only once with anti-Lyt-2 plus complement (174). Rolink et al. (175) have shown by repeated treatment of the allogeneic bone marrow cells with anti-Lyt-2 plus complement, that Lyt-2+ allosuppressor/killer cells do contribute to lethal GvH disease. Without elimination of the alloreactive Lyt-2+ T cells the recipients developed pancytopenia and consequently suffered from immunosuppression leading to fatal sepsis. In H-2 compatible donor-recipient combinations differing at multiple minor histocompatibility antigens it has been shown that purified Lyt-1-2+ T cells are potent inducers of lethal GvH disease, whereas Lyt-1+2- T cells fail to cause GvH disease (176).

In the splenomegaly and PLN assays the situation may be different: Mage et al. (177) have demonstrated that after separation of Lyt-2 positive and negative cells the GvH reactivity as determined in the splenomegaly assay resides in the Lyt-2 negative cell population and not in the Lyt-2 positive fraction. This data is complemented by the studies of Loveland et al. (84,178,179) about the Lyt phenotype of T cells involved in graft rejection. These investigators thymectomized CBA/H adult mice, irradiated them and injected them with syngeneic bone marrow cells depleted of T cells. Three to four weeks later they infused these mice with CBA/H lymphocytes of select Lyt phenotypes immunized against C57BL/6 cells. When the mice were grafted with C57BL/6 skins, it was found that skin rejection was completely dependent on the presence of Lyt-1+ cells in the inoculum and that Lyt-123+ and Lyt-23+ cells had no effect. Furthermore, administration of monoclonal anti-Lyt-1 antibodies to the mice resulted in prolongation of skin allograft survival, abolition of DTH and partial suppression of helper activity (180). Skin graft rejection correlated with DTH: mice that were unable to reject a skin graft produced no significant footpad swelling upon a challenge injection of cells syngeneic to the skin graft, whereas mice which had rejected a skin graft were found to exhibit specific foot swelling of 18-42% (84).

Similarly, in rats it has been demonstrated that the GvH inducing capacity, when tested in the PLN assay, resides in the helper T cell fraction and not in the cytotoxic T cell fraction (181,182).

However, in the mortality assay both fractions were effective, be it that the cytotoxic T cell fraction was less effective than the helper T cell fraction (see also the next section). It is unclear whether these data are confused by small contaminations by T cell subsets that were supposed to be eliminated.

Taken together, these data suggest that alloreactive Lyt-1+ ('helper type') T cells are well able to induce positive reactions in GvH assays based upon direct or indirect measurement of the proliferative activity of, or induced by, donor T cells, but that it still is an open question whether Lyt-1+ T cells by themselves are able to cause lethal GvH disease.

4. Cytotoxic T cells

Since the discovery that during MLC cytotoxic T cells are generated (76), it has been studied whether these cells are also involved in GvH reactions. Cerottini et al. (183) described the generation of cytotoxic cells in the spleens of irradiated DBA/2 and C57BL/6 mice, 4 days after transplantation of 5×10^7 spleen cells from C57BL/6 and DBA/2 mice, respectively. This cytotoxic activity could be abrogated by treatment with anti-Thy-1 antiserum plus complement, thus indicating that the cytotoxic cells are T cells. These cytotoxic T cells were antigen-specific: lysis occurred only with target cells syngeneic to the recipient strain.

For maximal responses, cytotoxic T lymphocytes need two signals for their activation: signal one, provided by H-2K and/or H-2D allo-antigens and/or minor histocompatibility antigens, and signal two,

given by helper T cells after their stimulation by H-2I alloantigen(s). The H-2I alloantigen(s) activates Lyt-1 positive T cells which release a soluble product (helper factor) that stimulates the proliferation and generation of cytolytic activity by Lyt-23+ cells (184-188). This T-T cell collaboration has also been demonstrated in vivo (189).

Elkins (190) has proposed that CML assays may predict the mortality due to GvH reactions. He showed that six mouse strain combinations differing from each other with regard to H-2 subregions (involving Class I as well as Class II antigens), were positive in both the CML assay and the mortality assay for GvH. Furthermore, eight out of nine CML-negative combinations displayed no GvH mortality. All these nine combinations were lacking H-2I differences. In combinations that did not involve H-2I differences, i.e., in MLC-negative combinations, there is no helper effect so that no cytotoxic T lymphocytes are generated. This would account for the absence of GvH disease. In cases without an H-2K or H-2D region difference, on the other hand, cytotoxic effector cells would neither be generated effectively nor would they encounter the most suitable (i.e., H-2K or H-2D) determinant on the target cells. Rouse and Wagner (191) have obtained similar data in the splenomegaly assay. They showed that CBA lymphoid cells enriched for cytotoxic T lymphocytes against BALB/c spleen cells, were inefficient in causing splenomegaly upon injection into neonatal (CBA x BALB/c) F1 hybrid mice. These results indicate that there is no direct relationship between cytotoxic activity and splenomegaly. The authors explain the

inability to produce splenomegaly as follows. During the in vitro generation of cytotoxic T cells, the incidence of helper T cells gradually decreases. Since these cells play a principle role in the splenomegaly assay, inocula poor in T helper cells would not induce GvH splenomegaly. Moreover, Schreier and Iscove (192) have shown that clones of helper T cells release colony-stimulating factors for granulocytic, macrophage and erythroid precursors, especially when they are cultured together with specific antigen and accessory cells. The resultant hematopoietic and granulopoietic activity may cause spleen enlargement in GvH. Thus, depletion of helper type T cells causes a decreased GvH reactivity when measured in a lymphoproliferative GvH assay. This conclusion is supported by the results reported by Pals et al. (193).

Spleen cells from non-irradiated F1 hybrid mice, in which a GvH reaction had been induced by lymphoid cells of parental origin, have been reported to contain donor-derived specific cytotoxic T cells (194) as well as host-derived natural killer cells (194,195). Spleen cells recovered from lethally irradiated F1 hybrids undergoing a GvH reaction due to reconstitution with parental lymphoid cells lysed specifically the appropriate parental target cells (196). Thus, during GvH there is not only sensitization of the donor cells to the antigens of the recipient, resulting in their ability to lyse specifically the recipient's cells, but certain host cells may also be activated so that they attain a state of nonspecific cytotoxicity. Thus, although the specific cytotoxic component of the anti-host response probably incites the most severe host tissue destruction (176), nonspecific effects by other donor and host cells might also play a role in the pathogenesis of GvH disease.

5. Synergistic and antagonistic activities of T cell subpopulations

Since the discovery that interaction between B and T lymphocytes is essential for the optimal induction of most antibody responses, several types of immune reactivity have been found to depend on cellular interactions, e.g., between macrophages and lymphocytes or between different T cell subpopulations. It has amply been demonstrated that in murine GvH reactions also T-T and T-B interactions occur, which can lead to stimulation as well as inhibition of the response. Interactions between donor T and donor B cells hardly or not contribute to GvH reactions, whereas interactions between donor T cells and recipient B cells play a central role in the pathogenesis of GvH disease in non-irradiated hosts. Thus, negative interaction between parental T cells and F1 hybrid B cells, leading to allosuppression, causes acute, lethal GvH disease, whereas positive interaction, generating allohelp, causes lymphoproliferation and SLE-like autoantibody formation (83,102,197).

Cantor and Asofsky have described synergism between lymph node cells and thymus cells in inducing a GvH reaction as measured by splenomegaly (198). The same holds for mixtures of spleen cells and thymus cells (198). Furthermore, when mixtures of spleen cells and thymus cells from 1-wk-old BALB/c mice were used, which by themselves did not display GvH-inducing capacity, a synergistic effect could be demonstrated (198). Cantor and coworkers (199,200) demonstrated the need of at least two types of T cells that were necessary for GvH reactions: T1 cells and T2 cells.

Both cell types were of thymic origin (200). The T1 cells, mainly occurring in spleen and thymus, were resistant to the in vivo effect of ATS, but sensitive to adult thymectomy. In contrast, the T2 cells were extremely susceptible to ATS in vivo and were found mainly in the peripheral blood and lymph nodes. Both cell populations act synergistically if they are allogeneic to the host (200,201). Synergism of T1 and T2 cells has been demonstrated in the splenomegaly assay, in the GvH mortality assay (202), in GvH-related DTH reactivity (203), and in the in vitro MLC (204,205) and CML (206,207) assays.

Cantor and coworkers concluded from secondary transfer experiments involving H-2 incompatible T1 and T2 cells that, in the splenomegaly assay, T1 cells determine the specificity of the immunologic injury leading to enlarged spleens and that these cells constitute the precursors of the GvH effector cells. The T2 cells were supposed to amplify the reactivity of the T1 cells (198,200).

Blomgren and Jacobsson (208), however, have demonstrated, also using the splenomegaly assay, that the thymus contains lymphoid cells which may synergize or antagonize the GvH reactivity mediated by mouse lymph node cells, indicating that lymph node T2 type cells are the effector cells and thymus cells the T1 type amplifier cells. Several studies have confirmed Blomgren and Jacobsson's conclusion that the T2 cells are the effector cells and the T1 cells the amplifiers (see ahead).

Andersson et al. (209) have found that, in MLC, spleen cells from adult thymectomized donor mice respond as well as spleen cells from sham-thymectomized mice, at least till 90 days after operation, thus indicating that these cells, which belong to the T2 class of lymphocytes, are not amplifier, but effector cells. Similar data have been reported by our own laboratory for anti-host DTH reactivity during acute and delayed GvH reactions in mice (61,203). These data suggest that synergism between T1 and T2 cells is not a requirement per se, provided a sufficiently high number of T cells is used for induction of GvH.

The conclusion that T2 cells are the precursors of the effector cells and that T1 cells provide the amplifier effect is further corroborated by Wright et al. (210). These authors demonstrated a synergistic interaction in the *in vitro* proliferative response to alloantigen for mixtures of rat thymus and lymph node cells. Irradiation was shown to have a differential effect on the response of the thymus and the lymph node cells in the mixtures. Thus it was found that irradiated thymus cells retained the capacity for synergy in the mixtures, whereas irradiated lymph node cells did not. Furthermore, it was shown that thymus cells can proliferate in response to alloantigen as well as have the capacity to amplify the proliferative response of lymph node cells - a capacity which requires *de novo* protein synthesis. Lymph node cells were found to lack the amplifier capacity.

The mechanism underlying this T-T interaction may be inferred from the data of Feldmann and Erb (211). These authors presented

evidence that T1-T2 cell synergism in the generation of helper T cells can occur across a cell-impermeable nucleopore membrane, indicating that it did not depend on cell contact, but was mediated by subcellular factors.

Our own laboratory has previously shown that after transplantation of limited numbers of spleen cells into lethally irradiated hosts, T1-T2 cell cooperation is required for optimal development of anti-host DTH reactivity (203). Anti-host DTH effector T cells were found to be the progeny of the pool of recirculating T2 cells, which are activated by H-2I region coded antigens. The response by these T2 cells is amplified by T1 cells, which are most likely activated by H-2K and/or H-2D region coded antigens. T1 cells themselves were found to be incapable to display anti-host DTH reactivity. Synergism between T1 and T2 cells in GvH also occurs across Mls differences (212). T2 cells respond to the Mls locus coded antigens and T1 cells amplify this response. Most likely, these T1 cells recognize other non-H-2 alloantigens. These data substantiate the parallel between the functional aspects of the H-2I subregion of the MHC and the Mls locus.

Interactions between T lymphocytes in GvH may also occur across H-2K plus H-Y differences (213). Thus, lymphocytes from female bm-1 mutant mice cause greater mortality in (bm-1 x b)F1 males than in F1 females. This greater mortality among males is related to recognition of both H-Y and $H\text{-}2K^b$ antigens by the different sets of donor lymphocytes. However, such a potentiating effect was not seen in other H-2K disparate combinations.

Furthermore, this potentiation was only observed within a limited cell dose range, around 10^7 lymph node cells and 2 x 10^7 spleen cells.

This phenomenon of potentiation of the reaction towards a minor H antigen plus a major H antigen may be a general phenomenon. Pritchard and Halle-Pannenko (214) described a greater severity of the GvH reaction after grafting C57BL/6 lymphoid cells into irradiated (C57BL x DBA/2)F1 recipients than into (C57BL/6 x BALB/c)F1 recipients. These authors showed that this observation arose from a potentiating effect of lymphocytes recognizing $H\text{-}2^d$ and DBA/2 minor H antigens and not from a difference in genetic resistance to the C57BL/6 grafts exhibited by the F1's. However, this synergistic effect depended on the assay used to detect GvH reactivity. The phenomenon could only be substantiated in the mortality assay and not in the splenomegaly assay. Moreover, the authors did not take into account the Mls differences between both F1 recipients. The BALB/c strain bears the Mls^b determinant, which is incapable of inducing proliferation of T lymphocytes, while the DBA/2 strain bears the Mls^a determinant which is very potent in eliciting proliferative responses (110). Furthermore, it has been proved by Rodey et al. that Mls incompatibilities by themselves already can cause lethal GvH disease (80). Thus, this synergism may be due to the Mls^a locus of DBA/2 and should be verified by using Mls identical strains.

An interesting form of T-T cooperation has been described by Cohen and Livnat (215-219).

When lymphocytes are sensitized to allogeneic fibroblasts in vitro for 16-18 hr and subsequently injected in the footpad of syngeneic mice, these sensitized lymphocytes recruit other, circulating lymphocytes to the draining popliteal lymph node. Subsequently, these recruited lymphocytes develop into effector T cells, e.g., cytotoxic cells or GvH-reactive cells. The in vitro sensitized lymphocytes are called 'initiator T lymphocytes' (ITL) and the lymphocytes which are recruited to the lymph node 'recruited T lymphocytes' (RTL). The ITL are present in the spleen and, to a smaller extent, in the thymus, but not in the lymph nodes. RTL are present in spleen and lymph nodes but not in the thymus. ITL are sensitive to adult thymectomy and resistant to ATS, thus resembling T1 cells. RTL are resistant to adult thymectomy and sensitive to ATS, thus resembling T2 cells. Hence, this conforms with Blomgren and Jacobsson (208), but not with Cantor and Asofsky (198).

When a recruitment response in popliteal lymph nodes is induced by injecting mouse footpads with syngeneic, sensitized ITL, GvH-reactive specific RTL are depleted from other lymphoid organs due to the trapping in the PLN. These lymphocytes reappeared in the other lymphoid organs after trapping ended in the PLN. Excising the PLN at the height of the recruitment phase significantly delayed reappearance of the specific GvH reactive lymphocytes in the other lymphoid organs.

Not only synergism of lymphocyte subpopulations may occur in GvH, but also antagonism.

In 1972 Gershon et al. (220), studying the regulation of murine antibody formation, demonstrated the existence of T cells with suppressor capacity. Some years later, the same authors demonstrated that these cells can also regulate GvH reactions (221). This was shown by injecting parental thymocytes into irradiated F1 hybrid mice and by measuring the DNA synthesis in the lymph nodes and spleen. Suppression of DNA synthesis by parental thymocytes could be modulated by the concomitant injection of F1 thymocytes. The F1 thymocytes increased the response when a small number of parental cells was injected and depressed the response when a large number of parental cells was injected.

Van Bekkum and Knaan (222,223) described amelioration of GvH disease in mice by T lymphocytes. They grafted lethally irradiated F1 mice with parental bone marrow and spleen cells and observed acute GvH disease with 100% mortality within 12 days. However, when thymocytes of 4-6 days old mice from the same parental strain were added to the graft, the mortality was reduced and delayed. This amelioration was mediated by T cells (223).

Halle-Pannenko et al. (224) have shown that mortality attributable to GvH could be delayed and reduced by treating the donors with soluble H-2 alloantigens. Both the delay of mortality and the cumulative mortality were found to depend on the dose of soluble H-2 alloantigens used to treat the donors.

Furthermore, these authors showed that lethal GvH reactions to minor H antigens could be decreased by simultaneous immunization of the donors with H-2 and non-H-2 alloantigens (225). This decrease varied as a function of the H-2 haplotype used for preimmunization. It is not clear whether suppressor T cells are involved in the delay and reduction of mortality observed.

Using the GvH-related DTH assay (see Fig. 1), we have demonstrated the regulating influence of suppressor T cells in GvH. The suppressor T cells were induced by iv preimmunization of spleen or bone marrow cell donors with recipient-type irradiated allogeneic lymphoid cells. These suppressor T cells were capable of suppressing the anti-host immune reactivity during acute as well as delayed GvH reactions (226,227) and were found to have the Lyt-12+ phenotype (228).

The suppressor T cells regulating the anti-host DTH reactivity require proliferation in order to display their suppressive effect. This can be deduced from the observation that treatment of spleen cells from suppressed donors with mitomycin C *in vitro*, which were subsequently transferred with untreated spleen cells from non-suppressed donors to irradiated allogeneic recipients, resulted in abolition of the suppression which normally occurs after combining non-suppressed and suppressed spleen cells (Fig. 2).

We have investigated the specificity of these suppressor T cells at two levels. Firstly, in the activation of suppressor T cells and secondly with regard to the suppressive effect displayed by the suppressor T cells once they are activated.

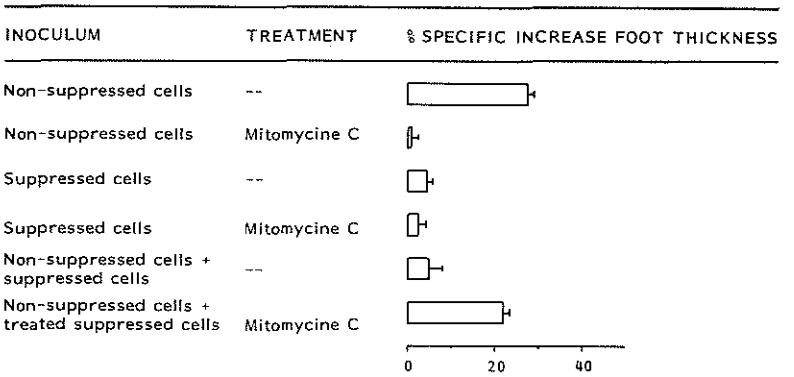

Fig. 2. Anti-host DTH reactivity in lethally irradiated (C57BL x CBA) F1 mice inoculated with either 1 x 10^7 non-suppressed, 1 x 10^7 suppressed, 1 x 10^7 non-suppressed plus 1 x 10^7 suppressed, 1 x 10^7 non-suppressed plus 1 x 10^7 mitomycin C-treated suppressed, or 1 x 10^7 mitomycin C-treated non-suppressed DBA/2 spleen cells. Recipients were tested for DTH reactivity 6 days after irradiation and reconstitution. DTH responses were measured 40 h after challenge. Each horizontal bar represent the arithmetic mean ± 1 SEM of 6 mice.

The specificity of activation of the suppressor T cells was investigated by iv preimmunization of the donors with irradiated allogeneic spleen cells of a particular H-2 haplotype and subsequent testing of the GvH-related DTH reactivity in irradiated recipients of another H-2 haplotype. Thus, BALB/c mice were pretreated with either irradiated BALB/c, BALB.B or BALB.K spleen cells iv and subsequently used as donors of spleen cells to induce GvH in lethally irradiated BALB.B and BALB.K recipient mice. We found that spleen cells from BALB/c mice suppressed to BALB.B or BALB.K do not react against BALB.B and BALB.K recipients, respectively (Fig. 3, upper part). However, BALB/c mice suppressed to BALB.K do respond to BALB.B. The same results were obtained in experiments with B10.D2 donor mice and B10.ScSn and B10.BR recipients (Fig. 3, lower part). Thus, H-2

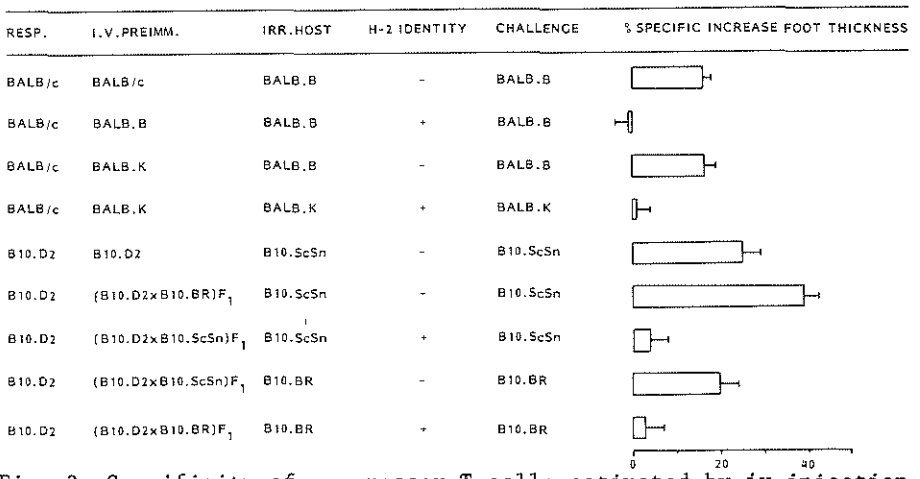

Fig. 3. Specificity of suppressor T cells activated by iv injection of irradiated spleen cells of different H-2 haplotypes. BALB/c mice were preimmunized with either BALB/c, BALB.B or BALB.K spleen cells. Four days later 2 x 10^7 spleen cells from these pretreated BALB/c mice were used to reconstitute irradiated BALB.B and BALB.K mice (upper part). According to the same schedule, B10.D2 mice were iv injected with either B10.D2, (B10.D2 x B10.BR)F1 or (B10.D2 x B10.ScSn)F1 irradiated spleen cells. Four days later 5 x 10^7 spleen cells from these pretreated B10.D2 mice were used to reconstitute lethally irradiated B10.ScSn and B10.BR recipients (lower part). In all groups of mice the anti-host DTH reactivity was determined in a transfer system 5 days after reconstitution of the recipients. DTH responses were measured 24 h after challenge (reprinted by kind permission from ref. 227).

identity is required between the irradiated allogeneic cells used to induce the suppressor T cells and the irradiated recipients in order to ensure optimal suppression. Thus, the suppressor T cells are antigen-specific with regard to their recognition of the alloantigen.

The specificity of the suppressive effect by the activated suppressor T cells was studied using spleen cells from BALB.B mice, suppressed to BALB/c alloantigens, to reconstitute irradiated (BALB/c x BALB.K)F1 mice. These recipients have in addition to the $H\text{-}2^d$ alloantigens of BALB/c that induced the suppressor T cells, 'third party' $H\text{-}2^k$ alloantigens of BALB.K origin. We found that the reaction against these so-called 'bystander' $H\text{-}2^k$ alloantigens was

effectively suppressed (Fig. 4).

Similar data were obtained in other strain combinations. Thus, the antigen-specific suppressor T cells are capable of non-specific suppression of the anti-host DTH reactivity to third party H-2 alloantigens of the recipients, provided these alloantigens are presented as bystanders to the alloantigens that had induced the suppressor T cells (229,230).

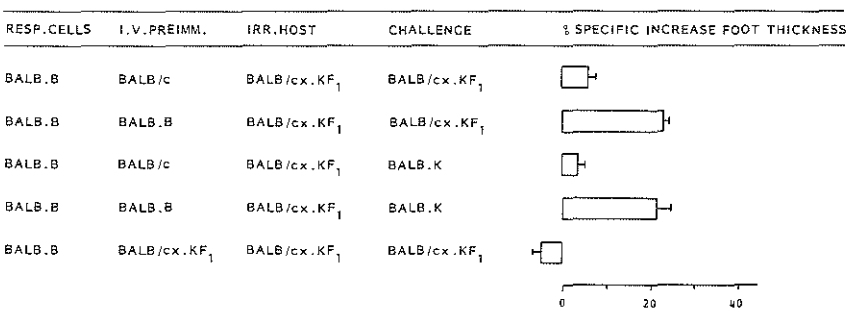

Fig. 4. Capacity of spleen cells from suppressed donors to suppress the GvH-related DTH reactivity to bystander H-2 alloantigens. BALB.B donor mice were preimmunized with either BALB/c, BALB.B or (BALB/c x BALB.K)F1 irradiated spleen cells. Four days later 2 x 10^7 spleen cells from these suppressed donors were used to reconstitute lethally irradiated (BALB/c x BALB.K)F1 mice. Five days after irradiation and reconstitution the anti-host DTH reactivity was determined by challenging secondary recipients with either (BALB/c x BALB.K)F1 or BALB.K spleen cells. DTH responses were measured 24 h after challenge (reprinted by kind permission from ref. 227).

Shand (231) demonstrated that spleen cells from F1 hybrid mice subjected to GvH by injection of parental spleen cells could suppress the *in vitro* antibody response of normal F1 spleen cells to chicken red blood cells and to levan. He demonstrated that this suppression was mediated by T cells of parental origin. By transfer of GvH cells to irradiated repopulated recipients challenged with

thymus-independent antigen he showed that the suppressor T cells most likely exert their suppression via an anti-mitotic influence on the antigen-stimulated B cells. In other studies, Elie and Lapp (232,233) demonstrated that during GvH also Thy-1 negative suppressor cells are generated which can suppress the *in vitro* plaque-forming cell response to heterologous erythrocytes.

The Lyt phenotype of suppressor T cells arising in mice during a GvH reaction was determined by Pickel and Hoffmann (234). These investigators showed that these cells bear the Lyt-12+ phenotype. Shand (235) reported that suppressor T cells, generated in his system, also bear the Lyt-12+ phenotype.

After total lymphoid irradiation (TLI) of adult BALB/c mice, allogeneic bone marrow cells injected into these animals evoke a state of tolerance rather than immunity (236).

This state of unresponsiveness to allogeneic bone marrow cells in TLI-treated mice is related to the presence of T cells that nonspecifically suppress the MLC *in vitro* and the GvH disease *in vivo* (237). It was shown that two subpopulations of suppressor cells may exist in TLI-treated mice: a TL^+ subpopulation that mediates suppression of antibody responses and a TL^- subpopulation that mediates suppression of MLC and thus possibly also of GvH. These suppressor cells could be demonstrated up to 5 months after TLI (238). The finite duration of the GvH reaction may be ascribed to such suppressor cells.

In humans, during the first four months after allogeneic bone marrow transplantation, a state of immunodeficiency occurs. Later on,

the patients who have recovered from GvH disease regain their immune reactivity, whereas patients with chronic GvH disease remain immunologically impaired. Evidence has been presented that the basis for this immunodeficiency may be a deficiency in some kind of immunoregulatory cell (239-244). Patients with chronic GvH disease possess circulating cells that suppress lymphocytes from the marrow donor to proliferate in response to unrelated lymphocytes (245), i.e., the suppression is nonspecific. This might explain why patients suffering from chronic GvH disease are predisposed to infections (246).

In contrast to patients with chronic GvH disease, longterm stable chimeras without GvH disease lack anti-host cellular immune responses (247-249a). Apparently, the donor derived cells in these chimeras are tolerant to the host antigens (see also Section VII).

VI. MECHANISMS UNDERLYING GVH

The cellular changes in most of the target organs of individuals subjected to GvH have already been described in Section II.3, 'Pathology of GvH'. Below we will describe and discuss the changes occurring in the lymphoid organs and the mechanisms underlying GvH. From these, the thymus has been most extensively investigated.

As the phenomenology and mechanisms of GvH reactions in irradiated and non-irradiated recipients differ in many respects, we will discuss below both conditions separately.

1. GvH in irradiated recipients

In the thymus of murine parent to F1 hybrid radiation chimeras, the number of cells increases approximately 10-fold between 7 and 14 days after reconstitution with bone marrow cells (250). At least 50% of the thymocytes on day 14 are of host origin. These cells can respond to virus presented in the context of both parental H-2 haplotypes when primed in irradiated, virus infected F1 hybrid recipients. The responder potential of day 14 thymocytes was retained after radiation doses as high as 1200 rads. The host component was no longer detectable by day 28. Similar data have been presented by Ceredig and MacDonald (251) for the AKR→CBA combination. After allogeneic bone marrow transplantation into lethally irradiated recipients the donor-derived CTL-precursor content of the thymus is initially very low but starts to increase between 10 and 20 days after bone marrow transplantation. (251).

The ratio between donor and host derived CTL precursors further increases with time. Thymocytes and peripheral lymphoid cells of donor origin can be activated by virus-infected cells 3 to 5 weeks after irradiation and reconstitution (251).

Onoé et al. (252) described sequential changes of thymocyte surface antigens in lethally irradiated mice reconstituted with syngeneic or allogeneic bone marrow cells.
They found an increased expression of H-2 and Lyt-1 surface antigens on thymocytes after allogeneic bone marrow transplantation and a decreased expression of Thy-1 and Lyt-2 surface antigens as compared to syngeneically reconstituted mice. Similar findings have been reported by Mathieson et al. (170).
It may be that these differences in expression are caused by changes in the adrenocorticoid hormone levels which are increased due to the stress caused by the GvH disease. Onoé et al. (252) did not correlate these changes in surface antigen-expression to the functional capabilities of the cells, i.e., it is not clear whether cells with increased Lyt-1 expression have a higher or lower helper-like activity, etc. Whether these changes also occur in the peripheral lymphoid organs is unknown as yet.

Kruisbeek et al. (253) demonstrated that chimeric thymocytes were entirely of donor origin approximately 4 weeks after irradiation and reconstitution. By that time, however, the thymocytes are unable of autonomously generating CTL responses to alloantigens and trinitrophenylated syngeneic spleen cells.

However, they do so at this time point in the presence of interleukin-2 (253). Hardt et al. (254) reported that during acute GvH disease Lyt-23+ cells produce interleukin-2 (IL-2) inhibitor. This IL-2 inhibitor has been claimed to counteract the activity of the IL-2 derived from the Lyt-1+ helper T cells and thereby inhibit the activity of other T cells that require IL-2 for their proliferation, e.g., cytotoxic T cells. This may explain the deficient anti-viral cytotoxic responses during acute GvH disease(23,31), which in turn may enhance the chance of fatal infections.

With regard to the kinetics of the anti-recipient cytotoxic T cell response in the spleen of mice subjected to GvH disease induced by bone marrow transplantation across minor histocompatibility antigens, the existence of two subpopulations of cytotoxic T cells was revealed, namely cytotoxic T lymphocytes and their precursors. The effector CTL were present in recipient spleens 2 and 3 weeks after transplantation but they disappeared from the spleen before the onset of clinical disease. CTL precursors were present in the spleen starting 2 weeks after transplantation and could be detected during clinical disease as well (255).

Acute GvH reactions are unanimously attributed to alloreactive T cells in the donor inoculum. With regard to the mechanism underlying delayed GvH reactions there is no general agreement. Several theories have been presented to explain the occurrence of delayed GvH reactions.

There are two lines of thoughts: either immunocompetent T lymphocytes which contaminate the bone marrow inoculum are responsible for delayed GvH reactions, just as for acute GvH, or the T cells which are newly formed under influence of the recipient's thymus are responsible for causing delayed GvH reactions.

The first notion is supported by the observation of many investigators that after velocity sedimentation or density separation of bone marrow cells, the lymphocyte-rich fractions (256) and the fractions with the greatest in vitro proliferative responsiveness to allogeneic cells (257) cause the severest GvH reactions. Furthermore, Löwenberg et al. (17) demonstrated the absence of acute GvH disease and a delay in the appearance of secondary GvH disease after transplantation of allogeneic fetal liver cells, which do not include morphologically and functionally distinguishable T lymphocytes. Korngold and Sprent (47) demonstrated that chronic GvH disease elicited in irradiated mice by allogeneic bone marrow transplantation across non-MHC antigenic differences could be prevented by thorough treatment of the bone marrow inoculum with anti-Thy-1 antiserum plus complement, thus indicating that mature T cells residing in the bone marrow inoculum are responsible for eliciting this syndrome.
Similar results were obtained by Muto et al. (258) and Norin and Emeson (259) for allogeneic bone marrow transplantation across MHC barriers.

There are other investigators who have presented data which can be taken as evidence that delayed GvH disease may be due to the lym-

phoid progeny of donor stem cells that have matured in the host's thymus.

For instance, thymectomy of the host before allogeneic (260) or xenogeneic (261) bone marrow transplantation can delay the mortality due to GvH disease.

Wolters et al. (61) observed a delay in the onset of GvH-related anti-host DTH reactivity after thymectomy of the hosts. Also the studies of Lydyard and Ivanyi (262) suggest that newly-formed T cells may contribute to the development of GvH disease. Chen et al. (138) have shown that marrow from thymectomized donors infused into thymectomized allogeneic hosts exhibited a markedly reduced capacity and rate of killing, as compared to non-thymectomized recipients of bone marrow cells from thymectomized donors. Moreover, Rabinowich et al. (263) showed that fetal liver chimeras, made by lethally irradiating thymectomized F1 mice and subsequent reconstitution with parental fetal liver cells possessed no T cell function. No GvH disease was reported in such animals.

These studies, however, do not prove that newly-formed T cells by themselves can cause GvH disease. Instead, the available data suggest that the anti-host reactivity during GvH disease is due to mature T cells in the graft, while T cells which have matured from pre-thymic donor T cells under influence of the recipient's thymus may somehow enhance or accelerate this reactivity.

Kindred (264-266) and Korngold and Sprent (267,268) demonstrated that in lethal delayed GvH reactions the donor T cells attack the host in a H-2 restricted manner. In the system used by Kindred, T

cell donors are established radiation chimeras (made by irradiation and reconstitution with syngeneic or allogeneic fetal liver cells). Irradiated recipient mice die after reconstitution with T cells from such donors if they share a restriction element with the donor T cells and also have one or more histocompatibility antigens which are recognized as foreign by the donor T cells.

It was found that H-2D and H-2K molecules, but not H-2I-A molecules can act as restriction elements. H-2K, H-2I-A and minor H could all serve as target histocompatibility antigen for the alloreactive T cells. Moreover, when lethally irradiated mice were reconstituted with T cells from normal A, A.SW and CBA donors, H-2 restricted mortality of these recipients could be demonstrated. In contrast, T cells from normal C57BL/6 donors caused only unrestricted killing of lethally irradiated recipients (265). The simplest explanation for these data is that normal mice possess two different populations of T cells, each being capable of causing lethal GvH disease. One is responsible for H-2 restricted GvH-reactivity and the other for unrestricted GvH. Dependent on the proportions of these two populations the aforementioned different killing patterns can be observed. It is suggested by Kindred that during the regeneration of radiation chimeras, the balance is in favor of the T cells that recognize the target antigens in a H-2 restricted manner.

In their elegant studies on lethal GvH disease after bone marrow transplantation across minor histocompatibility barriers, Korngold and Sprent (267,268), too, observed MHC-restricted killing. They used a system in which the donor T cells were exposed to minor H antigens

during blood to lymph recirculation through irradiated allogeneic mice. After collection of the thoracic duct lymphocytes these cells were transferred into irradiated secondary recipients and tested for their ability to cause mortality of these hosts. The investigators demonstrated that T cells producing chronic GvH disease to minor H antigens consist of separate subgroups of H-2K and H-2D restricted cells.

They found no evidence that H-2I restricted T cells contributed to GvH disease either as effector or as helper T cells: pretreatment of T cells with anti-Lyt-2 antibody plus complement, thus leaving the Lyt-1+ cells intact, failed to cause lethal GvH disease. If I-restricted T cells were needed to provide helper function for the H-2K/D restricted T cells, elimination of Lyt-1+ cells would be expected to abolish GvH.

This proved not to be the case: the surviving cells show little reduction in their potency. Hence, in terms of H-2 restriction and Lyt phenotype, GvH disease-inducing T cells are undistinguishable from minor H antigen-specific T killer/T suppressor cells. Both populations recognize the minor H antigens in the context of H-2K/D (but not H-2I) molecules and both express the Lyt-1-2+ phenotype. Moreover, it has been shown by Widmer and Bach (269) that there exist helper-independent cytotoxic T cells. This may explain why GvH disease inducing T cells are capable of functioning independent of helper T cells.

Since minor H antigens are present on virtually all cell types it is to be expected that any cell type can present minor H

antigens to the alloreactive T cells in vivo and thereby can elicit the GvH reaction. It has been shown, however, that in non-H-2 incompatible donor-recipient combinations the T cells ignored minor H antigens expressed on radioresistant non-hematopoietic stromal cells of the host (268). Thus, the minor H antigens have to be presented by marrow-derived cells and in order to induce T cell selection, marrow-derived cells have to share particular H-2 determinants with the T cells. However, in the effector phase non-marrow derived cells are the main targets for attack (see Section II.3).

In this instance it is noteworthy to mention the data of Mowat et al. concerning the intestinal phase of GvH: infiltration of the mucosa by intra-epithelial lymphocytes required Lyt-1+2- T cells: the development of full crypt hyperplasia required Lyt-1+2- as well as Lyt-1+2+ T cells, although Lyt-1+2- cells alone also display some activity. Lyt-1-2+ cells were found to be ineffective. These results were obtained by reconstitution of neonatal F1 mice with parental spleen cells depleted of particular Lyt subsets by monoclonal antibodies plus complement (270,271; Mowat, A.McI., Borland, A. and Parott, D.M.V., unpublished).

The acute form of GvH disease seen in MHC-incompatible combinations, and the chronic type of GvH seen in MHC-compatible, non-MHC incompatible combinations, (see Section II.3) have distinct histopathological features. Hence it might be possible that the pathogenetic events underlying anti-MHC and anti-non-MHC GvH are different. An alternative possibility is that the differences observed were caused by the intensity of the T cell response. Thus,

the incidence of acute and chronic GvH disease may vary depending on the number and function of donor lymphocytes grafted. This concept is supported by the data of Hamilton and Parkman (272). They showed that high doses of lymphocytes injected into MHC-compatible, non-MHC-incompatible lethally irradiated mice favored the development of acute type GvH disease, whereas low numbers favored the development of the chronic type (272). Similarly, Korngold and Sprent (176) described that injections of small numbers of T lymphocytes across H-2 barriers can cause chronic GvH disease.

A possible explanation for this is that small doses of donor T cells provide an amount of allohelp that is too small to induce the alloreactive T suppressor/T killer cells within the T cell circuit (273).

The concept that GvH disease in MHC-incompatible and non-MHC-incompatible combinations are pathogenetically different seems to be supported by the finding of Vallera et al. (174) and Korngold and Sprent (176) in mice that GvH disease to H-2 differences is elicited by Lyt-1+2- T cells, whereas Lyt-1-2+ cells elicit only weak reactions (174,176) or no reaction at all (175). Comparable data were found in rats (274). Furthermore, T cells that cause severe mortality in MHC-compatible, non-MHC-incompatible chimeras, do not cause mortality in MHC-incompatible, non-MHC-compatible chimeras (176).

2. GvH in non-irradiated recipients

After parental spleen cell transplantation into non-irradiated F1 hybrid mice two forms of GvH disease can occur: acute and chronic.

(83,275). The acute form is characterized by pancytopenia and is caused by allosuppressor/killer T cells bearing the Lyt-1+2+ phenotype (175,273). The chronic GvH disease in non-irradiated allogeneic mice is characterized by stimulatory pathological phenomena which include B cell hyperreactivity and the formation of autoantibodies (see Section IX). However, despite these stimulatory phenomena, animals and patients with chronic GvH disease are still partially immunodeficient (243-246). Helper T cells bearing the Lyt-1+2- phenotype play a dominant role in chronic GvH disease (175). Thus, the cellular requirements for different forms of GvH disease are as follows: parental lymphoid cells enriched for allohelper T cells favors chronic GvH disease in non-irradiated F1 recipients, regardless of the P→F1 combination tested, whereas inocula enriched for allosuppressor/killer T cells induce the more severe acute form of the disease with concomitant hypoplasia of the immune system. The genetic requirements for these types of GvH were as follows: Class II (I-A/I-E) alloantigenic differences between donor and host cause activation of the alloreactive donor helper T cells. Class I (K/D) alloantigens differences, on the other hand, stimulate alloreactive donor suppressor/killer T cells (102). However, the allosuppression induced across Class I alloantigens only is relatively weak, whereas strong allosuppression can be detected in those donor-recipient combinations which differ in both Class I and Class II alloantigens (102). It is noteworthy, that in the latter combinations the allosuppression is preceded by a brief phase of allohelp (276,277). It has been postulated that Class II

reactive Lyt-1+2- donor helper T cells activate Class I-reactive allosuppressor/killer effector T cells (276,277). Initially, these allosuppressor/killer T cells seem to bear the Lyt-1+2+ phenotype and later during the GvH reaction they are Lyt-1±2+ (273,278,279). The latter may act via the release of anti-mitotic factors or interleukin-2 inhibitors that hamper proliferation of e.g., the lymphohemopoietic tissue and of interleukin-2 dependent T cells (254). This causes a general pancytopenia and a state of immunodeficiency leading to sepsis and death.

In humans suffering from GvH disease it has been demonstrated that B and T lymphocytes do not interact properly (280). Furthermore, an increased incidence of immature lymphocytes (281) and of cells bearing the suppressor cell phenotype has been found (282). This may cause the profound state of immunodeficiency seen after bone marrow transplantation in man.

The resolution of acute or chronic GvH disease may be due to the appearance of specific suppressor T cells (248), which maintain a state of unresponsiveness of immune reactivities directed to host antigens. Alternatively, the alloreactive donor T cells may simply disappear or die out. During the state of chimerism, full immunocompetence may be attained, at least for thymus-independent responses (31). Damaging effects of cytotoxic T cells may be profound provided the appropriate antigenic differences and the stimulating signals delivered by helper T cells are present (see Section V-4).

To reconcile the aforementioned phenomena it should be borne in

mind that GvH disease presents a spectrum of abnormalities in which it is well possible that all lymphoid cell types are simultaneously involved (273,277,283). Furthermore, the different mechanisms underlying GvH reactions in irradiated and non-irradiated recipients indicate that host lymphohemopoietic cells influence the activity of the infused donor T cells.

VII. MECHANISMS UNDERLYING THE RESOLUTION OF GvH

After resolution of chronic GvH disease, stable chimeras are established. The principal theories to account for stable chimerism are the deletion of donor cells capable of reactivity against recipient histocompatibility antigens (clonal deletion theory) (284) and the induction of specific and nonspecific suppressor cells (suppressor T lymphocytes and perhaps macrophages) that regulate the reactivity of the hostreactive donor type lymphocytes (suppressor cell theory) (285). Thus, the suppressor T cells involved in the resolution of GvH, have other target cells than the donor-type suppressor T cells that cause lethal GvH disease (83).

Several studies have been performed to discriminate between these two alternatives and the results show that the suppressor cell theory might well be right, at least in several models. Weiden et al. (286) made stable canine radiation chimeras by total body irradiation (1200 rad) and subsequent infusion of bone marrow cells from littermate donors matched for the MHC. Attempts were made to perturb the stable chimeric state by infusion of large numbers of donor peripheral blood lymphocytes at 7 to 30 months post transplantation. However, none of the nine chimeras studied developed significant clinical or histological evidence of GvH disease. If clonal deletion of hostreactive lymphocytes would have taken place, infusion of donor lymphocytes should have resulted in a perturbation of stable graft-host tolerance and induction of fatal GvH disease should have occurred.

Since cytotoxicity of donor lymphocytes for fibroblasts of the chimera could not be detected, neither before nor after lymphocyte infusion and the presence of blocking serum factors was ruled out, the authors raised the possibility that a suppressor cell population might be responsible for the stable state of chimerism (286). However, these data can be equally well explained by supposing that during the chronic GvH disease the expression of alloantigenic determinants by the lymphohemopoietic host cells gradually decreased, so that, finally, the original allogeneic stimulus was lacking.

Atkinson et al. (287) described that cells from their long-term stable canine radiation chimeras reconstituted with bone marrow cells from MHC-incompatible littermates showed markedly diminished but still detectable reactivity to host lymphocytes in vitro, thus excluding permanent clonal deletion. Tutschka et al. (288-290) were able to identify suppressor T cells in stable rat radiation chimeras. These suppressor T cells specifically inhibited donor to host MLC and adoptively transferred suppression of GvH disease to secondary hosts. In stable murine irradiation chimeras, on the other hand, so far no specific suppressor cells could be demonstrated in vitro (291,292). In the studies of Tutschka et al. (288-290) with rat chimeras, nylon-wool fractionation of the chimeric spleen cells restored the response of the chimeric lymphocytes to host alloantigens, indicating that the state of tolerance must be attributed to the activity of nylon wool-adherent suppressor T cells. These authors also demonstrated that infusion of stable radiation chimeras with bone marrow cells or spleen cells from normal donors did not break the tolerance.

Tutschka et al. (289) demonstrated that in stable rat radiation chimeras, two years after grafting, suppressor T cells could no longer be identified by in vitro methods, but could still be demonstrated by adoptive transfer in vivo. After injection of irradiated host-type spleen cells into these chimeras, the suppressor cells could be demonstrated again in vitro. Thus, these suppressor cells undergo a gradual clonal reduction. This reduction can be enhanced by transferring lymphoid spleen or bone marrow cells from stable chimeras into irradiated recipients of the original donor strain, i.e., the suppressor cells are more or less 'parked' in a target-antigen-free environment. After 'parking' for 120 days in these secondary recipients, the suppressor cells are no longer capable of adoptive transfer of suppression of GvH disease. However, when chimeric cells in these secondary recipients are stimulated with original hosttype antigens, clonal expansion of the suppressor cells occurs.

The clonal deletion theory, on the other hand, is supported by the studies of Sprent et al. (284,293). They prepared semi-allogeneic radiation chimeras by lethally irradiating mice and reconstituting them with anti-Thy-1 treated semi-allogeneic bone marrow cells. After six months, thoracic duct lymphocytes from these chimeric mice were assessed for anti-host reactivity in MLC and CML. It was found that the lymphocytes were responsive to host-type determinants in MLC, but not in CML. These findings were explained as follows: stimuli for the MLC are controlled by the I region of the H-2 complex (i.e., Class II alloantigens) and are expressed predominantly by lymphohemopoietic cells.

Lymphocytes of radiation chimeras entirely repopulated with donor-type cells do not encounter these determinants. This lack of MLC determinants would fail to cause elimination of cells reactive to these determinants, thus donor-derived lymphocytes would still possess the potential to respond to host type MLC determinants. Host type CML determinants (i.e., Class I alloantigens), on the other hand, not being confined to lymphoid cells but present on virtually all somatic cells, would still exist in the chimeras and therefore be available to confront the CML reactive cells continuously, thereby causing a deletion of host-reactive lymphocytes, presumably by exhaustive proliferation. The presence of suppressor cells was ruled out in these studies since addition of chimera lymphocytes to normal donor-type lymphocytes did not prevent the latter from differentiating into cytotoxic lymphocytes. This could be confirmed by Pals et al. (193) for two other types of chimeras.

Using tetraparental bone marrow chimeras (TBMC) prepared by lethally irradiating F1 mice and subsequently reconstituting them with anti-Thy-1 treated bone marrow cells from each parental strain, it was demonstrated that thymus cells, spleen cells and thoracic duct lymphocytes failed to cause lysis of blast cells carrying host type CML determinants. These cell types were also unresponsive to host type MLC determinants (284). This might be explained by the fact that host type MLC determinants were presented to each of the populations of differentiating T cells by the lymphohemopoietic cells of the opposite parental strain, thus resulting in a clonal deletion of the specific MLC reactive cells from either parental strain due to exhaustive proliferation of the responding cells.

In these TBMC, also no evidence of suppressor cells was found as chimeric lymph node cells cultured in vitro with normal parental cells did not prevent the proliferative response of the latter; the same was found in the CML. Moreover, it was found that the tolerant state was not abrogated when chimeric lymphoid cells were maintained for two weeks in an environment lacking host type CML determinants. In case the tolerance had been maintained by suppressor cells, the tolerance should have been abrogated almost immediately after removal of the suppressor cells (294).

The clonal deletion theory and the suppressor cell theory are not mutually exclusive: Green et al. (295) showed that in mice activated suppressor T cells bearing the Lyt-2+ phenotype are capable of abrogating the functional activity of two T cell subsets, namely the specific helper T cells for antibody formation and the specific T cells that had induced the Lyt-2+ suppressor T cells. This clonal deletion could even be demonstrated after that the suppressor T cells had been removed.

Hence, immunological tolerance in stable chimeras due to clonal deletion and to T cell suppression might rest on the same mechanism.

In summary, the immune reactivity of stable radiation chimeras depends on the cellular and genetic background of the cells used for reconstitution as well as upon the type of immune reactivity evaluated. In general, after bone marrow transplantation across MHC barriers, the recipients may not become fully immunocompetent after resolution of GvH disease, especially with regard to MHC-restricted T cell dependent antibody responses and cytotoxic responses against virus-infected cells.

Witherspoon et al. (244) described in a study on immune functions of humans reconstituted with HLA-matched bone marow cells, that all patients tested displayed a very low level of antibody production at 180 days after transplantation. Later on, patients without chronic GvH disease showed antibody responses indistinguishable from those of normal controls. However, patients with chronic GvH disease still displayed impaired antibody responses to all antigens tested.

VIII. T CELL REPERTOIRE OF RADIATION CHIMERAS

Stable radiation chimeras are valuable tools to study the immune reactivity of T cells that have differentiated in an allogeneic thymus. The generation of mature T lymphocytes occurs for a large part in the thymus. Antigens encoded for by the MHC and expressed on thymic reticulum cells are often thought to determine the restriction specificity of the maturing T cells, i.e., murine T cells reactive to conventional antigens ('X') respond much better to X-plus-self-H-2 than they do to X-plus-nonself-H-2. In the case of viral antigens, the self antigens involved are being coded for by the H-2K or H-2D regions (296). When parental strain recipient mice (A) are irradiated and reconstituted with hemopoietic cells from (A x B)F1 mice and subsequently immunized with minor H antigens (297) or vaccinia virus (298), activation of cytotoxic T cells occurs preferentially when the antigen is presented in association with A-derived MHC-coded antigens and not in association with B-derived MHC-coded antigens. Thus, the H-2 type of the host determines the H-2 restriction specificity of cytotoxic T cells. In contrast, chimeras made by reconstituting irradiated A mice with adult spleen cells of (A x B)F1 origin, generate virus-specific cytotoxicity to infected A and B targets, indicating that mature T cells do not change their restriction specificity (298) and effectively cooperate with macrophages from the infused spleen cell inoculum.

Using $H\text{-}2^{bm1}$ mutant mice, which carry mutations in the H-2K region, it could be demonstrated that there exists also a restriction specificity for host type H-2 determinants: $(H\text{-}2^{bm1} \times H\text{-}2^{b})F1 \rightarrow H\text{-}2^{b}$

bone marrow chimeras immunized with vaccinia virus lyse infected H-2K^{b} target cells, whereas (H-2^{bm1} x H-2^{b})F1 $\rightarrow$ H-2^{bm1} chimeras cannot generate cytotoxic activity towards infected H-2K^{b} targets (299). Thus, the receptor for self-H-2K is exquisitely specific since the bm1 mutations cause only minor changes in the primary and tertiary structure of the H-2K^{b} molecule (107). Furthermore, it was shown that it is the thymus which determines the spectrum of receptor specificities of differentiating T cells for self-H-2: adult thymectomized, irradiated and (A x B)F1 bone marrow reconstituted (A x B)F1 mice, transplanted with an irradiated thymus of A origin, generate virus-specific cytotoxic T cells specific for infected A target cells but not for infected B target cells (300).

T lymphocytes from A $\rightarrow$ (A x B)F1 longterm chimeric mice can generate and display cytotoxic T cell activity to infected A cells but not to infected B cells. However, when these chimeric lymphocytes are sensitized in irradiated and acutely infected (A x B)F1 recipients, they respond to both infected A and infected B targets (300). This phenomenon can be explained as follows: in these irradiated and acutely infected recipient mice, all host lymphohemopoietic cells are still present, in contrast to longterm radiation chimeras in which the great part of the original lymphohemopoietic system has been replaced by cells descending from the transplanted hemopoietic stem cells. Thus, although the thymus determines the range of H-2 antigens that can be recognized as 'self' by the newly-formed T cells, it is mainly the H-2 of the lymphohemopoietic system and not of other somatic cells that determines the actual, phenotypically expressed and measurable specificity for self-H-2.

So, if T cells of any H-2 type learn to recognize H-2 from strain A as self, they cannot express any immune reactivity unless the same A molecules are expressed on the antigenpresenting cells (300). This can also explain why long-term A→B chimeras, completely reconstituted by donor cells, are severely T cell immunoincompetent (301). These animals do not eliminate virus during an acute virus infection, do not develop DTH after challenge with viral antigens, do not generate measurable numbers of virus-specific cytotoxic T cells and have low titers of antiviral antibodies. Furthermore, lymphocytes from these allogeneic chimeras fail to generate significant alloreactivity *in vitro* (301). Maestroni et al. (302), on the other hand, described that in stable, long-term A→B chimeras, made by injecting so-called 'marrow regulating factors' before irradiation and bone marrow reconstitution of allogeneic mice (303), alloreactivity as measured by skin graft rejection and MLC reactivity was within the normal range. However, the MHC-restricted T-cell-dependent primary response to sheep erythrocytes and viruses was clearly deficient. Similar data have been reported by Onoé et al. (304) and Krown et al. (305). The quantitative difference in immunocompetence when compared with syngeneically reconstituted recipients was at least in the order of ten-to-thirty fold (301). The reasons for the apparent discrepancy between alloreactivity *in vivo* and *in vitro* and MHC-restricted T cell responsiveness are unclear. However, Kruisbeek et al. showed that T cells that mediate cytotoxic responses to trinitrophenylmodified self determinants do acquire functional competence in allogeneic A→B radiation bone marrow chimeras and are restricted to the host haplotype (306).

Also, helper T cells of donor bone marrow origin from fully allogeneic bone marrow chimeras acquire functional competence and do recognize and respond to antigen presented by accessory cells that express thymic type H-2 determinants (307), thus stressing the role of the lymphohemopoietic cells in determining the actual MHC restriction.

Surprisingly, skin reactions of longterm A→B chimeras after sensitization with 2,4-dinitro-1-fluorobenzene (DNFB) were not different from those of normal mice (304). In fact, these reactions should have been absent because identity at the MHC has been shown to be necessary for transfer of DTH to DNFB (308,309). The explanation might be that host-derived Langerhans cells, which play an important role in the development of skin reactions in contact sensitivity (310,311), were still present and therefore could interact with host MHC-restricted donor-type T lymphoid cells.

Katz et al. (312) have demonstrated that T cells generated in lethally irradiated F1 mice reconstituted with anti-Thy-1 treated parental strain bone marrow cells did not collaborate with B cells from the opposite parental strain. These data could not be corroborated by Sprent and Von Boehmer (313). They irradiated F1 hybrid mice sublethally and reconstituted them also with anti-Thy-1-treated bone marrow cells from one parental strain. These mice were found to have two discrete subpopulations of H-2 restricted helper T cells, one restricted by the H-2 determinants of the bone marrow donor strain and the other restricted by H-2 determinants of the opposite parental strain, probably because the lymphohemopoietic system of these chimeric mice consists of donor strain cells as well as host cells.

The combined action of two subgroups of H-2 restricted T cells, each able to interact with B cells of only one parental strain, may explain the apparent unrestricted T helper function of parent to F1 chimeric T cells, as found by Von Boehmer and Sprent (313). The essential difference between the mice used by Katz et al. (312) and the parent to sublethally irradiated F1 chimeras used by Sprent and Von Boehmer (313) is that the antigen-presenting cells in the mice of Katz et al. were probably all of donor origin. Hence, direct immunization of these repopulated mice might stimulate only one of the two subgroups of T cells, namely the subgroup which recognizes the MHC antigens of the antigen-presenting cells as self. Consequently both cell populations would be stimulated by antigen in the presence of heterozygous macrophages. Indeed, this has proven to be the case. Waldmann et al. (314) demonstrated that T cells from A⟶(A x B)F1 chimeras primed under these conditions provide a good helper effect to B cells of the opposite parental strain.

MHC restriction specificity is not necessarily determined by thymic reticulum cells. Zinkernagel et al. (315) showed that nude mice grafted with an allogeneic fetal thymus were restricted exclusively for the nude H-2, thus indicating that precursors of T cells in nude mice have already acquired restriction specificity independently of a functioning thymus. Furthermore, several groups have shown that T cells of chimeras can respond in an unrestricted fashion (316-318).

Thus, the host thymus does not absolutely determine the MHC restriction specificity, although it is plausible that it is the most efficient and common pathway (319).

IX. GRAFT-VERSUS-HOST REACTIONS AND AUTOIMMUNITY

During the course of GvH disease induced by transplantation of parental lymphoid cells into non-irradiated F1 recipients, inhibitory as well as stimulatory pathological phenomena have been described (83,102,175). The inhibitory phenomena such as pancytopenia, aplastic anemia and hypogammaglobulinemia seem to be caused by alloreactive suppressor/killer T cells (see Sections II, V and VI) and is due to a negative allogeneic effect (197). The stimulatory phenomena, on the other hand, cause lymphoid hyperplasia, hypergammaglobulinemia and formation of autoantibodies and seem to be the result of alloreactive helper T cells triggering F1 B cells, which are normally switched off (320-323), to autoantibody formation (see Sections II, V, VI). This is due to a positive allogeneic effect and has originally been described by Katz et al. (324) to occur after injection of allogeneic lymphocytes into guinea pigs and has subsequently been demonstrated in mice and rats as well (325-330).

A variety of autoantibodies directed against different organs and tissues have been described. Gleichmann and Gleichmann (331) have demonstrated that autoimmune hemolytic anemia can arise when parental lymphoid cells are injected into non-irradiated F1 hybrid recipients. Cortisone-resistant thymocytes are very potent in inducing such anti-erythroid antibodies whereas bone marrow cells are ineffective. In subsequent studies they demonstrated that autoantibodies of the IgG1 and IgA subclasses are deposited along the basement membrane of the skin after transplantation of donor T lymphocytes of DBA/2 origin into (C57BL/10 x DBA/2)F1 recipients (332).

Furthermore, in this parent to F1 combination autoantibodies of the IgG and IgM class are generated to the F1 thymocytes (333) as well as IgG autoantibodies to nuclear antigens (334) and double-stranded DNA (335). Also immune complex glomerulonephritis could be shown to occur as a consequence of the deposition of these autoantibodies in the kidney (336). This pattern of autoantibodies closely resembles that of patients with systemic lupus erythematodes (SLE).

The genetic requirements for induction of these autoantibodies have also been investigated by the group of Gleichmann (102,337). It appeared that differences in the I-E and/or I-A subregion of the MHC were necessary. Interestingly, I-E seems to be the murine analogue of HLA-DR (338,339) and among patients with SLE particular HLA-DR alleles occur in an increased frequency (340-342). Gleichmann et al. (343) have postulated that the formation of autoantibodies in certain cases of SLE is caused by a GvH-like abnormal cooperation between autologous T and B cells. The generation of this abnormal T cell help would be due to the binding of drugs or viruses to B lymphocytes or macrophages, rendering these cells immunogenic to autologous T cells. Indeed, it has been demonstrated that drugs such as diphenylhydantoin are able to combine with murine lymphocytes, so that they stimulate autologous T cells, which in turn stimulate a massive IgG antibody production (344). Moreover, lymphoproliferation induced by footpad injection of these drugs is higher in adult thymectomized than in normal mice, suggesting that suppressor T cells might be involved in the constraint of this immunogenic stimulus (344).

The chronic GvH disease which develops in rats can also be used as a model for autoimmune disease. However, this model has not as extensively been explored as the murine model, yet. Beschorner et al. (345) showed that the histopathological lesions of such rats closely resemble those of scleroderma, Sjögren syndrome and chronic hepatitis.

Not only 'too much help' has been demonstrated during chronic GvH reactions, also 'too little help' can occur. Dosch and Gelfand (280) have reported a patient with aplastic anemia, who developed GvH disease and a transient state of immunodeficiency after bone marrow transplantation. During this period his B and T lymphocytes could cooperate with normal lymphocytes from several unrelated donors and generate specific antibody-forming cell responses to the T cell dependent antigen ovalbumin, but were incapable of cooperating with each other. Evidence for cell or serum mediated suppressor mechanisms were sought but could not be demonstrated. Similar data have been obtained in studies on murine GvH. These studies revealed, however, that such qualitative deficiencies in T and B cells are not related to the severity of GvH disease experienced (346).

X. GvH AND LYMPHOMA

It has been amply documented that non-irradiated animals suffering from GvH disease have an increased incidence of lymphoma (347-349). Runt disease caused by transplacental traffic of maternal lymphocytes might explain the high incidence of lymphoma in young animals and children. According to Gleichmann (350), most murine lymphomas consist of B lymphoblasts. The cells are often Thy-1 negative and contain immunoglobulins (350). The majority of lymphomas are derived from host cells and not from donor lymphocytes (350,351). This is probably due to a positive allogeneic effect by the infused lymphocytes.

The pathogenesis underlying lymphoma formation after parent to F1 lymphoid cell transplantation might be as follows. An H-2I subregion difference between donor and host is essential for inducing maximal donor lymphocyte proliferation (see Sections III and IV). During this proliferation, lymphokines are produced which may induce proliferation of other lymphoid cells, e.g., host B lymphocytes (351). The anti-H-2I reaction of donor T cells in mice subjected to GvH persists for a long period (197,352). Consequently, host B lymphocytes will progressively increase their proliferation rate. If one of these cells transforms malignantly, a malignant lymphoma may evolve. The oncogenic event might be due to unmasking and stimulation of normally latent oncogenic viruses (353). This is supported by the finding of Pals (personal communication) that lymphoma arising during GvH display rearrangements of the DNA coding for murine leukemia virus.

It has been described by Walford (354) that C3H and C3H.K mice injected at birth with H-1 incompatible spleen cells (i.e., a weak histoincompatibility) may lead to a 2- to 4-fold increase of the incidence of lymphomas, suggesting that weaker stimulation of the donor T cells essentially has the same effect.

XI. CONCLUDING REMARKS

Despite the considerable recent advances in immunology and hematology many questions remain to be resolved in the elucidation of the mechanisms underlying GvH disease. GvH disease represents a vast array of pathological aberrations in which all types of lymphocytes seem to play a role. These lymphocytes frequently act simultaneously and can synergize as well as antagonize, depending on the actual conditions and the subpopulations involved. This poses a major barrier to research aimed at establishing the cell types that cause the various aberrations. Furthermore, it is clear that, generally, GvH disease is most severe when donor and host differ at the MHC and much less severe when only minor histocompatibility differences are involved. Monoclonal antibodies against T cell subsets may offer therapeutical possibilities for the prevention and treatment of GvH disease (355,356).

Patients with acute leukemia may possibly benefit from bone marrow transplantation. The GvH which ensues often exerts an anti-leukemic effect, resulting in a prolongation of life and a higher chance of eradication of the residual leukemic cells (357).

GvH disease is not only of interest to the clinician. GvH reactions and GvH disease can also be used to study lymphocyte interactions and the necessary MHC restriction requirements in T cell activation. Furthermore, GvH disease can be used as a model to study autoimmune diseases, the development of tolerance, etc.

Many aspects of GvH disease still have to be resolved, but with the further delineation of the identity and function of all elements constituting the immune system we will also get more insight into the mechanisms underlying GvH disease and the pathology associated with this disorder.

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XII. REFERENCES

1. Billingham, R.E., Brent, L., and Medawar, P.B. Acquired tolerance of skin homografts. Ann. N.Y. Acad. Sci. 59, 409, 1955.
2. Trentin, J.J. Mortality and skin transplantability in X-irradiated mice receiving isologous, homologous or heterologous bone marrow. Proc. Soc. Exp. Biol. Med. 92, 688, 1956.
3. Trentin, J.J. Induced tolerance and homologous disease in X-irradiated mice protected with homologous bone marrow. Proc. Soc. Exp. Biol. Med. 96, 139, 1957.
4. Billingham, R.E., and Brent, L. A simple method for inducing tolerance of skin homografts in mice. Transplant. Bull. 4, 67, 1957.
5. Simonsen, M. The impact on the developing embryo and newborn animal of adult homologous cells. Acta Pathol. Microbiol. Scand. 40, 480, 1957.
6. Billingham, R.E. The biology of graft-versus-host reactions. The Harvey Lectures, p. 21, 1968.
7. Medawar, P.B. The immunologically competent cell. Ciba Found. Study Group 16, 1, 1963.
8. Gowans, J.L. Fate of parental strain small lymphocytes in F1 hybrid rats. Ann. N.Y. Acad. Sci. 99, 432, 1962.
9. Grebe, S.C. and Streilein, J.W. Graft-versus-Host reactions, a review. Adv. Immunol. 22. 119. 1976.
10. Beer, A.E., Billingham, R.E. and Yang, S.L. Maternally induced transplantation immunity, tolerance and runt disease in rats. J. Exp. Med. 135, 808, 1972.
11. Beer, A.E. and Billingham, R.E. Maternally acquired runt disease. Science 179, 240, 1973.
12. Niethammer, D., Goldmann, S.F., Flad, H.D., Bienzle, U., Dieterle, U., Haas, R.J., Heymer, B., Meigel, W., Belohradsky, B.H., and Kleihauer, E. Nature of reconstitution with histo-incompatible maternal marrow in a case of severe combined immunodeficiency with GvH disease following maternofetal transfusion. Clin. Immunol. Immunopathol. 18, 387, 1981.
13. Tuffrey, M., Bishun, N.P. and Barnes, R.D. Porosity of the mouse placenta to maternal cells. Nature 221, 1029, 1969.
14. Tuffrey, M., Bishun, N.P. and Barnes, R.D. Porosity of the placenta to maternal cells in normally derived mice. Nature 224, 701, 1969.
15. Van Bekkum, D.W. Immunological basis of graft-versus-host disease. In: 'Biology of bone marrow transplantation', eds. R.P. Gale and C.F. Fox, Academic Press, New York, p. 173, 1980.
16. Weiden, P.L. Graft-versus-Host disease in allogeneic marrow transplantation. In: 'Biology of bone marrow transplantation', eds. R.P. Gale and C.F. Fox. Academic Press, New York, p. 37, 1980.

17. Löwenberg, B., De Zeeuw, H.M.C., Dicke, K.A. and Van Bekkum, D.W. Nature of the delayed graft-versus-host reactivity of fetal liver cell transplants in mice. J. Natl. Cancer Inst. 58, 959, 1977.
18. Congdon, C.C. and Urso, I.S. Homologous bone marrow in the treatment of radiation injury in mice. Am. J. Pathol. 33, 749, 1957.
19. De Vries, M.J. and Vos, O. Delayed mortality of radiation chimaeras. A pathological and haematological study. J. Natl. Cancer Inst. 23, 1403, 1959.
20. Nowell, P.C. and Cole, L.J. Pathological changes in old non-irradiated F1 hybrid mice injected with parental strain spleen cells. Transplant. Bull. 6, 435, 1959.
21. Van Bekkum, D.W., De Vries, M.J. and Van der Waay, D. Lesions characteristic of secondary disease in germ-free heterologous radiation chimeras. J. Natl. Cancer Inst. 38, 223, 1967.
22. Krüger, G.R.F., Berard, C.W., Elias, P.M. and Graw, R.G. Morphology of graft-versus-host reaction in HL-A bone marrow transplantation and its differential diagnosis. Exp. Hematol. 21, 4, 1971.
23. Slavin, R.E. and Santos, G.W. The graft-versus-host reaction in man after bone marrow transplantation: pathology, pathogenesis, clinical features and implication. Clin. Immunol. Immunopathol. 1, 472, 1973.
24. Glucksberg, H., Storb, R., Fefer, A., Buckner, C.D., Neimann, P.E., Clift, R.A., Lerner, K.G. and Thomas, E.D. Clinical manifestations of graft-versus-host disease in human recipients of marrow from HLA matched sibling donors. Transplantation 18, 295, 1974.
25. De Vries, M.J., Crouch, B.G., Van Putten, L.M. and Van Bekkum, D.W. Pathologic changes in irradiated monkey treated with bone marrow. J. Natl. Cancer Inst. 27, 67, 1961.
26. Kolb, H., Sale, G.E., Lerner, K.G., Storb, R. and Thomas, E.D. Pathology of acute graft-versus-host disease in the dog. Am. J. Pathol. 96, 581, 1979.
27. Walker, K.Z., Schoefl, G.I. and Lafferty, K.J. The pathogenesis of graft-versus-host reaction in chicken embryos. Pathological changes in yolk sac, thymus, bone marrow and bursa of Fabricius. Aust. J. Exp. Biol. Med. Sci. 50, 675, 1972.
28. Walker, K.Z., Schoefl, G.I., Adams, E.P. and Lafferty, K.J. The pathogenesis of graft-versus-host reaction in chicken embryos. The development of haemorrhagic lesions. Aust. J. Exp. Biol. Med. Sci. 51, 93, 1973.
29. Beschorner, W.E., Tutschka, P.J. and Santos, G.W. Sequential morphology of graft-versus-host disease in the rat radiation chimera. Clin. Immunol. Immunopathol. 22, 203, 1982.
30. Woodruff, J.M., Eltringham, J.R., and Casey, H.W. Early secondary disease in the rhesus monkey. I. A comparative histopathologic study. Lab. Invest. 20, 499, 1969.
31. Thomas, E.D., Storb, R., Clift, R.A., Fefer, A., Johnson, F.L., Neiman, P.E., Lerner, K.G., Glucksberg, H. and Buckner, C.D. Bone marrow transplantation. N. Engl. J. Med. 292, 832, 1975.

32. Atkinson, K., Farewell, V., Storb, R., Tsoi, M.S., Sullivan, K.M., Witherspoon, R.P., Fefer, A., Clift, R., Goodell, B. and Thomas, E.D. Analysis of late infections after human bone marrow transplantation: role of genotypic non-identity between marrow donor and recipient and of non-specific suppressor cells in patients with chronic graft-versus-host disease. Blood 60, 714, 1982.
33. Meyers, J.D., and Thomas, E.D. Infection complicating bone marrow transplantation. In: 'Clinical approach to infection in the immunocompromised host', eds. R.H. Rubin and L.S. Young, Plenum Press, New York, p. 507, 1982.
34. Lerner, K.G., Kao, G.F., Storb, R., Buckner, C.D., Clift, R.A. and Thomas, E.D. Histopathology of graft-vs-host reaction (GvHR) in human recipients of marrow from HL-A-matched sibling donors. Transplant. Proc. 6, 367, 1974.
35. Beschorner, W.E., Pino, J., Boitnott, J.K., Tutschka, P.J. and Santos, G.W. Pathology of the liver with bone marrow transplantation. Am. J. Pathol. 99, 369, 1980.
36. Sale, G.E., Storb, R., and Kolb, H. Histopathology of hepatic acute graft-versus-host disease in the dog. Transplantation 26, 103, 1978.
37. Sullivan, K.M., Shulman, H.M., Weiden, P.L., Storb, R., Tsoi, M.S. and Thomas, E.D. The spectrum of chronic graft-versus-host disease in man. In: 'Biology of bone marrow transplantation', eds. R.P. Gale and C.F. Fox, Academic Press, New York, p. 69, 1980.
38. Graze, P.R., and Gale, R.P. Chronic graft-versus-host disease: a syndrome of disordered immunity. Am. J. Med. 66, 611, 1979.
39. Shulman, H.M., Sale, G.E., Lerner, K.G., Barker, E.A., Weiden, P.L., Sullivan, K., Gallucci, B., and Thomas, E.D. Chronic cutaneous graft-versus-host disease in man. Am. J. Pathol. 92, 545, 1978.
40. Bergan, J.J. Report from the ACS/NIH bone marrow transplant registry. Exp. Hemat. 3, 149, 1975.
41. Sale, G.F., Lerner, K.G., Barker, E.A., Shulman, H.M. and Thomas, E.D. The skin biopsy in the diagnosis of acute graft-versus-host disease in man. Am. J. Pathol. 89, 621, 1977.
42. Lampert, I.A., Suitters, A.J. and Chisholm, P.M. Expression of Ia antigen on epidermal keratinocytes in graft-versus-host disease. Nature 293, 149, 1981.
43. Mason, D.W., Dallman, M. and Barclay, A.N. Graft-versus-host disease induces expression of Ia antigen in rat epidermal cells and gut epithelium. Nature 293, 150, 1981.
44. Van Bekkum, D.W. and Knaan, S. Role of bacterial microflora in development of intestinal lesions from graft-versus-host reaction. J. Natl. Cancer Inst. 58, 787, 1977.
45. Storb, R.L., Prentice, R.L., Buckner, C.D., Clift, R.A., Appelbaum, F., Deeg, H.J., Doney, K., Hansen, J.A., Mason, M., Sanders, J.E., Singer, J., Sullivan, K.E., Witherspoon, R.P. and Thomas, E.D. Graft-versus-host disease and survival in patients with aplastic anemia treated by marrow grafts from HLA-identical siblings. Beneficial effect of a protective environment. N. Engl. J. Med., 308, 302, 1983.

45a. Vossen, J.M., Heidt, P.J., Guiot, H.F.L., and Dooren, L.J. Prevention of acute graft-versus-host disease in clinical bone marrow transplantation: complete versus selective intestinal decontamination.
In: 'Recent advances in germfree research', eds. S. Sasaki, A. Ozawa and K. Hashimoto, Tokai Univ. Press, Tokio, p. 573, 1981.
46. Elkins, W.L. Cellular immunology and the pathogenesis of graft-versus-host reactions. Progr. Allergy 15, 78, 1971.
47. Korngold, R., and Sprent, J. Lethal graft-versus-host disease after bone marrow transplantation across minor histocompatibility barriers in mice. Prevention by removing mature T cells from marrow. J. Exp. Med. 148, 1687, 1978.
48. Sprent, J. and Korngold, R. Immunogenetics of graft-versus-host reactions to minor histocompatibility antigens. Immunology Today 2, 189, 1981.
49. Simonsen, M. and Jensen, E. The graft-versus-host assay in transplantation chimaeras. In: 'Biological problems of grafting', eds. F. Albert and G. Lejeune-Ledant, Blackwell, Oxford, p. 214, 1959.
50. Piguet, P.F., Dewey, H.K. and Vassalli, P. Origin and nature of the cells participating in the popliteal graft-versus-host reaction in mouse and rat. Cell. Immunol. 31, 242, 1977.
51. Levine, S. Local and regional forms of graft-versus-host disease in lymph nodes. Transplantation 6, 799, 1968.
52. Ford, W.L., Burr, W. and Simonsen, M. A lymph node weight assay for the graft-versus-host activity of rat lymphoid cells. Transplantation 10, 258, 1970.
53. Hardt, F. and Claësson, M.H. Graft-versus-host reactions mediated by spleen cells from amyloidotic and nonamyloidotic mice. Transplantation 12, 36, 1971.
54. Démant, P. and Graff, R.J. Transplantation analysis of the H-2 complex. Transplant. Proc. 5, 267, 1973.
55. Huber, B., Pena-Martinez, J., Festenstein, H. Spleen cell transplantation in mice: influence of non-H-2 M locus on graft-vs-host and host-vs-graft reactions. Transplant. Proc. 5, 1373, 1973.
56. Twist, V.W. and Barnes, R.D. Popliteal lymph node weight gain assay for graft-versus-host reactivity in mice. Transplantation 15, 182, 1973.
57. Rolstad, B. The host component of the graft-versus-host reaction. A study on the popliteal lymph node reactions in the rat. Transplantation 21, 117, 1976.
58. Emeson, E.E. and Thursh, D.R. Mechanism of graft-versus-host induced lymphadenopathy in mice. Trapping versus proliferation. J. Exp. Med. 137, 1293, 1973.
59. Ford, W.L., Rolstad, B., Fossum, S., Hunt, S.V., Smith, M.E. and Sparshott, S.M. The stimulus to host cell proliferation in graft-versus-host reactions. Scand. J. Immunol. 14, 705, 1981.
60. Wolters, E.A.J. and Benner, R. Immunobiology of the graft-versus-host reaction. I. Symptoms of graft-versus-host disease are preceded by delayed-type hypersensitivity to host histocompatibility antigens. Transplantation 26, 40, 1978.

61. Wolters, E.A.J., Brons, N.H.C., Benner, R. and Vos, O. Anti-host immune reactivity after allogeneic bone marrow transplantation. In: 'Experimental Hematology Today 1979', eds. S.J. Baum and G.D. Ledney, Springer Verlag, New York, p. 163, 1979.
62. Wolters, E.A.J., Van der Kwast, Th.H., Odijk, L.M. and Benner, R. Differential responsiveness to H-2 subregion coded antigens in graft-versus-host and host-versus-graft reactions. Cell. Immunol. 57, 389, 1981.
63. Blomgren, H. and Andersson, B. Inhibition of erythroid cell growth in irradiated mice by allogeneic lymphoid cells: a quantitative method for graft-versus-host reactivity of lymphoid cells. Cell. Immunol. 3, 318, 1972.
64. Howard, J.G. Changes in the activity of reticuloendothelial system following the injection of parental spleen cells into F1 hybrid mice. Brit. J. Exp. Path. 42, 72, 1961.
65. Murphy, J.B. The effect of adult chicken organ grafts on the chicken embryo. J. Exp. Med. 24, 1, 1916.
66. Burnet, F.M. and Boyer, G.S. The chorio-allantoic lesion in the Simonsen phenomenon. J. Path. Bact. 81, 141, 1961.
67. Wilson, D.B. and Nowell, P.C. Quantitative studies on the mixed lymphocyte interaction in rats. IV. Immunologic potentiality of the responding cells. J. Exp. Med. 131, 391, 1970.
68. Wilson, D.B. Quantitative studies on the mixed lymphocyte interaction in rats. I. Conditions and parameters of the response. J. Exp. Med. 126, 625, 1967.
69. Bach, F.H., Widmer, M.B., Segall, M., Bach, M.L. and Klein, J. Genetic and immunological complexity of major histocompatibility regions. Science 176, 1024, 1972.
70. Bach, F.H., Widmer, M.B., Bach, M.L. and Klein, J. Serologically defined and lymphocyte defined components of the major histocompatibility complex in the mouse. J. Exp. Med. 136, 1430, 1972.
71. Klein, J., Widmer, M.B., Segall, M. and Bach, F.H. Mixed lymphocyte culture reactivity and H-2 histocompatibility loci differences. Cell. Immunol. 4, 442, 1972.
72. Meo, T., Vives, G., Rijnbeek, A.M., Miggiano, V.C., Nabholz, M., and Shreffler, D.C. A bipartite interpretation and tentative mapping of H-2 associated MLR determinants in the mouse. Transplant. Proc. 5, 1339, 1973.
73. Eijsvoogel, V.P., Koning, L., De Groot-Kooy, L., Huismans, L., Van Rood, J.J., Van Leeuwen, A. and Du Toit, E.D. Mixed lymphocyte culture and HL-A. Transplant. Proc. 4, 199, 1972.
74. Gowans, J.L. and McGregor, D.D. The immunological activities of lymphocytes. Progr. Allergy 9, 1, 1965.
75. Wilson, D.B., Silvers, W.K. and Nowell, P.C. Quantitative studies on the mixed lymphocyte interaction in rats. II. Relationship of the proliferative response to the immunologic status of the donors. J. Exp. Med. 126, 655, 1967.
76. Cerottini, J.C., Nordin, A.A. and Brunner, K.T. Specific in vitro cytotoxicity of thymus-derived lymphocytes sensitized to alloantigens. Nature 228, 1308, 1970.

77. Van der Kwast, Th.H., Bianchi, A.T.J., Bril, H. and Benner, R. Suppression of antigraft immunity by preimmunization. I. Kinetic aspects and specificity. Transplantation 31, 79, 1981.
78. Liew, F.Y. Regulation of delayed-type hypersensitivity. I. T suppressor cells for delayed-type hypersensitivity to sheep erythrocytes in mice. Eur. J. Immunol. 7, 714, 1977.
79. Rich, S.S. and Rich, R.R. Regulatory mechanisms in cell-mediated immune response. I. Regulation of mixed lymphocyte reactions by alloantigen-activated thymus-derived lymphocytes. J. Exp. Med. 140, 1588, 1974.
80. Rodey, G.E., Bortin, M.M., Bach, F.H. and Rimm, A.A. Mixed leucocyte culture reactivity and chronic graft-versus-host reactions (secondary disease) between allogeneic H-2^k mouse strains. Transplantation 17, 84, 1974.
81. Cerottini, J.C. and Brunner, K.T. Cell-mediated cytotoxicity, allograft rejection and tumor immunity. Adv. Immunol. 18, 67, 1974.
82. Rouse, B.T. and Wagner, H. The in vivo activity of in vitro immunized mouse thymocytes. II. Rejection of skin allografts and graft-versus-host reactivity. J. Immunol. 109, 1282, 1972.
83. Rolink, A.G., Radaszkiewicz, T., Pals, S.T., Van der Meer W.G.J. and Gleichmann, E. Allosuppressor and allohelper T cells in acute and chronic graft-vs-host disease. I. Alloreactive suppressor cells rather than killer T cells appear to be the decisive effector cells in lethal graft-vs-host disease. J. Exp. Med. 155, 1501, 1982.
84. Loveland, B.E., Hogarth, P.M., Ceredig, Rh. and McKenzie, I.F.C. Cells mediating graft rejection in the mouse. I. Lyt-1 cells mediate skin graft rejection. J. Exp. Med. 153. 1044. 1981.
85. Hildemann, W.H. Rathbun, W.E. and Walford, R.I. Early manifestation of acute transplantation (allogeneic) disease in mice. Transplantation 5, 504, 1967.
86. Simonsen, M. Graft-vs-host reactions. Their natural history and applicability as tools of research. Progr. Allergy 6, 349, 1962.
87. Kretschmer, R., Jeannet, M. and Mereu, T.R. Hereditary thymic dysplasia: a graft-versus-host reaction induced by bone marrow cells with partial 4a series histoincompatibility. Pediat. Res. 3, 34, 1969.
88. Jaffe, W.P. and Payne, L.N. Genetic basis for graft-against-host reaction between inbred lines of fowls. Immunology 5, 399, 1962.
89. Klein, J. Biology of the mouse histocompatibility-2 complex. Springer Verlag, New York, 1975.
90. Klein, J. and Park, J.M. Graft-versus-host reactions across different regions of the H-2 complex of the mouse. J. Exp. Med. 137, 1213, 1973.
91. Livnat, S., Klein, J. and Bach, F.H. Graft-versus-host reactions in strains of mice identical for H-2K and H-2D antigens. Nature New Biol. 243, 42, 1973.
92. Elkins, W.L., Kavathas, P. and Bach, F.H. Activation of T cells by H-2 factors in the graft-versus-host reaction. Transplant. Proc. 5, 1759, 1973.

93. Klein, J., Juretic, A., Baxevanis, C.N. and Nagy, Z.A. The traditional and a new version of the mouse H-2 complex. Nature 291, 455, 1981.
94. Klein, J. Relative importance of H-2 regions in the development of graft-versus-host reactions. Transplant. Proc. 8, 335, 1976.
95. Prud'homme, G.J., Sohn, U. and Delovitch, T.L. The role of H-2 and Ia antigens in graft-versus-host reactions (GvHR). Presence of host alloantigens on donor cells after GvHR and suppression of GvHR with an anti-Ia antiserum against host Ia antigens. J. Exp. Med. 149, 137, 1979.
96. Klein, J. Antigens and receptors involved in bone marrow transplantation. Transplant. Proc. 10, 5, 1978.
97. Klein, J. and Chiang, C.L. Ability of H-2 regions to induce graft-versus-host disease. J. Immunol. 117, 736, 1976.
98. Clark, E.A. and Hildemann, W.H. Genetics of graft-versus-host reactions. I. Production of splenomegaly and mortality in mice disparate at H-2I subregions. Immunogenetics 4, 281, 1977.
99. Clark, E.A. and Hildemann, W.H. Genetics of graft-versus-host reactions. II. Interallelic effects and regulation of GvHR by antirecipient alloantibodies. Immunogenetics 5, 309, 1977.
100. Steinmetz, M., Minard, K., Horvath, S., McNicholas, J., Frelinger, J., Wake, C., Long, E., Mach, B. and Hood, L. A molecular map of the immune response region from the major histocompatibility complex of the mouse. Nature 300, 35, 1982.
101. Oppltova, L. and Démant, P. Genetic determinants for the graft-vs-host reaction in the H-2 complex. Transplant. Proc. 5, 1367, 1973.
102. Rolink, A.G., Pals, S.T. and Gleichmann, E. Allosuppressor and allohelper T cells in acute and chronic graft-vs-host disease. II. F1 recipients carrying mutations at H-2K and or I-A. J. Exp. Med. 157, 755, 1983.
103. Schendel, D.J., Alter, B.J. and Bach, F.H. The involvement of LD- and SD-region differences in MLC and CML: a three-cell experiment. Transplant. Proc. 5, 1651, 1973.
104. Wagner, H., Götze, D., Ptschelinzew, W. and Rollinghoff, M. Induction of cytotoxic T lymphocytes against I-region-coded determinants: in vitro evidence for a third histocompatibility locus in the mouse. J. Exp. Med. 142, 1477, 1975.
105. Nabholz, M., Young, H., Rijnbeek, A., Boccardo, R., David, C.S., Meo, T., Miggiano, V. and Shreffler, D.C. I-region-associated determinants: expression on mitogen-stimulated lymphocytes and detection by cytotoxic T cells. Eur. J. Immunol. 5, 594, 1975.
106. Klein, J. and Egorov, I.K. Graft-vs-host reaction with an H-2 mutant. J. Immunol. 111, 976, 1973.
107. Klein, J. H-2 mutations: their genetics and effect on immune functions. Adv. Immunol. 26, 55, 1978.
108. Rychlikova, M. and Ivanyi, P. Mixed lymphocyte cultures and histocompatibility antigens in mice. Folia Biol. (Prague) 15, 126, 1969.

109. Götze, D. (ed) The major histocompatibility system in man and animals. Springer Verlag, Berlin, 1977.
110. Festenstein, H. Immunogenetic and biological aspects of in vitro lymphocyte allotransformation (MLR) in the mouse. Transplant. Rev. 15, 62, 1973.
111. Coutinho, A., Meo, T. and Watanabe, T. Independent segregation of two functional markers expressed on the same B-cell subset in the mouse: the Mls determinants and LPS receptors. Scand. J. Immunol. 6, 1005, 1977.
112. Ahmed, A., Scher, I., Smith, A.H. and Sell, K.W. Studies on non-H-2 linked lymphocyte activating determinants. I. Description of the cell type bearing the Mls product. J. Immunogenetics 4, 201, 1977.
113. Von Boehmer, H. and Sprent, J. Expression of M locus differences by B cells but not T cells. Nature 249, 363, 1974.
114. Schirrmacher, V., Pena-Mertinez, J. and Festenstein, H. Specific lymphocyte-activating determinants expressed on mouse macrophages. Nature 255, 155, 1975.
115. Pena-Martinez, J., Huber, B. and Festenstein, H. The influence of H-2 and non-H-2 M locus on spleen colony transformation after allogeneic bone marrow transplantation in irradiated mice. Transplant. Proc. 5, 1393, 1973.
116. Bartova, J. and Ivanyi, D. The influence of M locus incompatibility on tooth germ allografts. J. Immunogenetics 2, 365, 1975.
117. Rychlikova, M., Demant, P. and Ivanyi, P. The mixed lymphocyte reaction in H-2K, H-2D and non-H-2 incompatibility. Biomedicine 18, 401, 1973.
118. Wolters, E.A.J., Brons, N.H.C., Van der Kwast, Th.H. and Benner, R. Differential responsiveness to Mls locus antigens in graft-versus-host and host-versus-graft reactions. Cell. Immunol. 51, 215, 1980.
119. Peck, A.B. and Bach, F.H. Mouse cell-mediated lympholysis assay in serum-free and mouse serum-supplemented media: culture conditions and genetic factors. Scand. J. Immunol. 4, 53, 1975.
120. Leben, L. and Festenstein, H. H-2 restricted T cell killing against Mls^b-associated target specificity. Transplant. Proc. 15, 189, 1983.
121. Trentin, J.J. Tolerance and homologous disease in irradiated mice protected with homologous bone marrow. Ann. N.Y. Acad. Sci. 73, 799, 1958.
122. Storb, R., Rudolph, R.H., Kolb, H.J., Graham, T.C., Mickelson, E., Erickson, V., Lerner, K.G., Kolb, H. and Thomas, E.D. Marrow grafts between DLA matched canine littermates. Transplantation 15, 92, 1973.
123. Cantrell, J.L. and Hildemann, W.H. Characteristics of disparate histocompatibility barriers in congenic strains of mice. I. Graft-versus-host reactions. Transplantation 14, 761, 1972.
124. Rappeport, J., Reinherz, E., Mihm, M., Lopansri, S. and Parkman, R. Acute graft-versus-host disease in recipients of bone marrow transplants from identical twin donors. Lancet· ii, 717, 1979.

124a. Glazier, A., Tutschka, P.J., Farmer, E.R., and Santos, G.W. Graft-versus-host disease in cyclosporin A-treated rats after syngeneic and autologous bone marrow reconstitution J. Exp. Med. 158, 1, 1983.
125. Fathman, C.G., and Nabholz, M. In vitro secondary mixed leukocyte reaction (MLR). Interaction MLR determinants expressed by F1 cells. Eur. J. Immunol. 7, 370, 1977.
126. Fathman, C.G., Watanabe, T. and Augustin, A. In vitro secondary MLR.III. Hybrid histocompatibility determinants. J. Immunol. 121, 259, 1978.
127. Lafuse, W.P., McCormick, J.F. and David, C.S. Serological and biochemical identification of hybrid Ia antigens. J. Exp. Med. 151, 709, 1980.
128. Lafuse, W.P., McCormick, J.F., Corser, P.S. and David, C.S. Gene complementation to generate Ia antigens (Ia.23) on hybrid molecules. Transplantation 30, 341, 1980.
129. Sandrin, M.S., Tobias, G.H., McKenzie, I.F.C. and Hämmerling, G.J. Alterations in the expression of Ia antigens in F1 hybrid mice. Immunogenetics 14, 507, 1981.
130. Cantor, H. The effects of anti-theta antiserum upon graft-versus-host activity of spleen and lymph node cells. Cell. Immunol. 3, 461, 1972.
131. Golub, E.S. Brain associated Thy-antigen: reactivity of rabbit anti-mouse brain with lymphoid cells. Cell. Immunol. 2, 353, 1971.
132. Yoshida, Y. and Osmond, D.G. Graft-versus-host activity of rat bone marrow, marrow fractions and lymphoid tissues quantitated by a popliteal lymph node weight assay. Transplantation 12, 121, 1971.
133. Heim, L.R., McGarry, M.P., Montgomery, J.R., Trentin, J.J. and South, M.A. Potentials of spleen, lymph node and Peyer's patches to reconstitute lymphoid tissue and produce graft-versus-host reaction. Transplantation 14, 418, 1972.
134. Kerckhaert, J.A.M., Benner, R., and Willers, J.M.N. Cells involved in the graft-versus-host reaction in vitro. Immunology 25, 103, 1973.
135. McGregor, D.D. Bone marrow origin of immunologically competent lymphocytes in the rat. J. Exp. Med. 127, 953, 1968.
136. Umiel, T. Thymus-influenced immunological maturation of embryonic liver cells. Transplantation 11, 531, 1971.
137. Umiel, T. Requirements for development of immunocompetence of embryonic liver cells: the graft-versus-host response. Differentiation 1, 295, 1973.
138. Chen, M.G., Price, G.B. and Makinodan, T. Incidence of delayed mortality (secondary disease) in allogeneic radiation chimeras receiving bone marrow from aged mice. J. Immunol. 108, 1370, 1972.
139. Krohn, P.L. Review lectures on senescence. II. Heterochronic transplantation in the study of ageing. Proc. R. Soc. Ser. B. 157, 128, 1962.
140. Benner, R. and Haaijman, J.J. Ageing of the lymphoid system at the organ level. Developm. Comp. Immunol. 4, 591, 1980.

141. Miller, J.F.A.P. and Osoba, D. Current concepts of immunological function of the thymus. Physiol. Rev. 47, 437, 1967.
142. Miller, J.F.A.P., Mitchell, G.F. and Weiss, N.S. Cellular basis of the immunological defects in thymectomized mice. Nature 214, 992, 1967.
143. Rieke, W.O. Lymphocytes from thymectomized rats. Immunological, proliferative and metabolic properties. Science 152, 535, 1966.
144. Law, L. and Agnew, H.D. Effect of thymic extracts on restoration of immunologic competence in thymectomized mice. Proc. Soc. Exp. Biol. N.Y. 127, 953, 1968.
145. Trainin, N., Small, M. and Globerson, A. Immunocompetence of spleen cells from neonatally thymectomized mice conferred in vitro by a syngeneic thymus effect. J. Exp. Med. 130,765, 1969.
146. Sprent, J. and Basten, A. Circulating T and B lymphocytes of the mouse. II. Lifespan. Cell. Immunol. 7, 40, 1973.
147. Blomgren, H. and Andersson, B. Characteristics of the immunocompetent cells in the mouse thymus: cell population changes during cortisone-induced atrophy and subsequent irradiation. Cell. Immunol. 1, 545, 1971.
148. Cohen, J.J., Fischbach, M. and Claman, H.N. Hydrocortisone resistance of graft-vs-host activity in mouse, thymus, spleen and bone marrow. J. Immunol. 105, 1146, 1970.
149. Blomgren, H. and Andersson, B. Recirculating lymphocytes in the mouse thymus are part of the relatively cortisone resistent cell population. Clin. exp. Immunol. 10, 297, 1972.
150. Tigelaar, R.E. and Asofsky, R. Graft-versus-host reactivity of mouse thymocytes: effects of cortisone pretreatment of donors. J. Immunol. 110, 567, 1973.
151. Yamashita, A., Hattori, Y., Mori, F. and Kosaka, A. Acquisition of graft-versus-host reactivity by immature thymocytes in the coexistence of activated macrophages. Transplantation 33, 80, 1982.
152. Boak, J.L. and Wilson, R.E. Modification of the graft-versus-host syndrome by anti-lymphocyte serum treatment of the donor. Clin. exp. Immunol. 3, 795, 1968.
153. Ledney, G.D. and Van Bekkum, D.W. Secondary disease in irradiated mice grafted with allogeneic bone marrow from anti-lymphocyte serum-treated donors. J. Natl. Cancer Inst. 42, 633, 1969.
154. Fox, M. Cytological estimation of proliferating donor cells during graft-vs-host disease in F1 hybrid mice injected with parental spleen cells. Immunology 5, 489, 1962.
155. Meuwissen, H.J. and Good, R.A. Suppression of graft-versus-host reaction by Mitomycin C. Nature 215, 634, 1967.
156. Cheever, M.A., Einstein, A.B., Kempf, R.A. and Fefer, A. Reduction of fatal graft-versus-host disease by ^{3}H-thymidine suicide of donor cells cultured with host cells. Transplantation 23, 299, 1977.
157. Wolters, E.A.J. and Benner, R. Immunobiology of the graft-versus-host reaction. II. The role of proliferation in the development of specific anti-host immune responsiveness. Transplantation 27, 39, 1979.

158. Lafferty, K.J., Walker, K.Z., Scollay, R.G. and Killby, V.A.A. Allogeneic interactions provide evidence for a novel class of immunological reactivity. Transplant. Rev. 12, 198, 1972.
159. Scollay, R.G., Hofman, F., and Globerson, A. Graft-versus-host reaction in F1 recipients in the absence of donor parental cell proliferation. Eur. J. Immunol. 4, 490, 1974.
160. Spach, C. and Motta, R. Lymphoid cell proliferation in non-lymphoid organs during graft-versus-host reactions (GVHR). I. Lack of correlation between mortality and grafted cell proliferation. Exp. Hematol. 9, 531, 1981.
161. Cantor, H. and Boyse, E.A. Functional subclasses of T lymphocytes bearing different Ly antigens. I. The generation of functionally distinct T cell subclasses is a differentiative process independent of antigen. J. Exp. Med. 141, 1376, 1975.
162. Cantor, H. and Boyse, E.A. Functional subclasses of T lymphocytes bearing different Ly antigens. II. Cooperation between subclasses of Ly+ cells in the generation of killer activity. J. Exp. Med. 141, 1390, 1975.
163. Shen, F.W., Boyse, E.A. and Cantor, H. Preparation and use of Ly antisera. Immunogenetics 2, 591, 1975.
164. Cantor, H. and Boyse, E.A. Regulation of the immune response by T cell subclasses. In: 'Contemporary topics in immunobiology' 7, 47, 1977.
165. Kisielow, P., Hirst, J., Shiku, H., Beverley, P.C.L., Hoffman, M.K., Boyse, E.A. and Oettgen, H.F. Ly antigen markers for functionally distinct subsets of thymus-derived lymphocytes of the mouse. Nature 253, 219, 1975.
166. Shiku, H., Kisielow, P., Bean, M.A., Takahashi, T., Boyse, E.A., Oettgen, H.F. and Old, L.J. Expression of T cell differentiation antigens on effector cells in cell-mediated cytotoxicity in vitro: evidence for functional heterogeneity related to surface phenotype of T cells. J. Exp. Med. 141, 227, 1975.
167. Cantor, H., Shen, F.W. and Boyse, E.A. Separation of helper T cells from suppressor T cells expressing different Ly components. II. Activation by antigen: after immunization, antigen-specific suppressor and helper activities are mediated by distinct T cell subclasses. J. Exp. Med. 143, 1391, 1976.
168. Jandinski, J., Cantor, H., Tadakuma, T., Peavy, D.L., and Pierce, C.W. Separation of helper T cells from suppressor T cells expressing different Ly components. I. Polyclonal activation: suppressor and helper activities are inherent properties of distinct T cell subclasses. J. Exp. Med. 143, 1382, 1976.
169. Scollay, R., Kochen, M., Butcher, E. and Weissman, I. Lyt markers on thymus cell migrants. Nature 276, 79, 1978.
170. Mathieson, B.J., Sharrow, S.O., Campbell, P.S. and Asofsky, R. A Lyt differentiated thymocyte subpopulation detected by flow microfluorometry. Nature 277, 478, 1979.
171. Stutman, O. and Shen, F.W. Postthymic precursor cells give rise to both Lyt-1 and Lyt-23 subsets of T cells. Transplant. Proc. 11, 907, 1979.

172. Feldmann, M., Beverley, P.C.L., Woody, J. and McKenzie, I.F.C. T-T interactions in the induction of suppressor and helper T cells: analysis of membrane phenotype of precursor and amplifier cells. J. Exp. Med. 145, 793, 1977.
173. Scollay, R. Adult thymectomy does not alter the proportion of T cells of the Lyt-123 subclass. Nature 300, 529, 1982.
174. Vallera, D.A., Soderling, C.C.B., and Kersey, J.H. Bone marrow transplantation across major histocompatibility barriers in mice. III. Treatment of donor grafts with monoclonal antibodies directed against Lyt determinants. J. Immunol. 128, 871, 1982.
175. Rolink, A.G. and Gleichmann, E. Allosuppressor and allohelper T cells in acute and chronic graft-vs-host disease. III. Different Lyt subsets of donor T cells induce different pathological syndromes. J. Exp. Med. 158, 546, 1983.
176. Korngold, R. and Sprent, J. Lethal GvHD across minor histocompatibility barriers: nature of the effector cells and role of the H-2 complex. Immunol. Rev. 71, 5, 1983.
177. Mage, M., Mathieson, B., Sharrow, S., McHugh, L., Hämmerling, U., Kanellopoulos-Langevin, C., Brideau, D. and Thomas, C.A. Preparative nonlytic separation of Lyt-2+ and Lyt-2- T lymphocytes, functional analysis of the separated cells and demonstration of synergy in graft-vs-host reaction of Lyt-2+ and Lyt-2- cells. Eur. J. Immunol. 11, 228, 1981.
178. Loveland, B.E. and McKenzie, I.F.C. Cells mediating graft rejection in the mouse. III. Ly-1+ precursor T cells generate skin graft rejection. Transplantation 33, 407, 1982.
179. Loveland, B.E. and McKenzie, I.F.C. Delayed-type hypersensitivity and allograft rejection in the mouse: correlation of effector cell phenotype. Immunology 46, 313, 1982.
180. Michaelides, M., Hogarth, P.M. and McKenzie, I.F.C. The immunosuppressive effect of monoclonal anti-Lyt-1.1 antibodies in vivo. Eur. J. Immunol. 11, 1005, 1981.
181. White, R.A.H., Mason, D.W., Williams, A.F., Galfre, G. and Milstein, C. T lymphocyte heterogeneity in the rat: separation of functional subpopulations using a monoclonal antibody. J. Exp. Med. 148, 664, 1978.
182. Mason, D.W., Subpopulations of T cells in the rat that mediate graft-versus-host reactions and lethal graft-versus-host disease.
Adv. Exp. Med. Biol. 149, 545, 1982.
183. Cerottini, J.C., Nordin, A.A. and Brunner, K.T. Cellular and humoral response to transplantation antigens. I. Development of alloantibody forming cells and cytotoxic lymphocytes in the graft-versus-host reaction. J. Exp. Med. 134, 553, 1971.
184. Bach, F.H., Bach, M.L. and Sondel, P.M. Differential function of major histocompatibility complex antigens in T lymphocyte activation. Nature 259, 273, 1976.
185. Wagner, H., Starzinski-Powitz, A., Pfizenmaier, K. and Röllinghof, M. T-T cell collaboration during in vivo responses to antigens coded by the peripheral and central region of the MHC. Nature 263, 235, 1976.

186. Wagner, H., and Röllinghoff, M. T-T cell interaction during in vitro cytotoxic allograft responses. I. Soluble products from activated Lyt-1+ T cells trigger autonomously antigen-primed Ly-23+ T cells to cell proliferation and cytolytic activity. J. Exp. Med. 148, 1523, 1978.
187. Wagner, H., Röllinghoff, M., Hardt, C. and Pfizenmaier, K. T-cell-derived helper factor allows Lyt-123 thymocytes to differentiate into cytotoxic T lymphocytes. Nature 280, 405, 1979.
188. Wagner, H., Röllinghoff, M., Pfizenmaier, K., Hardt, C. and Johnscher, G. T-T cell interactions during in vitro cytotoxic T lymphocytes (CTL) responses. II. Helper factor from activated Lyt-1+ T cells is rate limiting i) in T cell responses to non-immunogenic alloantigen, ii) in thymocyte responses to allogeneic stimulator cells and iii) recruits allo- or H-2 restricted CTL precursors from the Lyt123+ T subset. J. Immunol. 124, 1058, 1980.
189. Gose, J.E. and Bach, F.H. H-2I-region encoded targets in allograft rejection. J. Exp. Med. 149, 1254, 1979.
190. Elkins, W.L. Correlation of graft-versus-host mortality and positive CML assay in the mouse. Transplant. Proc. 8, 343, 1976.
191. Rouse, B.T. and Wagner, H. The in vivo activity of in vitro immunized mouse thymocytes. II. Rejection of skin allografts and graft-vs-host activity. J. Immunol. 109, 1282, 1972.
192. Schreier, M.H. and Iscove, N.N. Haematopoietic growth factors are released in cultures of H-2 restricted helper T cells, accessory cells and specific antigen. Nature 287, 228, 1980.
193. Pals, S.T., Gleichmann, H. and Gleichmann, E. Allosuppressor and allohelper T cells in acute and chronic graft-versus-host disease.
V. F1 mice with secondary chronic GvHD contain activated allohelper but no allosuppressor T cells. J. Exp. Med. 1983, in press.
194. Kubota, E., Ishikawa, H. and Saito, K. Modulation of F1 cytotoxic potentials by GvHR. Host- and donor-derived cytotoxic lymphocytes arise in the unirradiated F1 host spleens under the condition of GvHR-associated immunosuppression. J. Immunol. 131, 1142, 1983.
195. Pattengale, P., Ramstedt, U., Gidlund, M., Orn, A., Axberg, I. and Wigzell, H. Natural killer (NK) activity in mice undergoing acute and chronic graft-versus-host (GvH) disease. Immunobiol. 159, 140, 1981.
196. Singh, J.N., Sabbadini, E., and Sehon, A.H. Cytotoxicity in graft-versus-host reaction. I. Role of donor and host spleen cells. J. Exp. Med. 136, 39, 1972.
197. Gleichmann, E., Van Elven, F. and Gleichmann, H. Immunoblastic lymphadenopathy, systemic lupus erythematosus and related disorders. Possible pathogenetic pathways. Am. J. Clin. Pathol. 72, 708, 1979.
198. Cantor, H., and Asofsky, R. Synergy among lymphoid cells mediating the graft-versus-host response. II. Synergy in graft-versus-host reactions produced by BALB/c lymphoid cells of differing anatomic origin. J. Exp. Med. 131, 235, 1970.

199. Raff, M.C. and Cantor, H. Subpopulations of thymus cells and thymus-derived lymphocytes. In: 'Progress in Immunology', B. Amos, ed., Academic Press, New York, p. 83, 1971.
200. Cantor, H. and Asofsky, R. Synergy among lymphoid cells mediating the graft-versus-host response. III. Evidence for interaction between two types of thymus derived cells. J. Exp. Med. 135, 764, 1972.
201. Tigelaar, R.E. and Asofsky, R. Synergy among lymphoid cells mediating the graft-versus-host response. V. Derivation by migration in lethally irradiated recipients of two interacting subpopulations of thymus-derived cells from normal spleen. J. Exp. Med. 137, 239, 1973.
202. Tigelaar, R.E. and Asofsky, R. Synergy among lymphoid cells mediating the graft-versus-host response. IV. Synergy in the GvH reaction quantitated by a mortality assay in sublethally irradiated recipients. J. Exp. Med. 135, 1059, 1972.
203. Wolters, E.A.J. and Benner, R. Different H-2 subregion coded antigens as targets for T cell subsets synergizing in graft-versus-host reaction. Cell. Immunol. 59, 115, 1981.
204. Cohen, D. and Howe, M.L. Synergism between subpopulations of thymus-derived cells mediating the proliferation and effector phases of the mixed lymphocyte reaction. Proc. Natl. Acad. Sci. USA 70, 2707, 1973.
205. Tittor, W., Gerbase-Delima, M. and Walford, R.L. Synergy among responding lymphoid cells in the one-way mixed lymphocyte reaction. Interaction between two types of thymus-dependent cells. J. Exp. Med. 139, 1488, 1974.
206. Wagner, H. Synergy during in vitro cytotoxic allograft responses. I. Evidence for cell interaction between thymocytes and peripheral T cells. J. Exp. Med. 138, 1379, 1973.
207. Wagner, H. Cell-mediated immune responses in vitro: interaction of thymus-derived cells during cytotoxic allograft responses in vitro. Science 181, 1170, 1973.
208. Blomgren, H. and Jacobsson, H. Interaction between thymocytes and lymph node cells in the graft-versus-host response: evidence for a synergistic and an antagonistic activity mediated by mouse thymocytes. Cell. Immunol. 12, 296, 1974.
209. Andersson, L.C., Häyry, P., Bach, M.A. and Bach, J.F. Differences in the effects of adult thymectomy on T cell mediated responses in vitro. Nature 252, 252, 1974.
210. Wright, P.W., Loop, S.M. and Bernstein, I.D. Synergy among rat T cells in the proliferative response to alloantigen. Cell. Immunol. 43, 245, 1979.
211. Feldmann, M. and Erb, P. Requirement for interactions between two subpopulations of T cells for helper cell induction in vitro. Z. Immun. Forsch. 153, 217, 1977.
212. Bril, H., Molendijk-Lok, B.D., Hussaarts-Odijk, L.M. and Benner, R. Synergism of T lymphocyte subsets in the response to Mls-locus coded antigens during graft-versus-host reaction. Cell. Immunol. in press.
213. Elkins, W.L. and Silvers, W.K. Synergistic interaction of H-Y and H-2K in elicitation of graft-versus-host response. Transplantation 34, 71, 1982.

214. Pritchard, L.L. and Halle-Pannenko, O. Interaction of major and minor histocompatibility antigens in the graft-versus-host reaction. Transplantation 31, 257, 1981.
215. Cohen, I.R. The recruitment of specific effector lymphocytes by antigen-reactive lymphocytes in cell-mediated autosensitization and allosensitization reactions. Cell. Immunol. 8, 209, 1973.
216. Cohen, I.R. and Livnat, S. The cell-mediated immune response: interactions of initiator and recruited T lymphocytes. Transplant. Rev. 29, 24, 1976.
217. Livnat, S. and Cohen, I.R. Recruitment of effector lymphocytes by initiator lymphocytes. Circulating lymphocytes are trapped in the reacting lymph node. J. Immunol. 117, 608, 1976.
218. Livnat, S. and Cohen, I.R. Recruitment of effector lymphocytes by initiator lymphocytes. Recruited lymphocytes are immunospecific and include graft-versus-host reactive lymphocytes. J. Immunol. 117, 614, 1976.
219. Cohen, I.R., Livnat, S. and Waksal, S.D. Initiator and recruited T lymphocytes are distinct subclasses of T lymphocytes. Eur. J. Immunol. 8, 35, 1978.
220. Gershon, R.K., Cohen, P., Hencin, R. and Liebhaber, S.A. Suppressor T cells. J. Immunol. 108, 586, 1972.
221. Gershon, R.K., Lance, E.M. and Kondo, K. Immunoregulatory role of spleen localizing thymocytes. J. Immunol. 112, 546, 1974.
222. Van Bekkum, D.W. and Knaan-Shanzer, S. Characterization of the thymocyte involved in the suppression of graft-versus-host disease in mice. Exp. Haematol. 9, suppl. 9, 160, 1981.
223. Van Bekkum, D.W. and Knaan-Shanzer, S. Characterization of a subpopulation in neonatal thymus which suppresses the graft-vs-host reaction. Eur. J. Immunol. 13, 403, 1983.
224. Halle-Pannenko, O., Martyre, M.C. and Mathé, G. Prevention of graft-versus-host reaction by donor pretreatment with soluble H-2 antigens. Transplantation 11, 414, 1971.
225. Halle-Pannenko, O., Pritchard, L.L., Motta, R. and Mathé, G. Lethal graft-versus-host reactions to minor histocompatibility antigens is decreased by donor immunization against H-2 and varies as a function of the H-2 haplotype. Transplant. Proc. 11, 652, 1979.
226. Bril, H. and Benner, R. Specific suppression of anti-host immune reactivity in graft-versus-host reactions. Adv. Exp. Med. Biol. 149, 177, 1982.
227. Bril, H., Molendijk-Lok, B.D. and Benner, R. Specific and non-specific T cell mediated suppression of anti-host immune reactivity in graft-versus-host reaction. Transplantation 36, 323, 1983.
228. Bril, H., Molendijk-Lok, B.D., Hussaarts-Odijk, L.M. and Benner, R. Characterization of suppressor T cells in graft-versus-host reactions. Cell. Immunol., submitted.
229. Bianchi, A.T.J., Hussaarts-Odijk, L.M., and Benner, R. Antigen-specific suppressor T cells suppress in vivo the cellular immune response to unrelated 'bystander' antigens. Transplant. Proc. 15, 760, 1983.

230. Bianchi, A.T.J., Bril, H. and Benner, R. Alloantigen-specific suppressor T cells suppress also the in vivo immune response to 'bystander' alloantigens. Nature 301, 614, 1983.
231. Shand, F.L. Analysis of immunosuppression generated by the graft-versus-host reaction. I. A suppressor T cell component studied in vivo. Immunology 29, 953, 1975.
232. Elie, R. and Lapp, W.S. Apparent T cell function of bone marrow cells from mice experiencing a graft-versus-host reaction. Cell. Immunol. 21, 185, 1976.
233. Elie, R. and Lapp, W.S. Graft-versus-host immunosuppression: depressed T helper cell function in vitro. Cell. Immunol. 34, 38, 1977.
234. Pickel, K. and Hoffmann, M.K. The Lyt phenotype of suppressor T cells arising in mice subjected to a graft-versus-host reaction. J. Exp. Med. 145, 1169, 1977.
235. Shand, F.L. Ly and Ia phenotype of suppressor T cells induced by graft-versus-host reactions. Eur. J. Immunol. 7, 746, 1977.
236. Strober, S., Slavin, S., Gottlieb, M., Zan-Bar, I., King, D.P., Hoppe, R.T., Fuks, Z., Grumet, F.C. and Kaplan, H.S. Allograft tolerance after total lymphoid irradiation (TLI). Immunol. Rev. 46, 87, 1979.
237. King, D.P., Strober, S. and Kaplan, H.S. Suppression of the mixed leukocyte response and of graft-vs-host disease by spleen cells following total lymphoid irradiation. J. Immunol. 126, 1140, 1981.
238. King, D.P. and Strober, S. Immunoregulatory changes induced by total lymphoid irradiation. II. Development of thymus-leukemia antigen-positive and -negative suppressor T cells that differ in their regulatory function. J. Exp. Med. 154, 13, 1981.
239. Fass, L., Ochs, H.D., Thomas, E.D., Mickelson, E., Storb, R. and Fefer, A. Studies on immunological reactivity following syngeneic or allogeneic marrow grafts in man. Transplantation 16, 630, 1973.
240. Storb, R., Ochs, H.D., Weiden, P.L. and Thomas, E.D. Immunologic reactivity in marrow graft recipients. Transplant. Proc. 8, 637, 1976.
241. Elfenbein, G.J., Anderson, P.N., Humphrey, R.L., Mullins, G.M., Sensenbrenner, L.L., Wands, J.R. and Santos, G.W. Immune system reconstitution following allogeneic bone marrow transplantation in man: a multiparameter analysis. Transplant. Proc. 8, 641, 1976.
242. Neely, J.E., Neely, A.N. and Kersey, J.H. Immunodeficiency following human marrow transplantation: in vitro studies. Transplant. Proc. 10, 229, 1978.
243, Noel, D.R., Witherspoon, R.P., Storb, R., Atkinson, K., Doney, K., Mickelson, E.M., Ochs, H.D., Warren, R.P., Weiden, P.L. and Thomas, E.D. Does graft-versus-host disease influence the tempo of immunologic recovery after allogeneic human marrow transplantation? An observation of 56 long-term survivors. Blood 51, 1087, 1978.

244. Witherspoon, R.P., Storb, R. and Ochs, H.D. Recovery of antibody production in human allogeneic marrow graft recipients: influence of time posttransplantation, the presence or absence of chronic graft-versus-host disease, and antithymocyte globulin treatment. Blood 58, 360, 1981.
245. Tsoi, M.S., Storb, R., Dobbs, S., Kopecky, K.J., Santos, E., Weiden, E. and Thomas, E.D. Nonspecific suppressor cells in patients with chronic graft-vs-host disease after marrow grafting. J. Immunol. 123, 1970, 1979.
246. Atkinson, K., Farewell, V. and Storb, R. Analysis of late infections after human bone marrow transplantation: role of genotypic non-identity between marrow donor and recipient and of nonspecific suppressor cells in patients with chronic graft-versus-host disease. Blood 60, 714, 1982.
247. Tsoi, M.S., Storb, R., Weiden, P.L. and Thomas, E.D. Studies on cellular inhibition and serum-blocking factors in 28 human patients given marrow grafts from HLA identical siblings. J. Immunol. 118, 1799, 1977.
248. Tsoi, M.-S., Storb, R., Dobbs, S. and Thomas, E.D. Specific suppressor cells in graft-host tolerance of HLA-identical marrow transplantation. Nature 292, 355, 1981.
249. Tsoi, M.-S., Storb, R., Weiden, P., Santos, E., Kopecky, K.J. and Thomas, E.D. Sequential studies of cell inhibition of host fibroblasts in 51 patients given HLA-identical marrow grafts. J. Immunol. 128, 239, 1982.
249a. Van Bekkum, D.W., Van Putten, L.M., and De Vries, M.J. Anti-host reactivity and tolerance of the graft in relation to secondary disease in radiation chimeras. Ann. N.Y. Acad.Sci. 99, 550, 1962.
250. Korngold, R., Bennink, J.R., and Doherty, P.C. Early dominance of irradiated host cells in the responder profile of thymocytes from P⟶F1 radiation chimeras. J.Immunol. 127, 124, 1981.
251. Ceredig, R., and MacDonald, H.R. Phenotypic and functional properties of murine thymocytes. 11. Quantitation of host- and donor-derived cytolytic T lymphocyte precursors in regenerating radiation bone marrow chimeras. J.Immunol. 128, 614, 1982.
252. Onoé, K., Fernandes, G., Shen, F.W., and Good, R.A. Sequential changes of thymocyte surface antigens with presence or absence of graft-versus-host reaction following allogeneic bone marrow transplantation. Cell. Immunol. 68, 207, 1982.
253. Kruisbeek, A.M., Hodes, R.J., and Singer, A. Cytotoxic T lymphocyte responses by chimeric thymocytes. Self-recognition is determined early in T cell development. J.Exp.Med. 153, 13, 1981.
254. Hardt, C., Röllinghoff, M., Pfizenmaier, K., Mosmann, H., and Wagner, H. Lyt-23$^+$ cyclophosphamide-sensitive T cells regulate the activity of an interleukin 2 inhibitor in vivo. J.Exp.Med. 154, 262, 1981.
255. Hamilton, B.L., and Parkman, R. Kinetics of the anti-recipient cytotoxic cell response of mice with minor histocompatibility antigen graft-vs-host disease. J.Immunol. 128, 376, 1982.

256. Dicke, K.A., and Van Bekkum, D.W. Allogeneic bone marrow transplantation after elimination of immunocompetent cells by means of density gradient centrifugation. Transplant.Proc. 3, 666, 1971.
257. Amato, D., Cowan, D.H., and McCulloch, E.A. Separation of immunocompetent cells from human and mouse hemopoietic cell suspensions by velocity sedimentation. Blood 39, 472, 1972.
258. Muto, M., Sado, T., Aizawa, S., Kamisaku, H., and Kubo, E. Bone marrow transplantation across the major histocompatibility barrier in specific-pathogen-free mice: effects of intact versus T cell-depleted bone marrow on the expression of anti-host reaction in the recipient spleens. J.Immunol. 127, 2421, 1981.
259. Norin, A.J., and Emeson, E.E. Effects of restoring lethally irradiated mice with anti Thy-1.2 treated bone marrow: graft-vs-host, host-vs-graft and mitogen reactivity. J.Immunol. 120, 754, 1978.
260. Goedbloed, J.F., and Vos, O. Influences on the incidence of secondary disease in radiation chimeras: thymectomy and tolerance. Transplantation 3, 603, 1965.
261. Van Putten, L.M. Thymectomy: effect on secondary disease in radiation chimeras. Science 145, 935, 1964.
262. Lydyard, P.M. and Ivanyi, J. Chimaerism of immunocompetent cells in allogeneic bone marrow reconstituted lethally irradiated chickens. Transplantation 20, 155, 1975.
263. Rabinowich, H., Umiel, T., and Globerson, A. T cell progenitors in the mouse fetal liver. Transplantation 35, 40, 1983.
264. Kindred, B. H-2-restricted lethal graft-versus-host disease. Immunogenetics 14, 527, 1981.
265. Kindred, B. H-2-restricted GvH reaction caused by T cells from normal donors of certain strains. Immunogenetics 17, 203, 1983.
266. Kindred, B. H-2-restricted GvH reaction: foreign determinants and restriction elements. Immunogenetics 18, 57, 1983.
267. Korngold, R. and Sprent, J. Negative selection of T cells causing lethal graft-versus-host disease across minor histocompatibility barriers. Role of the H-2 complex. J. Exp. Med. 151, 1114, 1980.
268. Korngold, R. and Sprent, J. Features of T cells causing H-2 restricted lethal graft-vs-host disease across minor histocompatibility barriers. J. Exp. Med. 155, 872, 1982.
269. Widmer, M.B., and Bach, F.H. Antigen-driven helper cell-independent cloned cytolytic T lymphocytes. Nature 294, 750, 1981.
270. Mowat, A.McI. and Ferguson, A. Hypersensitivity reactions in the small intestine. 6. Pathogenesis of the graft-versus-host reaction in the small intestinal mucosa of the mouse. Transplantation 32, 238, 1981.
271. Mowat, A.McI. and Ferguson, A. Intra-epithelial lymphocyte count and crypt hyperplasia measure the mucosal component of the graft-versus-host reaction in mouse small intestine. Gastroenterology 83, 417, 1982.

272. Hamilton, B.L. and Parkman, R. Acute and chronic graft-versus-host disease induced by minor histocompatibility antigens in mice. Transplantation 36, 150, 1983.
273. Gleichmann, E., Rolink, A.G., Pals, S.T. and Gleichmann, H. Graft-versus-host reactions (GvHRs): clues to the pathogenesis of a broad spectrum of immunologic diseases. Transplant. Proc. 15, 1436, 1983.
274. Mason, D.W. Subsets of T cells in the rat mediating lethal graft-versus-host disease. Transplantation 32, 222, 1981.
275. Van Elven, E.H., Rolink, A.G., Van der Veen, F., Van Heertum, J.G. and Gleichmann, E. Capacity of genetically different T lymphocytes to induce lethal graft-versus-host disease correlates with their capacity to generate suppression but not with their ability to generate anti-Fl T-killer cells. A dominant non-H-2 locus determines the inability to induce lethal graft-versus-host disease. J.Exp.Med. 153, 1474, 1981.
276. Pals, S.T., Radaszkiewicz, T., and Gleichmann, E. Induction of either acute or chronic graft-versus-host disease due to genetic differences among donor T cells. Adv. Exp. Med. Biol. 149, 537, 1982.
277. Pals, S.T. and Gleichmann, E. A sequential alloactivation of subsets of donor T cells underlies the transition from acute graft-versus-host disease (GvHD) to chronic GvHD. Transplant. Proc. 15, 1480, 1983.
278. Pals, S.T., Radasziekwicz, T. and Gleichmann, E. Allosuppressor and allohelper T cells in acute and chronic graft-versus-host disease. IV. Activation of donor allosuppressor cells is confined to acute GvHD. J. Immunol., submitted.
279. Hurtenbach, U. and Shearer, G.M. Analysis of murine T lymphocyte markers during the early phases of GvH-associated suppression of cytotoxic T lymphocyte responses. J. Immunol. 130, 1561, 1983.
280. Dosch, H.M., and Gelfand, E.W. Failure of T and B cell cooperation during graft-versus-host disease. Transplantation 31, 48, 1981.
281. De Bruin, H.G., Astaldi, A., Leupers, T., Van de Griend, R.J., Dooren, L.J., Schellekens, P.Th.A., Tanke, H.J., Roos, M., and Vossen, J.M. T lymphocyte characteristics in bone marrow-transplanted patients. 11. Analysis with monoclonal antibodies. J.Immunol. 127, 244, 1981.
282. Reinherz, E.L., Parkman, R., Rappeport, J., Rosen, F.S., and Schlossman, S.F. Aberrations of suppressor T cells in human graft-versus-host disease. N.Engl.J.Med. 300, 1061, 1979.
283. OKunewick, J.P., Meredith, R.F., Raikow, R.B., Buffo, M.J., and Jones, D.L. Possibility of three distinct and separable components to fatal graft-vs-host reaction. Exp.Hematol. 10, 277, 1982.
284. Von Boehmer, H., Sprent, J., and Nabholz, M. Tolerance to histocompatibility determinants in tetraparental bone marrow chimeras. J.Exp.Med. 141, 322, 1975.

285. Phillips, S.M., and Wegmann, T.G. Active suppression as a possible mechanism of tolerance in tetraparental mice. J.Exp.Med. 137, 291, 1973.
286. Weiden, P.I., Storb, R., Tsoi, M.-S., Graham, T.C., Lerner, K.G. and Thomas, E.D. Infusion of donor lymphocytes into stable canine radiation chimeras: implications for mechanisms of transplantation tolerance. J. Immunol. 116, 1212, 1976.
287. Atkinson, K., Storb, R., Weiden, P.L., Deeg, H.J., Kopecky, K.J., Graham, T.C., and Thomas, E.D. Studies on transplantation tolerance in canine radiation chimeras. In 'Biology of bone marrow transplantation' eds. R.P. Gale and C.F. Cox, Academic Press, New York, p. 271, 1980.
288. Tutschka, P.J., Hess, A.D., Beschorner, W.E. and Santos, G.W. Suppressor cells in transplantation tolerance. I. Suppressor cells in the mechanism of tolerance in radiation chimeras. Transplantation 32, 203, 1981.
289. Tutschka, P.J., Ki, P.F., Beschorner, W.E., Hess, A.D. and Santos, G.W. Suppressor cells in transplantation tolerance. II. Maturation of suppressor cells in the bone marrow chimera. Transplantation 32, 321, 1981.
290. Tutschka, P.J., Hess, A.D., Beschorner, W.E. and Santos, G.W. Suppressor cells in transplantation tolerance. III. The role of antigen in the maintenance of transplantation tolerance. Transplantation 33, 510, 1982.
291. Auchincloss, H. and Sachs, D.H. Mechanisms of tolerance in murine radiation bone marrow chimeras. I. Nonspecific suppression of alloreactivity by spleen cells from early, but not late, chimeras. Transplantation 36, 436, 1983.
292. Auchincloss, H., and Sachs, D.H. Mechanisms of tolerance in murine radiation bone marrow chimeras. II. Absence of nonspecific suppression in mature chimeras. Transplantation 36, 442, 1983.
293. Sprent, J., Von Boehmer, H., and Nabholz, M. Association of immunity and tolerance to host H-2 determinants in irradiated F1 hybrid mice restored with bone marrow cells from one parental strain. J. Exp. Med. 142, 321, 1975.
294. Von Boehmer, H., and Sprent, J. T cell function in bone marrow chimeras: absence of host-reactive T cells and cooperation of helper T cells across allogeneic barriers. Transplant.Rev. 29, 3, 1976.
295. Green, D.R., Gershon, R.K., and Eardley, D.D. Functional deletion of different Ly-1 T-cell-inducer subset activities by Ly-2 suppressor T lymphocytes. Proc.Natl.Acad.Sci USA 78, 3819, 1981.
296. Zinkernagel, R.M., and Doherty, P.C. H-2 compatibility requirement for T-cell-mediated lysis of target cells infected with lymphocytic choriomeningitis virus. Different cytotoxic T-cell specificities are associated with structures coded for in H-2K or H-2D. J.Exp.Med. 141, 1427, 1975.
297. Fink, P.J., and Bevan, M.J. H-2 antigens of the thymus determine lymphocyte specificity. J.Exp.Med. 148, 766, 1978.

298. Zinkernagel, R.M., Callahan, G.N., Althage, A., Cooper, S., Klein, P.A. and Klein, J. On the thymus in the differentiation of 'H-2 self-recognition' by T cells: evidence for dual recognition? J.Exp.Med. 147, 882, 1978.
299. Zinkernagel, R.M., Klein, P.A., and Klein, J. Host-determined T cell fine specificity for self H-2 in radiation bone marrow chimeras of standard C57BL/6 ($H\text{-}2^{b}$), mutant Hz1 ($H\text{-}2^{ba}$), and F1 mice. Immunogenetics 7, 73, 1978.
300. Zinkernagel, R.M., Callahan, G.N., Althage, A., Cooper, S., Streilein, J.W., and Klein, J. The lymphoreticular system in triggering virus plus self-specific cytotoxic T cells: evidence for T help. J.Exp.Med. 147, 897, 1978.
301. Zinkernagel, R.M., Althage, A., Callahan, G., and Welsh Jr., R.M. On the immunocompetence of H-2 incompatible irradiation bone marrow chimeras. J.Immunol. 124, 2356, 1980.
302. Maestroni, G.J.M., Pierpaoli, W., and Zinkernagel, R.M. Immunoreactivity of long-lived H-2 incompatible irradiation chimeras ($H\text{-}2^{d} \rightarrow H\text{-}2^{b}$). Immunology 46, 253, 1982.
303. Pierpaoli, W., Maestroni, G.J.M., and Sache, E. Enduring allogeneic marrow engrafment via nonspecific bone marrow derived regulating factors (MRF), Cell.Immunol. 57, 219, 1981.
304. Onoé, K., Fernandes, G., and Good, R.A. Humoral and cell mediated immune-responses in fully allogeneic bone marrow chimera in mice. J.Exp.Med. 151, 115, 1980.
305. Krown, S.E., Coico, R., Scheid, M.P., Fernandes, G. and Good, R.A. Immune function in fully allogeneic mouse bone marrow chimeras. Clin. Immunol. Immunopathol. 19, 268, 1981.
306. Kruisbeek, A.M., Hathcock, K.S, Hodes, R.J., and Singer, A. T cells from fully H-2 allogeneic (A→B) radiation bone marrow chimeras are functionally competent and host restricted but are alloreactive against hybrid Ia determinants expressed on (AxB)F1 cells. J.Exp.Med. 155, 1864, 1982.
307. Singer, A., Hathcock, K.S., and Hodes, R.J. Self recognition in allogeneic radiation bone marrow chimeras. A radiation-resistant host element dictates the self-specificity and immune response gene phenotype of T-helper cells. J.Exp.Med. 153, 1286, 1981.
308. Miller, J.F.A.P., Vadas, M.A., Whitelaw, A., and Gamble, J. Role of major histocompatibility complex gene products in delayed-type hypersensitivity. Proc.Natl.Acad.Sci. USA 73, 2486, 1976.
309. Asherson, G.L., Mayhew, B., and Perera, M.A.C.C. The production of contact sensitivity by the injection into footpads of recipients of the lymph nodes from mice 1 day after painting the skin with contact-sensitizing agent: requirement for matching at major histocompatibility complex between donor and recipient mice. Immunology 32, 241, 1979.
310. Shelley, W.B., and Juhlin, L. Langerhans cells form a reticulo-epithelial trap for external contact antigens. Nature 261, 46, 1976.
311. Klareskog, L., Tjernlund, V.M., Forsum, U. and Peterson, P.A. Epidermal Langerhans cells express Ia antigens. Nature 268, 248, 1977.

312. Katz, D.H., Skidmore, B.J., Katz, L.R., and Bogowitz, C.A. Adaptive differentiation of murine lymphocytes. I. Both T and B lymphocytes differentiating in F1→parental chimeras manifest preferential cooperative activity for partner lymphocytes derived from the same parental strain type corresponding to the chimeric host. J.Exp.Med. 148, 727, 1978.
313. Sprent, J., and Von Boehmer, H. T-helper function of parent→F1 chimeras. Presence of a separate T-cell subgroup able to stimulate allogeneic B cells but not syngeneic B cells. J.Exp.Med. 149, 387, 1979.
314. Waldmann, H., Pope, H., Brent, L., and Bighouse, K. Influence of the major histocompatibility complex on lymphocyte interactions in antibody formation. Nature 274, 166, 1978.
315. Zinkernagel, R.M., Althage, A., Waterfield, E., Kindred, B., Welsh, R.M., Callahan, G., and Pincetl, P. Restriction specificities, alloreactivity and allotolerance expressed by T cells from nude mice reconstituted with H-2-compatible or -incompatible thymus grafts. J. Exp.Med. 151, 376, 1980.
316. Matzinger, P., and Mirkwood, G. In a fully H-2 incompatible chimera, T cells of donor origin can respond to minor histocompatibility antigens in association with either donor or host H-2 type. J. Exp. Med. 148, 84, 1978.
317. Doherty, P.C. and Bennink, J.R. Vaccinia specific cytotoxic T cell responses in the context of H-2 antigens not encountered in thymus may reflect aberrant recognition of a virus H-2-complex. J. Exp. Med. 149, 150, 1979.
318. Blanden, R.V., and Andrew, M.E. Primary anti-viral cytotoxic T cell responses in semiallogeneic chimeras are not absolutely restricted to host H-2 type. J.Exp.Med. 149, 535, 1979.
319. Ando, I., and Hurme, M. Self-MHC-restricted cytotoxic T-cell response without thymic influence. Nature 289, 494, 1981.
320. Streilein, J.W., and Stone, M.J. GvH disease: unmasking of forbidden clones. Transplant. Proc. 5, 861, 1973.
321. Streilein, J.W., Stone, M.J. and Duncan, W.R. Studies of the specificity of autoantibodies produced in systemic graft-vs-host disease. J. Immunol. 114, 255, 1975.
322. Allison, A.C., and Denman, A.M. Self-tolerance and autoimmunity. Brit. Med. Bull. 32, 124, 1976.
323. Primi, D., Smith, C.I.E., Hammarström, L., and Möller, G. Polyclonal B cell activators induce immunological response to autologous serum proteins. Cell. Immunol. 34, 367, 1977.
324. Katz, D.H., Paul, W.E., and Benacerraf, B. Carrier function in anti-hapten antibody responses. V. Analysis of cellular events in the enhancement of antibody responses by the allogeneic effect in DNP-OVA-primed guinea pigs challenged with a heterologous DNP-conjugate. J. Immunol. 107, 1319, 1971.
325. Osborne Jr., D.P., and Katz, D.H. The allogeneic effect in inbred mice. I. Experimental conditions for the enhancement of hapten-specific secondary antibody responses by the graft-versus-host reaction. J.Exp.Med. 136, 439, 1972.

326. Katz, D.H., and Osborne Jr., D.P. The allogeneic effect in inbred mice. II. Establishment of the cellular interactions required for enhancement of antibody production by the graft-versus-host reaction. J.Exp.Med. 136, 455, 1972.
327. Katz, D.H. The allogeneic effect on immune responses: model for regulatory influences of T lymphocytes on the immune system. Transplant. Rev. 12, 141, 1972.
328. Ornellas, E.P., and Scott, D.W. Allogeneic interactions in the immune response and tolerance. I. The allogeneic effect in unprimed rats and mice. Cell.Immunol. 11, 108, 1974.
329. Scott, D.W., and Ornellas, E.P. Allogeneic interactions in the immune response and tolerance. II. Mechanism of stimulation of TNP plaque forming cells in normal rats. Cell. Immunol. 11, 116, 1974.
330. Ornellas, E.P., Sanfilippo, F., and Scott, D.W. Cellular events in tolerance. IV. The effect of graft-versus-host reaction and endotoxin on hapten- and carrier-specific tolerance. Eur.J.Immunol. 4, 587, 1974.
331. Gleichmann, E., and Gleichmann, H. Diseases caused by reactions of T lymphocytes to incompatible structures of the major histocompatibility complex. I. Autoimmune hemolytic anemia. Eur.J.Immunol. 6, 899, 1976.
332. Van Elven, E.H., Agterberg, J., Sadal, S., and Gleichmann, E. Diseases caused by reactions of T lymphocytes to incompatible structures of the major histocompatibility complex. II. Autoantibodies deposited along the basement membrane of skin and their relationship to immune-complex glomerulonephritis. J. Immunol. 126, 1684, 1981.
333. Van der Veen, J.P.W., Rolink, A.G., and Gleichmann, E. Diseases caused by reactions of T lymphocytes to incompatible structures of the major histocompatibility complex. III. Autoantibodies to thymocytes. J. Immunol. 127, 1281, 1981.
334. Van der Veen, F.M., Rolink, A.G., and Gleichmann, E. Diseases caused by reactions of T lymphocytes to incompatible structures of the major histocompatibility complex. IV. Autoantibodies to nuclear antigens. Clin.exp.Immunol. 46, 589, 1981.
335. Van Elven, E.H., Van der Veen, F.M., Rolink, A.G., Issa, P., Duin, T.M., and Gleichmann, E. Diseases caused by reactions of T lymphocytes to incompatible structures of the major histocompatibility complex. V. High titers of IgG autoantibodies to double-stranded DNA. J.Immunol. 127, 2435, 1981.
336. Rolink, A.G., Gleichmann, H., and Gleichmann, E. Diseases caused by reactions of T lymphocytes to incompatible structures of the major histocompatibility complex. VII. Immune-complex glomerulonephritis. J. Immunol. 130, 209, 1983.
337. Rappard-Van der Veen, F.M., Rolink, G., and Gleichmann, E. Diseases caused by reactions of lymphocytes towards incompatible structures of the major histocompatibility complex. VI. Autoantibodies characteristic of systemic lupus erythematosus induced by abnormal T-B cell cooperation across I-E. J.Exp.Med. 155, 1555, 1982.

338. Delovitch, T.L., and Falk, J.A. Evidence for structural homology between murine and human Ia antigens. Immunogenetics 8, 405, 1979.
339. Silver, J., Walker, L.E., Reisfeld, R.A., Pellegrino, M.A., and Ferrone, S. Structural studies of murine I-E and human DR antigens. Mol.Immunol. 16, 37, 1979.
340. Celada, A., Barras, C., Benzonana, G., and Jeannet, M. Increased frequency of HLA-DRw3 in systemic lupus erythematosus. New Engl.J. Med. 301, 1398, 1979.
341. Gladman, D.D., Terasaki, P.I., Park, M.S., Iwaki, Y., Louie, S., Quismorio, F.P., Barnett, E.V., and Liebling, M.R. Increased frequency of HLA-DRw2 in SLE. Lancet i, 902, 1979.
342. Batchelor, J.R., Welsh, K.I., and Mansilla Tinoco, R. Hydralazine-induced systemic lupus erythematosus: influence of HLA-DR and sex on susceptibility. Lancet i, 1107, 1980.
343. Gleichmann, E., Van Elven, E.H., and Van der Veen, J.P.W. A systemic lupus erythematosus (SLE)-like disease in mice induced by abnormal T-B cell cooperation. Preferential formation of autoantibodies characteristic of SLE. Eur.J.Immunol. 12, 152, 1982.
344. Gleichmann, H., Pals, S., Radaszkiewicz, T., and Wasser, M. T cell dependent B cell lymphoproliferation and activation induced by the drug diphenylhydantoin. Adv.Exp.Med.Biol. 149, 617, 1982.
345. Beschorner, W.E., Tutschka, P.J., and Santos, G.W. Chronic graft-versus-host disease in the radiation chimera. Transplantation 33, 393, 1982.
346. Urso, P., and Gengozian, N. Immune competence of splenic lymphocytes following graft-vs-host disease in mouse allogeneic radiation chimeras. J. Immunol. 118, 657, 1977.
347. Schwartz, R.S., Beldotti, L. Malignant lymphomas following allogeneic disease: transition from an immunological to a neoplastic disorder. Science 149, 1511, 1965.
348. Armstrong, M.Y.K., Schwartz, R.S. and Beldotti, L. Neoplastic sequelae of allogeneic disease. III. Histological events following transplantation of allogeneic spleen cells. Transplantation 5, 1380, 1967.
349. Armstrong, M.Y.K., Gleichmann, E., Gleichmann, H., André-Schwartz, J., and Schwartz, R.S. Chronic allogeneic disease. II. Development of lymphomas. J.Exp.Med. 132, 417, 1970.
350. Gleichmann, E., Gleichmann, H., Schwartz, R.S., Weinblatt, A., and Armstrong, M.Y.K. Immunologic induction of malignant lymphoma: identification of donor and host tumors in the graft-versus-host model. J.Natl.Cancer Inst. 54, 107, 1975.
351. Gleichmann, E., Peters, K., Lattmann, E., and Gleichmann, H. Immunologic induction of reticulum cell sarcoma: donor-type lymphomas in the graft-versus-host model. Eur. J. Immunol. 5. 406. 1975.
352. Gleichmann, E., Gleichmann, H., and Wilke, W. Autoimmunization and lymphomagenesis in parent→F1 combinations differing at the major histocompatibility complex: model for spontaneous disease caused by altered self antigens? Transplant.Rev. 31, 156, 1976.

353. Hirsch, M.S., Philips, S.M., Solnik, C., Black, P.H., Schwartz, R.S., and Carpenter, C.B. Activation of leukemia viruses by graft-versus-host and mixed lymphocyte reactions in vitro. Proc.Natl.Acad.Sci. USA 69, 1069, 1972.
354. Walford, R. Increased incidence of lymphoma after injections of mice with cells differing at weak histocompatibility loci. Science 152, 78, 1966.
355. Reinherz, E.L., Geha, R., Rappeport, J.M., Wilson, M., Penta, A.C., Hussey, R.E., Fitzgerald, H.A., Daley, J.F., Levine, H., Rosen, F.S., and Schlossman, S.F. Reconstitution after transplantation with T-lymphocyte-depleted HLA haplotype-mismatched bone marrow for severe combined immunodeficiency. Proc.Natl.Acad.Sci.USA 79, 6047, 1982.
356. Prentice, H.G., Janossy, G., Skeggs, D., Blacklock, H.A. Bradstock, K.F., Goldstein, G. and Hoffbrand, A.V. Use of anti-T-cell monoclonal antibody OKT3 to prevent acute graft-versus-host disease in allogeneic bone marrow transplantation for acute leukemia. Lancet i, 700, 1982.
357. OKunewick, J.P., and Meredith, R.F.(eds.). Graft-versus-leukemia in man and animal models. Boca Raton, CRC Press Inc., 1981.

INTRODUCTION AND DISCUSSION OF THE APPENDIX PUBLICATIONS

Delayed-type hypersensitivity (DTH) is classified as a cell-mediated immune phenomenon and can be caused by antigen-reactive T lymphocytes without any essential involvement of B lymphocytes or antibodies. Skin reactions due to DTH are distinguished from antibody-mediated skin reactions of immediate type hypersensitivity by the delayed onset of the former type of skin lesions.

A state of DTH will arise after appropriate sensitization with antigen and its expression can be elicited locally or systemically by subsequent challenge with that particular antigen. Skin testing and, in experimental animals, footpad challenge and ear testing are examples of local cutaneous elicitation of DTH, whereas shock, changes in body temperature or hemorrhagic changes in the lung represent the systemic part of the DTH reaction (Crowle, 1975). DTH can be induced in mice by mycobacteria and other bacteria, by fungi, viruses, parasites, proteins, polysaccharides, contact-sensitizing agents, heterologous erythrocytes and MHC and non-MHC coded antigens (Crowle, 1975; Van der Kwast, 1979).

During the past six years our laboratory has been studying the cellular and genetic requirements of alloreactivity *in vivo* by employing experimental models that make use of the large variety of available inbred mouse strains with well-defined histocompatibility antigens (Van der Kwast, 1979; Wolters, 1980). In these studies DTH to alloantigens has been studied under HvG as well as GvH conditions.

Evidence was presented that under HvG conditions H-2 as well as non-H-2 alloantigens can induce DTH (Van der Kwast et al., 1979; Wolters et al., 1981). Also H-2 subregion coded alloantigens and a single minor histocompatibility antigen (i.e., the H-Y antigen) can induce specific DTH following subcutaneous (sc) administration of the antigen (Van der Kwast et al., 1979; Wolters et al., 1981). Under GvH conditions, the situation appeared to be completely different. Only H-2I and Mls-locus coded alloantigens were able to evoke anti-host DTH reactivity (Wolters et al., 1980, 1981).

The purpose of the Appendix papers I and II was to provide more insight into the cellular and genetic requirements for DTH reactivity to H-2 subregion coded alloantigens and non-H-2 coded alloantigens under GvH conditions.

Early in the seventies it has been amply documented that different T cell subpopulations can cooperate in GvH reactions. Thus, it has been demonstrated that short-lived, sessile T1 cells and long-lived, recirculating T2 cells synergize in the splenomegaly GvH assay (Cantor and Asofsky, 1970, 1972) and in the in vitro MLC (Tittor et al., 1974; Wright et al., 1979). Our laboratory has recently shown that such synergistic activity of T1 and T2 cells can also be demonstrated in the development of anti-host DTH reactivity to H-2 alloantigens (Wolters and Benner, 1981). This activity only occurred when the donor/recipient combination differed not only at H-2I, but also at the H-2K/D subregion of the H-2 complex.

It was found that the T2 cells responded to the H-2I-coded alloantigens, while the T1 cells, which were activated by the H-2K/D alloantigens, amplified their response. These T1 cells by themselves were unable to mount anti-host DTH reactivity (Wolters and Benner, 1981). In Appendix paper I, the role of T1 and T2 cells was studied in the development of anti-Mls DTH reactivity in lethally irradiated hosts. It could be demonstrated that anti-Mls antigen directed DTH effector T cells are the progeny of the T2 cells and that T1 cells amplified this response. The latter, however, were by themselves incapable of displaying anti-Mls DTH reactivity. Thus there is a parallel between the anti-Mls and the anti-H-2I DTH response by T2 cells. Furthermore, non-H-2 alloantigens, other than those encoded for by the Mls locus, and H-2K/D alloantigens induce a similar T1 cell mediated amplifying activity that enhances the anti-host response by the T2 cells. (see Fig. 5 for a model for T1-T2 cooperation. This model has been proposed by E.A.J. Wolters in his thesis and is proved right in this).

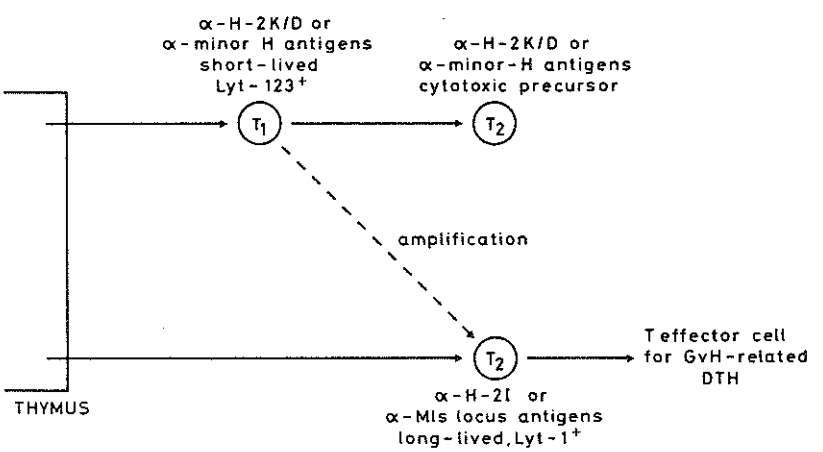

Fig. 5. Model for T1 cell amplification of T2 cell-derived GvH-related DTH reactivity.

Phenotyping of the T cell subsets cooperating in the anti-Mls response showed that the T1 cells have the Lyt-12$^+$ phenotype and the T2 cells the Lyt-1$^+$ phenotype. The same was found for the T1 and T2 cells synergizing in the development of anti-host DTH reactivity after transplantation of lymphoid cells into irradiated H-2 incompatible recipients (Appendix paper I) and for T cells interacting in the induction of helper T cells (Feldmann et al., 1977). Also in the generation of cytotoxic T cells there is a synergistic activity of different T cell subsets.

Bach et al. (1976) have found that Lyt-1$^+$ helper T cells, upon reacting to H-2I region coded alloantigens, proliferate and produce mediators that amplify the proliferation and differentiation of cytotoxic Lyt-23$^+$ T cells. Wagner et al. (1979) found that <u>in vitro</u> the rate-limiting factor for the differentiation of Lyt-123$^+$ cortical lymphocytes into Lyt-23$^+$ CTL is the presence of a helper factor from activated Lyt-1$^+$ T cells.

Most immune reactions occur in a MHC restricted fashion, i.e., T cells need to recognize the specific antigen in the context of self-MHC coded antigens. In Appendix paper II we investigated whether the same holds for sc induced DTH and GvH-related DTH reactivity to H-2 subregion coded alloantigens. The results of these experiments indicate that T cells reacting to H-2I region coded alloantigens need to recognize these antigens in the context of syngeneic H-2K/D molecules, while T cells reacting to H-2K/D region coded alloantigens

need to recognize these antigens in the context of syngeneic H-2I molecules. Thus, DTH reactive T cells recognize H-2 subregion coded alloantigens in a H-2 restricted fashion, just as they recognize other types of antigens (proteins, viruses, bacteria, etc.).

Suppression of DTH reactions to contact sensitizing agents and heterologous erythrocytes has also been described (Zembala and Asherson, 1973; Liew, 1977). In these studies suppression was induced by an intravenous (iv) injection of the specific antigen. It was shown in these studies that the state of suppression was due to suppressor T cells.

We investigated whether iv injection of mice with allogeneic spleen cells can suppress the development of DTH to MHC and non-MHC coded alloantigens which normally arises after sc immunization with such antigens (Appendix paper III). This was found to be the case. The iv route was found to be obligatory for inducing maximal suppression. Furthermore, the extent of suppression was clearly dependent on the cell dosages used. Suppression could be demonstrated after iv preimmunization with irradiated as well as with unirradiated spleen cells and was found to be mediated by T cells (Appendix paper IV). The induced suppression was found not to be due to a shift in peak reactivity and could be demonstrated up to 40 days after its induction. The suppression of DTH to H-2 alloantigens was haplotype specific. Moreover, iv injection of non-H-2 incompatible cells could not suppress the DTH to H-2 alloantigens and vice versa (Appendix paper III).

As suppression may affect the induction (Liew, 1977; Miller et al., 1978b; Germain and Benacerraf, 1981) as well as the expression phase of DTH (Miller et al., 1978a; Asherson and Zembala, 1980; Germain and Benacerraf, 1981) we investigated which phase of the DTH reaction was affected by our protocol of iv induction of suppressor T cells. It was found that in our system the suppression affected the induction phase of DTH. Most likely the suppressor cells also affect the expression phase, since the immune lymphocyte transfer (ILT) reactivity could be blocked by simultaneous transfer of suppressor T cells.

The mechanism by which suppressor T cells exert their influence might well be the inhibition of proliferation of DTH reactive T cells as immunosuppressed mice do not respond to sc immunization with the specific antigen with a proliferative activity in contrast to non-suppressed mice (Appendix paper III).

In Appendix paper IV the suppressive effect was further evaluated, and the T cells responsible for the suppression characterized. Different dosages of irradiated and unirradiated spleen cells were used to induce suppressor T cells. It was found that suppressor T cells were optimally induced by a dose of 1×10^6 unirradiated cells and by a dose of 5×10^7 irradiated cells. The spleen did not seem obligatory for the optimal induction of suppressor T cells as lymph node cells from splenectomized suppressed animals could transfer suppression to naive secondary recipients.

In subsequent studies we investigated whether suppressor T cells

induced according to the above protocol of iv preimmunization with irradiated allogeneic spleen cells could also suppress acute and delayed GvH reactions in mice. The assay employed in these studies for measuring GvH reactivity was originally developed by Wolters and Benner (1978) and is based upon transfer of spleen and lymph node cells from mice subjected to GvH to naive secondary recipients, which are syngeneic to the donors of the cells, used to reconstitute the irradiated recipients (Fig. 1). Immediately after transfer the secondary recipients are challenged in the right hind foot with spleen cells syngeneic to the irradiated recipients to test for DTH reactive T cells. If there were anti-host reactive T cells in the spleen and/or lymph nodes of the mice subjected to GvH, this results in a footpad swelling of the locally challenged secondary recipients, which can be measured 24, 48 and 72 h after challenge. Using this assay, we evaluated whether suppressor T cells induced by means of iv preimmunization of donor mice with irradiated allogeneic spleen cells were also able to minimize the anti-host DTH reactivity during GvH. Indeed, spleen cells and bone marrow cells from donors that had been iv preimmunized with recipient-type alloantigens induced weaker anti-host DTH reactions. Furthermore, such spleen cells were able to suppress the anti-host immune reactivity by spleen cells from non-suppressed donors (Appendix paper V). Suppressor T cells could be demonstrated at least 40 days after induction. Subsequently we showed that the generation of active suppressor T cells is associated with proliferation, and furthermore, that after restimulation these cells require further proliferation to display a maximal suppressive

effect. These suppressor cells were found to bear Lyt-1 and/or Lyt-2 molecules on their cell surface (Appendix paper V).

In recent years several suppressor T cell pathways have been described in the literature. Germain and Benacerraf (1981) have tried to integrate these different models. They advocate that the suppressor pathway is initiated by a Lyt-1^+ or Lyt-123^+ antigen-specific T cell, which produces a suppressor factor called TsF1. This factor triggers precursors of second order suppressor T cells (Lyt-123^+) to develop into Lyt-23^+ cells, which in turn produce another suppressor factor called TsF2.
The latter acts on a third suppressor cell precursor (Lyt-123^+) which develops afterwards to a Lyt-23^+ cell and produces a third suppressor factor called TsF3. The ultimate suppressive effect may be mediated by the third order suppressor T cell or the TsF3. Feldmann et al. (1977) have shown that suppressor precursor cells and suppressor effector cells are both Lyt-23^+ and reside in the T2 subpopulation. For differentiation of these precursor cells an amplifier T cell is required. This cell type is Lyt-123^+ and resides in the T1 subpopulation. Although there are some parallels between certain aspects of these studies and our own investigations, it is difficult to find clear parallels. So far, our studies do not indicate the involvement of more than one type of suppressor T cells in the suppression of in vivo allograft reactions (HvG and GvH). As far as the phenotype of this subset has been delineated, it resembles mostly the third order suppressor T cell described by Germain and Benacerraf (1981).

In the Appendix papers VI and VII the specificity of the suppressive effect by the activated suppressor T cells was investigated. In both DTH arising in sc immunized mice and in GvH-related DTH we found that suppressor T cells, although they are antigen-specific as far as their activation is concerned, also suppress DTH reactions to third party alloantigens, provided that these alloantigens are presented in combination with the alloantigens that had induced the suppressor T cells. This 'bystander-suppression' phenomenon might explain the beneficial effect of preoperative blood transfusion upon the survival of kidney transplants in man (Opelz et al., 1973; Persijn et al., 1977).

Sharing histocompatibility antigens by the transfused blood cells and the transplanted kidney and/or passenger blood cells present in the kidney might reactivate antigen-specific suppressor T cells after transplantation and that might suppress the antigraft reaction in a non-specific way.

Iv immunization with alloantigens always induced suppressor T cell activity in our system, independent of whether it concerned H-2 alloantigens, a H-2 subregion coded alloantigen or non-H-2 coded alloantigens. The I-J subregion, which is believed by several authors to play a cardinal role in mediating suppression (Germain and Benacerraf, 1981; Liew, 1981; Zembala et al., 1982) does not seem to play any role in our system (Appendix paper VII).

T lymphocytes are not only heterogeneous with regard to their surface

phenotype, but also with regard to the enzymes involved in the purine metabolism (Dosch et al., 1980). In man, deficiencies for these enzymes have profound effects on the immune system (Giblett et al., 1972, 1975) due to the intracellular accumulation of toxic purine metabolites in lymphocytes. In Appendix paper VIII it was investigated whether the injection of one of these metabolites, namely 2'-deoxyguanosine (dGuo) has a differential effect upon DTH reactive T cells and suppressor T cells. This proved to be the case under HvG as well as GvH conditions. It was found that proliferating DTH reactive T cells, which are at least partly identical to helper T cells (Bianchi et al., 1981), were not affected by moderate doses of dGuo, whereas proliferating suppressor T cells were. Therefore we suggest that DTH-reactive T cells and suppressor T cells might have a different purine metabolism.

It was shown by Carson et al. (1977) that in man the enzyme deoxycytidine kinase is abundantly present in resting and dividing T lymphocytes, while the level of the enzyme deoxynucleotidase is very low (Carson et al., 1981). The combination of a high level of deoxycytidine kinase and a low level of deoxynucleotidase implies that thymocytes and T lymphocytes phosphorylate dGuo to the nucleotides deoxyadenosine triphosphate (dATP) and deoxyguanosine triphosphate (dGTP) causing an accumulation of these until an equilibrium is reached or the cell dies. Human B cells, on the other hand, are relatively resistant to the effects of dGuo because they possess high levels of intracellular deoxynucleotidase, which can prevent the accumulation of dATP and dGTP (Carson et al., 1979;

Edwards et al., 1979; Kazmers et al., 1981). It might well be that helper T cells and DTH reactive T cells resemble B cells in this respect.

The above data can be summarized as follows:

a. T cells recognize alloantigens *in vivo* in a MHC-restricted fashion.

b. The anti-host DTH response by long-lived Lyt-1^+ T cells can be amplified by short-lived Lyt-12^+ T cells.

c. The induction of DTH reactivity to alloantigens is under control of suppressor T cells, independent of whether this DTH reactivity is induced under HvG or GvH conditions.

d. Alloantigen-specific suppressor T cells can also suppress the response to third party alloantigens, provided the latter are presented as 'bystanders' to the alloantigens that had induced the suppressor T cells.

e. The activities of enzymes involved in the purine metabolism of DTH reactive T cells and suppressor T cells are different.

SUMMARY

Graft-versus-Host (GvH) disease is characterized by weight loss, diarrhea, skin lesions, hypofunction of the immune system with concomitant infections, etc. This syndrome is potentially lethal. GvH reactions, which underly this disease, may occur when immunocompetent T lymphocytes are transplanted into a host, which is unable to eliminate the intrusive donor lymphocytes and which confronts the graft with a sufficient degree of histoincompatibility. The type of T lymphocyte which plays a central role in GvH reactions seems to be the Lyt-1$^+$ T cell, which probably is analogous to the helper T cell. This cell type may activate other subsets of T lymphocytes, viz., cytotoxic T cells and suppressor T cells, which may lead to GvH disease, depending on the experimental conditions and antigenic differences between donor and host.

The GvH reaction is not only interesting from the point of view of the ensuing syndrome, but can also be used to study certain fundamental immunological problems, such as which histocompatibility antigens evoke the strongest immune reactions, which cell types are involved, do these cell types synergize, etc. We approached these questions in experimental studies with mice. To study such problems one has to have an appropriate assay. We made use of a delayed-type hypersensitivity (DTH) assay which is appropriate to measure the anti-host immune reactivity during acute and delayed GvH reactions in mice.

Anti-host DTH reactivity can be demonstrated by means of transfer of spleen and lymph node cells from mice subjected to GvH to normal secondary recipients, syngeneic to the original lymphoid cell donor. These secondary recipients are challenged in the right hind foot with spleen cells syngeneic to the irradiated host. The increase in foot thickness, measured 24 h later, is a measure for the height of the anti-host immune response.

The GvH related DTH assay was previously proved to be a valuable tool to study the cellular and genetic requirements for anti-host immune reactivity. By using this assay we investigated the cells involved in the anti-host DTH response directed to Mls-locus coded antigens. It was found that for the development of maximal DTH reactivity two subsets of T cells must cooperate. Anti-Mls-locus antigen directed effector T cells turned out to be the progeny of long-lived recirculating T2 cells. This response could be amplified by short-lived, sessile T1 cells. The latter were incapable of displaying anti-Mls DTH reactivity, and were probably activated by other minor histocompatibility antigens (Appendix paper I).

The GvH related DTH assay was also used to study the genetic requirements for immune reactivity, directed to major histocompatibility complex (MHC) coded antigens. T cells differ fundamentlly from B cells in that they have to recognize not only the foreign antigen in order to become activated, but also one of the body's own MHC antigens. This phenomenon is called MHC restriction.

Most reactions to conventional antigens occur in a MHC restricted manner, e.g., reactions against viruses, bacteria and non-MHC coded alloantigens. The present study provides evidence that responses to MHC-coded antigens in vivo are also MHC restricted: T cells reacting to H-2I region coded antigens need to recognize these antigens in the context of syngeneic H-2K/D molecules and T lymphocytes reacting to H-2K/D antigens need to recognize these antigens in the context of syngeneic H-2I molecules. Thus reactions to MHC-subregion-coded antigens in vivo do not differ fundamentally from those directed to the more conventional thymus-dependent antigens (Appendix paper II).

The normal DTH reaction which occurs after subcutaneous (sc) immunization of mice with heterologous erythrocytes and contact sensitizing agents can be suppressed by an intravenous (iv) preimmunization with a high dose of the same antigen. In the Appendix papers III and IV it is shown that iv preimmunization of mice with allogeneic spleen cells also induces a state of suppression of DTH reactivity, which is apparent after sc immunization of these mice with the same allogeneic spleen cells. The induction of suppression was dependent on the route used for preimmunization (the iv route was obligatory) and the cell dose used for preimmunization (the higher the dose, the stronger the suppressive effect). Suppression could be demonstrated for at least 40 days after preimmunization and was antigen-specific: iv injection of cells incompatible for minor histocompatibility antigens could not suppress the DTH to MHC antigens and vice versa. The mechanism underlying this suppressive effect was found to be the inhibition of the proliferation of DTH effector T cells after the sc immunization.

The suppressive effect was found to be mediated by suppressor T cells, which bear, at least in part, Lyt-1 as well as Lyt-2 surface markers. When mice, that had been iv suppressed with allogeneic spleen cells, were used as spleen or bone marrow cell donors to reconstitute irradiated allogeneic recipient mice, no or only marginal anti-host DTH reactivity could be detected. However, this only occurred when the allogeneic recipients were compatible with the allogeneic spleen cells used to induce the state of suppression. This suppressive effect was a dominant phenomenon since spleen cells from 'suppressed' donors could inhibit the reaction by spleen cells from non-suppressed donors. The suppressive effect generated in this sytem was dependent on the proliferation of the activated suppressor cells, which have, just as suppressor T cells that regulate anti-graft DTH reactions, at least in part the Lyt-12^{+} phenotype. Suppressor cells could be generated against MHC plus non-MHC antigens as well as against H-2I subregion coded antigens only and Mls-locus coded antigens (Appendix papers V and VII).

Experiments aimed at unravelling the specificity of suppressor T cells showed that these cells were strictly antigen-specific as far as their activation is concerned, but that they could also suppress the in vivo immune response to unrelated alloantigens, provided that the latter were presented in combination with the antigens that had originally induced the suppressor T cells. This 'bystander suppression' occurred even when the specific and the 'bystander' antigens were not physically associated (Appendix papers VI and VII).

Finally, we characterized the suppressor T cells with regard to their sensitivity for deoxynucleotides. We found that the in vivo administration of micromolar quantities of 2'-deoxyguanosine (dGuo), a metabolite involved in the synthesis and degradation of DNA, to suppressed responder mice prevented the induction of suppressor T cells. The differentiation as well as the proliferation of DTH reactive T cells, on the other hand, were found to be resistant to such doses of dGuo (Appendix paper VIII). These results support the notion that DTH reactive T cells and suppressor T cells have different activities of enzymes involved in the purine metabolism. This observation may open new avenues to manipulate different subpopulations of T lymphocytes selectively.

SAMENVATTING

Transplantaat-anti-gastheer (Graft-versus-Host, GvH) reacties worden gekarakteriseerd door gewichtsverlies, diarree, huidafwijkingen en een slecht functioneren van het afweersysteem, wat resulteert in allerlei infecties. Deze constellatie van afwijkingen kan tot de dood van de gastheer leiden. Deze reacties kunnen ontstaan wanneer immunocompetente T lymfocyten worden getransplanteerd naar een gastheer, die niet in staat is zich te verdedigen tegen de binnendringende donor lymfocyten (bijv. wegens een bepaalde ziekte), en die andere transplantatie-antigenen heeft dan de donor. Het type lymfocyten dat een centrale rol speelt bij deze reacties lijkt een Lyt-1 positieve cel te zijn, die waarschijnlijk identiek is aan de helper T cel. Dit celtype kan, afhankelijk van de experimentele omstandigheden en van de genetische verschillen tussen donor en gastheer, andere typen lymfocyten, zoals de cytotoxische T cel of the suppressor T cel, activeren. Dit kan leiden tot het GvH syndroom.

De GvH reactie kan worden gebruikt om fundamentele problemen binnen de immunologie te bestuderen, zoals welke transplantatie antigenen de sterkste GvH reacties opwekken, welke celtypen daarbij betrokken zijn, en welke celsamenwerkingsverbanden daarbij kunnen bestaan. Wij hebben deze vraagstellingen benaderd in dierexperimenteel onderzoek dat van muizen gebruik maakte. Om dat te kunnen doen, dient men de beschikking te hebben over een goed en betrouwbaar testsysteem, dat kan worden toegepast in dat diermodel. Wij maakten gebruik van een vertraagd type overgevoeligheidsreactie (delayed-type

hypersensitivity, DTH) welke geschikt is om de tegen de gastheer optredende immuunreactie te meten.
Deze anti-gastheer DTH reactie kan worden aangetoond door middel van 'transfer' van milt- en lymfkliercellen van bestraalde gastheer-muizen, die een vreemd milt- of beenmergtransplantaat hadden gekregen, naar normale secundaire ontvangers. Deze ontvangers dienen van dezelfde muizestam te zijn als het milt- of beenmergtransplantaat dat gebruikt is voor transplantatie van de bestraalde muizen. Deze secundaire ontvangers worden in de rechterachterpoot gespoten met een testdosis miltcellen afkomstig van muizen die van dezelfde stam zijn als de bestraalde gastheer. De pootzwelling die 24 uur later optreedt, is dan een maat voor de sterkte van de transplantaat-anti-gastheer reactie.

De GvH-gerelateerde DTH test werd eerder in ons laboratorium gebruikt om de cellulaire en genetische voorwaarden voor het ontstaan van anti-gastheer immuunreactiviteit te bestuderen. Wij gebruikten deze test om te onderzoeken welke celtypen betrokken zijn bij de anti-gastheer immuunreactiviteit tegen Mls-locus gecodeerde antigenen. Het bleek dat twee soorten T cellen moesten samenwerken om een maximale DTH reactie te veroorzaken. De DTH effector T cellen gericht tegen Mls-antigenen bleken de nakomelingen te zijn van lang-levende, recirculerende T2 cellen. Hun reactie kon worden versterkt door kort-levende, sessiele T1 cellen. Deze laatste waren zelf niet in staat tot een DTH reactie tegen de Mls-antigenen, maar worden waarschijnlijk geactiveerd door andere, zwakke transplantatie-antigenen (Appendix publikatie I).

Daarnaast werd de GvH-gerelateerde DTH test gebruikt om de genetische voorwaarden te bestuderen voor het ontstaan van immuunreactiviteit tegen sterke transplantatie-antigenen die worden gecodeerd door het H-2 complex van de muis. T cellen verschillen n.l. fundamenteel van B cellen in het feit dat zij niet alleen het lichaamsvreemde antigeen moeten herkennen om te worden geactiveerd, maar dat ze daarnaast nog één van de lichaamseigen transplantatie-antigenen moeten herkennen. Dit verschijnsel wordt H-2 restrictie genoemd en treedt op bij de reactie tegen bacteriën, virussen, zwakke transplantatie-antigenen, en allerlei andere 'thymus-afhankelijke' antigenen. Het bleek dat wanneer de DTH reactie wordt bestudeerd tegen transplantatie-antigenen die worden gecodeerd door subregionen van het H-2 complex, de daartegen reagerende lymfocyten ook deze antigenen in vivo moeten herkennen in associatie met de eigen transplantatie-antigenen: reacties tegen H-2I-antigenen bleken afhankelijk te zijn van de gelijktijdige herkenning van de eigen H-2K/D moleculen en reacties tegen H-2K/D antigenen bleken afhankelijk te zijn van de gelijktijdige herkenning van de eigen H-2I moleculen. Het lijkt er dus op, dat in vivo reacties, gericht tegen transplantatie-antigenen die worden gecodeerd door subregionen van het H-2 complex, niet fundamenteel verschillen van de reacties gericht tegen de meer conventionele 'thymus-afhankelijke' antigenen (Appendix publikatie II).

Een DTH reactie kan bij muizen ook worden opgewekt door hen onderhuids (subcutaan, sc) te immuniseren met een thymus-afhankelijk antigeen en 6 dagen later met hetzelfde antigeen te 'challengen' in de rechterachterpoot.

Door andere onderzoekers is aangetoond, dat het ontstaan van een staat van DTH reactiviteit ten gevolge van immunisatie met rode bloedcellen van een andere diersoort kan worden onderdrukt door aan de muizen tevoren een hoge dosis van zulke rode bloedcellen via een staartader toe te dienen (intraveneuze (iv) preimmunisatie). In de Appendix publicaties III en IV wordt aangetoond dat iv preimmunisatie met miltcellen van een andere muizestam de DTH reactie tegen een-zelfde miltceltransplantaat ook kan onderdrukken. De opwekking van dit onderdrukkend effect bleek afhankelijk van de route van preimmunisatie (de iv route bleek noodzakelijk) en de celdosis die werd gebruikt (hoe hoger de celdosis, des te sterker het onderdrukkend effect). De suppressie kon worden aangetoond tot 40 dagen na iv preimmunisatie en is antigeen-specifiek: iv injectie van cellen afkomstig van donoren, die van de ontvangers alleen in non-H-2-antigenen verschilden, kon de DTH reactie tegen H-2-antigenen niet onderdrukken en vice versa. Het supprimerende effect berust op onderdrukking van de celdeling van de DTH effector T cellen na sc immunisatie. Het supprimerende effect wordt veroorzaakt door langlevende suppressor T lymfocyten, die tenminste voor een deel zowel Lyt-1 als Lyt-2 moleculen op hun celoppervlak dragen.

Wanneer responder muizen, die iv gepreimmuniseerd zijn met miltcellen van een andere stam, worden gebruikt om milt- of beenmergcellen te leveren om hiermee bestraalde muizen te transplanteren, kon er geen of een zeer beperkte anti-gastheer DTH reactiviteit worden aangetoond. Deze suppressie trad alleen op wanneer de bestraalde

ontvangers identiek waren aan de donoren van de miltcellen die waren gebruikt om de suppressie op te wekken.
Dit supprimerende effect is een 'dominant' verschijnsel, omdat miltcellen van gesupprimeerde donoren de anti-gastheer reactie door miltcellen van niet-gesupprimeerde donoren konden onderdrukken.
Het supprimerende effect in dit systeem wordt eveneens veroorzaakt door T lymfocyten, die tenminste voor een deel zowel Lyt-1 als Lyt-2 positief zijn. De suppressor T lymfocyten moeten zich delen om een maximaal supprimerend effect te kunnen ontplooien. Suppressor T cellen kunnen worden opgewekt door gelijktijdige iv toediening van H-2 en non-H-2 transplantatie-antigenen, door iv toediening van transplantatie-antigenen die worden gecodeerd door de H-2I subregio, en door Mls-locus gecodeerde antigenen (Appendix publicaties V en VII).

Onderzoek naar de specificiteit van de suppressor T cellen onthulde dat deze cellen antigeen-specifiek zijn voor wat betreft hun activatie, maar dat ze in staat zijn de immuunreactie tegen niet-verwante antigenen mede te onderdrukken, mits deze antigenen aan deze cellen worden aangeboden in combinatie met de specifieke antigenen, die de suppressor cellen hadden opgewekt. De suppressie van de DTH reaktie tegen deze zgn. 'bystander' antigenen is ook aantoonbaar, wanneer de specifieke en de 'bystander' antigenen niet op dezelfde cellen voorkomen (Appendix publikaties VI en VII).

Tenslotte bepaalden we de gevoeligheid van de suppressor T cellen voor

deoxynucleotiden. Wij vonden dat toediening in vivo van micromolaire hoeveelheden 2'-deoxyguanosine (dGuo, een metaboliet die betrokken is bij de opbouw en afbraak van DNA), aan gesupprimeerde muizen de inductie van suppressor T cellen voorkwam.

De differentiatie en proliferatie van DTH effector T cellen waren echter ongevoelig voor deze hoeveelheden dGuo (Appendix publikatie VIII). Deze resultaten ondersteunen het concept dat DTH effector T cellen en suppressor T cellen verschillende activiteiten van enzymen, die betrokken zijn bij het purine metabolisme, ten toon spreiden. Deze waarneming kan nieuwe wegen ontsluiten voor het selectief beïnvloeden van subpopulaties van T lymfocyten.

REFERENCES

Asherson, G.L. and Zembala, M. T suppressor cells and suppressor factor which act at the efferent stage of the contact sensitivity skin reaction: their production by mice injected with water-soluble, chemically reactive derivatives of oxazolone and picryl chloride. Immunology 42, 1005, 1980.

Bach, F.H., Bach, M.L. and Sondel, P.M. Differential function of major histocompatibility complex antigens in T lymphocyte activation. Nature 259, 273, 1976.

Bianchi, A.T.J., Hooijkaas, H., Benner, R., Tees, R., Nordin, A.A. and Schreier, M.H. Clones of helper T cells mediate antigen-specific H-2 restricted DTH. Nature 290, 62, 1981.

Cantor, H. and Asofsky, R. Synergy among lymphoid cells mediating the Graft-versus-Host response. II. Synergy in Graft-versus-Host reactions produced of BALB/c lymphoid cells of differing anatomic origin. J. Exp. Med. 131, 235, 1970.

Cantor, H. and Asofsky, R. Synergy among lymphoid cells mediating the Graft-versus-Host response. III. Evidence for interaction between two types of thymus-derived cells. J. Exp. Med. 135, 764, 1972.

Carson, D.A., Kaye, J. and Seegmiller, J.E. Lymphospecific toxicity in adenosine deaminase deficiency and purine nucleoside phosphorylase deficiency: possible role of nucleoside kinase(s). Proc. Natl. Acad. Sci. 74, 5677, 1977.

Carson, D.A., Kaye, J., Matsumoto, S., Seegmiller, J.E. and Thompson, L. Biochemical basis for the enhanced toxicity of deoxyribonucleotides towards malignant human T cell lines. Proc. Natl. Acad. Sci. 76, 2430, 1979.

Carson, D.A., Kaye, J. and Wasson, D.B. The potential importance of soluble deoxynucleotidase activity in mediating deoxyadenosine toxicity in human lymphoblasts. J. Immunol. 126, 348, 1981.

Crowle, A.J. Delayed hypersensitivity in the mouse. Adv. Immunol. 20, 197, 1975.

Dosch, H.M., Mansour, A., Cohen, A., Shore, A. and Gelfand, E.W. Inhibition of suppressor T cell development following deoxyguanosine administration. Nature 285, 494, 1980.

Edwards, N.L., Gelfand, E.W., Burk, L., Dosch, H.M. and Fox, I.H. Distribution of 5'-nucleotidase in human lymphoid tissues. Proc. Natl. Acad. Sci. 76, 3474, 1979.

Feldmann, M., Beverley, P.C.L., Woody, J. and McKenzie, I.F.C. T-T interactions in the induction of suppressor and helper T cells: analysis of membrane phenotype of precursor and amplifier cells. J. Exp. Med. 145, 793, 1977.

Germain, R.N. and Benacerraf, B. A single major pathway of T lymphocyte interactions in antigen-specific immune suppression. Scand. J. Immunol. 13, 1, 1981.

Giblett, E.R., Anderson, J.E., Cohen, F., Pollara, B. and Meuwissen, H.J. Adenosine-deaminase deficiency in two patients with severely impaired cellular immunity. Lancet ii, 1067, 1972.

Giblett, E.R., Amman, A.J., Sandman, R., Wara, D.W. and Diamond, L.K. Nucleoside-phosphorylase deficiency in a child with severely defective T cell immunity and normal B cell immunity. Lancet i, 1010, 1975.

Kazmers, I.S., Mitchell, B.S., Dadonna, P.E., Wotring, L.L., Townsend, L.B. and Kelley, W.N. Inhibition of purine nucleoside phosphorylase by 8-aminoguanosine: selective toxicity for T lymphoblasts. Science 214, 1137, 1981.

Liew, F.Y. Regulation of delayed-type hypersensitivity. I. T suppressor cells for delayed-type hypersensitivity to sheep erythrocytes in mice. Eur. J. Immunol. 7, 714, 1977.

Liew, F.Y. Regulation of delayed-type hypersensitivity. VII. The role of I-J subregion gene products in the inhibition of delayed-type hypersensitivity to major histocompatibility antigens by specific suppressor T cells. Eur. J. Immunol. 11, 883, 1981.

Miller, S.D., Sy, M.S. and Claman, H.N. Suppressor T cell mechanisms in contact sensitivity. II. Afferent blockade by allo-induced suppressor T cells. J. Immunol. 121, 274, 1978.

Miller, S.D., Sy, M.S. and Claman, H.N. Suppressor T cell mechanisms in contact sensitivity. I. Efferent blockade by syn-induced suppressor cells. J. Immunol. 121, 365, 1978.

Opelz, G., Sengar, D.P.S., Mickey, W.R. and Terasaki, P.I. Effect of blood transfusions on subsequent kidney transplants. Transplant. Proc. 5, 253, 1973.

Persijn, G.G., van Hooff, J.P., Kalff, M.W., Lansbergen, Q. and van Rood, J.J. Effect of blood transfusions and HLA matching on renal transplantation in the Netherlands. Transplant. Proc. 9, 503, 1977.

Tittor, W., Gerbase-Delima, M. and Walford, R.L. Synergy among responding lymphoid cells in the one-way mixed lymphocyte reaction. Interaction between two types of thymus-dependent cells. J. Exp. Med. 139, 1488, 1974.

Van der Kwast, Th.H. Cellular and genetic requirements for delayed-type hypersensitivity. Thesis, Erasmus University Rotterdam, 1979.

Wagner, H., Röllinghoff, M., Schawaller, R., Hardt, C. and Pfizenmaier, K. T-cell-derived helper factor allows Lyt-123 thymocytes to differentiate into cytotoxic T lymphocytes. Nature 280, 405, 1979.

Wolters, E.A.J. and Benner, R. Immunobiology of the Graft-versus-Host reaction. I. Symptoms of Graft-versus-Host disease are preceded by delayed type hypersensitivity to host histocompatibility antigens. Transplantation 26, 40, 1978.

Wolters, E.A.J. Cellular and genetic requirements for Graft-versus-Host reactivity. Thesis, Erasmus University Rotterdam, 1980.

Wolters, E.A.J. and Benner, R. Different H-2 subregion coded antigens as targets for T cell subsets synergizing in Graft-versus-Host reaction. Cell. Immunol. 59, 115, 1981.
Wright, P.W., Loop, S.M. and Bernstein, I.D. Synergy among rat T cells in the proliferative response to alloantigen. Cell. Immunol. 43, 245, 1979.
Zembala, M. and Asherson, G.L. T cell suppression of the T cell phenomenon of contact sensitivity. Nature 244, 227, 1973.
Zembala, M., Asherson, G.L. and Colizzi, W. Hapten-specific T suppressor factor recognizes both hapten and I-J region products on haptenized spleen cells. Nature 297, 411, 1982.

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CURRICULUM VITAE

De schrijver van dit proefschrift aanschouwde op 3 maart 1957 het levenslicht te Vlaardingen en groeide nadien voorspoedig op.
Hij begon op 4-jarige leeftijd aan een zeer lange schoolcarrière op achtereenvolgens de Ds. Hendrik de Cock kleuterschool, de Ds. Hendrik de Cockschool voor lager onderwijs en de Christelijke Scholengemeenschap 'Groen van Prinsterer'. Aan deze laatste legde hij in 1975 met goed gevolg het examen VWO-gymnasium β af.
Na een gelukkige loting kon hij in datzelfde jaar de studie Geneeskunde aanvangen aan de Medische Faculteit van de Erasmus Universiteit Rotterdam. In het kader van het keuzepraktikum deed hij in 1978 bij Drs. (toen nog) Theo van der Kwast onderzoek naar de regulatie van vertraagd type overgevoeligheidsreacties tegen transplantatie-antigenen bij muizen. Dit onderzoek werd daarna voortgezet in het kader van een student-assistentschap.
In oktober 1980 legde hij met goed gevolg het doktoraal examen Geneeskunde af waarna hij op 1 november 1980 in tijdelijke dienst werd aangesteld bij de afdeling Celbiologie en Genetica van de Erasmus Universiteit Rotterdam. Op deze afdeling werd onder leiding van Prof.Dr. R. Benner het in dit proefschrift beschreven onderzoek verricht.

SYNERGISM OF T LYMPHOCYTE SUBSETS IN THE RESPONSE TO MLS-LOCUS CODED ANTIGENS DURING GRAFT-VERSUS-HOST REACTION

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SUMMARY

After transplantation of lymphoid cells into lethally irradiated (semi)allogeneic mice specific anti-host directed effector T cells are generated. This can be demonstrated using a delayed type hypersensitivity (DTH) assay. In H-2 compatible combinations, Mls-locus coded antigens, but no other minor histocompatibility antigens, can induce the generation of such effector T cells. This paper shows that maximal anti-host DTH responses are obtained when the lymphoid cells transplanted constitute of a mixture of long-lived, recirculating T2 cells and short-lived, sessile T1 cells. It was demonstrated that anti-Mls locus-directed DTH effector T cells are the progeny of T2 cells, and that T1 cells amplify this response. The latter, however, are by themselves incapable of displaying anti-Mls DTH reactivity. The T1 cells were found to be of the Lyt-1+2+ phenotype, and the T2 cells of the Lyt-1+2- phenotype. The same Lyt phenotypes were found for T1 and T2 cells synergizing in the GvH reaction against H-2 alloantigens.

INTRODUCTION

Cantor and Asofsky (1) have shown synergism between parental peripheral blood lymphocytes and thymocytes in the production of splenomegaly in neonatal F1 recipients. According to this observation Raff and Cantor (2) proposed to subdivide T lymphocytes into two subpopulations, namely, T1 cells which are shortlived, sessile T cells, sensitive to adult thymectomy (ATx), and T2 cells, which are long-lived, recirculating T cells, sensitive to anti-thymocyte serum (ATS) *in vivo*.

We have previously shown in donor-recipient combinations differing at the major histocompatibility complex (MHC) that the effector T cells mediating anti-host directed delayed type hypersensitivity (DTH) are the progeny of T2 cells, activated by antigens encoded by the I region of the MHC (3). Their response was amplified by T1 cells, which are activated by H-2K and/or H-2D region coded antigens.

In other studies (4,5) we have shown that in H-2 compatible combinations Mls-locus coded antigens can also induce the generation of GvH-related effector T cells. It was found that Mls^a and Mls^c antigens evoked distinct anti-host DTH reactivity. Mls^b antigens, on the other hand, caused only a marginal and short-lasting anti-host DTH reactivity. Furthermore, Mls^a and Mls^c locus products can induce strong mixed lymphocyte culture (MLC) responses (6), resembling responses induced by I region products of the MHC.

The DTH assay we have developed for measuring the development of anti-host effector T cells during GvH is based upon secondary transfer of lymphoid cells from the animals undergoing the GvH reaction (7). The secondary recipients are subsequently tested for DTH reactivity to the histocompatibility antigens which evoked the GvH reaction. Using this assay we studied the contribution of T1 and T2 cells in the response to Mls-locus coded antigens during GvH reactions. The results further substantiate the functional similarity of H-2I and Mls antigens.

MATERIALS AND METHODS

Animals. (BALB/c x DBA/2)F1 ($H\text{-}2^d$; $Mls^{b/a}$) female mice were purchased from the Radiobiological Institute TNO, Rijswijk, The Netherlands. DBA/2 ($H\text{-}2^d$; Mls^a), AKR ($H\text{-}2^k$; Mls^a) and C3H/He ($H\text{-}2^k$; Mls^c) female mice were purchased from Bomholtgard, Ry, Denmark and from OLAC Ltd., Bicester, United Kingdom.
BALB/c ($H\text{-}2^d$; Mls^b) female mice were bred at the Laboratory Animals Centre of the Erasmus University, Rotterdam, The Netherlands. The age of the mice varied between 12 and 20 weeks.

Preparation of cell suspensions. Mice were killed by carbon dioxide. Immediately after killing, the lymphoid organs to be used (spleen and inguinal, axillary and mesenteric lymph nodes) were prepared for single cell suspensions as described previously (7). Nucleated cells were counted with a Coulter counter model B.

Irradiation. The recipient mice received 7.5 Gy whole body irradiation, except the DBA/2 mice, which received 6 Gy. The irradiation was generated in a Philips Müller MG 300 X-ray machine as described previously (7). Radiation control mice died in 14 to 21 days.

Selective elimination of Thy-1.2 positive cells. Monoclonal IgM anti-Thy-1.2 antibodies (clone F7D5) were purchased from OLAC Ltd., Bicester, United Kingdom. Cell suspensions were treated with anti-Thy-1.2 antibodies for 30 min at 4°C. The amount of anti-Thy-1.2 antibodies used was three times more than required to kill at least 95% of corticosteroid-resistant thymocytes (8). After incubation, the cells were centrifuged, resuspended in balanced salt solution (BSS) and incubated with guinea pig complement (Behringwerke AG, Marburg, W.-Germany) for 15 min at 37°C. Thereafter the cells were washed three times and resuspended in BSS.

Selective elimination of Lyt-1.1 and Lyt-2 positive cells. Monoclonal IgG2a anti-Lyt-1.1 antibodies (clone 7-20.6/3) were purchased from Cedarlane Laboratories Ltd., Hornby, Canada. Cell suspensions were treated in vitro with anti-Lyt-1.1 for 60 min at 4°C. After incubation, the cells were centrifuged, resuspended in BSS and incubated with guinea pig complement (Behringwerke AG) for 40 min at 37°C. Thereafter, the cells were washed three times and resuspended in BSS. Monoclonal IgM anti-Lyt-2 antibodies were produced

in our own laboratory from clone 3.168.8 which was kindly provided by Dr. F.W. Fitch, Department of Pathology, University of Chicago, USA. For elimination of Lyt-2 positive cells in vitro the same protocol was used as described for depletion of Lyt-1.1 positive cells.

Anti-thymocyte serum. Anti-thymocyte serum (ATS) was prepared and absorbed as described previously (8,9). Mice received two subcutaneous (sc) injections of 0.2 ml ATS to eliminate T2 cells, or 0.2 ml normal rabbit serum (NRS) as a control. This amount was equally distributed over the inguinal and axillary areas. These injections were given 5 and 2 days before the lymphoid organs were used.

Spleen cells from ATS and NRS-treated mice are referred to in the text as 'ATS spleen cells' and 'NRS spleen cells', respectively.

Lymph node seeking cells. Lymph node seeking T2 cells were isolated according to the method of Tigelaar and Asofsky (10) with some minor modifications. The experimental design was as follows: 15×10^7 pooled cells from spleen, peripheral and mesenteric lymph nodes were intravenously (iv) injected into lethally irradiated syngeneic recipients. At 24 hr after inoculation the peripheral lymph nodes were harvested from these recipients. These lymph nodes contained about 4×10^6 more nucleated cells than lymph nodes from irradiated recipients which had not received

spleen and lymph node cells. The cell yield from all peripheral lymph nodes of a single irradiated recipient was called one lymph node seeking equivalent (1 LNS eq.).

Acute GvH reactions. Acute GvH reactions were elicited by iv injection of irradiated recipients with an appropriate inoculum of allogeneic lymphoid cells within 4 hr after irradiation.

Assay for delayed type hypersensitivity. The assay for anti-host DTH reactivity has been described in detail in a previous paper (7). Briefly, at 4, 5 or 6 days after (semi)allogeneic reconstitution a number of cells equivalent to one whole spleen or the total cell yield from spleen, inguinal, axillary and mesenteric lymph nodes from an irradiated and allogeneically reconstituted recipient mouse was transferred iv into a normal secondary recipient. This secondary recipient was syngeneic to the original lymphoid cell donor. DTH reactivity of these secondary recipients was determined by measuring the difference in thickness of the hind feet 24, 48 and 72 hr after a challenge injection of 2×10^7 spleen cells, syngeneic to the irradiated recipients, into the dorsum of the right hind foot. In the figures the 24 hr values are presented. The DTH responses at 48 and 72 hr were in harmony with those at 24 hr, but lower. The percentage specific increase in foot thickness was calculated as the percentage increase in foot thickness of the immune mice minus the percentage increase in foot thickness of control mice which only received the challenge. The swelling of challenged control mice varied between 15 and 25%.

RESULTS

Dependence of anti-Mls DTH reactivity on T1 and T2 cells

We have previously shown that in H-2 compatible, Mls locus incompatible donor-recipient combinations maximal GvH-related anti-host DTH reactivity is found at 4 to 7 days after irradiation and reconstitution, dependent on the strain combination (5). In GvH induced by transplantation of BALB/c spleen cells into irradiated (BALB/c x DBA/2)F1 or DBA/2 mice, maximal anti-Mls^a DTH responses are found on day 4. After transplantation of C3H spleen cells into irradiated AKR mice, on the other hand, maximal anti-Mls^a DTH responses are found around day 6 (5). We have also shown that under such conditions the anti-host DTH response indeed is mainly directed towards the Mls-locus coded antigens (5).

In order to investigate whether both ATx-sensitive, sessile T1 cells and long-lived, recirculating T2 cells are involved in the GvH-related DTH reactivity to Mls antigens, elimination and mixing experiments were done. Spleen cells were depleted of T2 cells by *in vivo* treatment with ATS, while lymphoid cell suspensions were enriched for T2 cells by isolating the LNS cells according to the method of Tigelaar and Asofsky (10).

The involvement of T1 and T2 cells was investigated in the following donor-recipient combinations: BALB/c-anti-(BALB/c x DBA/2)F1, BALB/c-anti-DBA/2, and C3H/He-anti-AKR/J. It was found that elimination of T2 cells by ATS treatment completely prevented

the anti-Mls DTH response. After depletion of T1 cells by preparing LNS T2 cells, on the other hand, a dose dependent DTH response was found (Fig. 1). So, T2 cells can mediate anti-Mls DTH responses, whereas T1 cells, at least when tested in a dose of 10^8 ATS spleen cells, cannot.

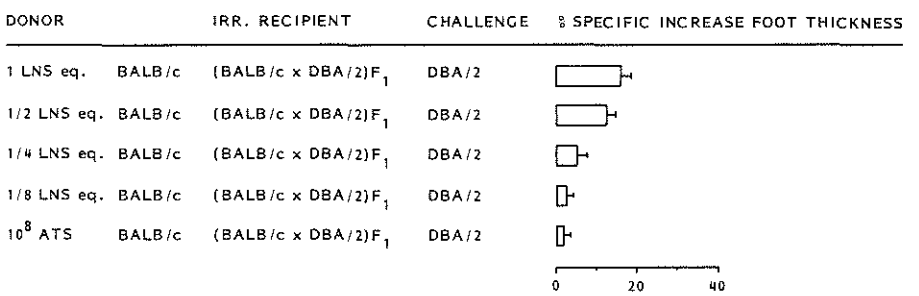

Fig. 1. Dependence of anti-Mls[a] GvH-related DTH reactivity on T2 cells. GvH reactions were elicited by transplantation of BALB/c lymphoid cells into lethally irradiated (BALB/c x DBA/2)F1 mice. T1 cells were obtained from the spleen (SPL) of ATS-treated BALB/c mice. Lymph node seeking (LNS) T2 cells were made in BALB/c mice according to the method of Tigelaar and Asofsky (10). Irradiated (BALB/c x DBA/2)F1 mice were inoculated with either different numbers of LNS cells or with 10^8 ATS-treated spleen cells. Anti-DBA/2 DTH reactivity was determined 4 days after reconstitution. Horizontal bars represent 1 SEM (n=6).

Cooperation between T1 and T2 cells in the development of anti-Mls DTH

To investigate whether the putative T1 and T2 cells have an additive or synergistic activity in the development of anti-Mls DTH, BALB/c cell suspensions containing T1 cells (ATS spleen cells) and T2 cells (LNS cells) were transferred either alone or together into lethally irradiated (BALB/c x DBA/2)F1 recipients. It was found

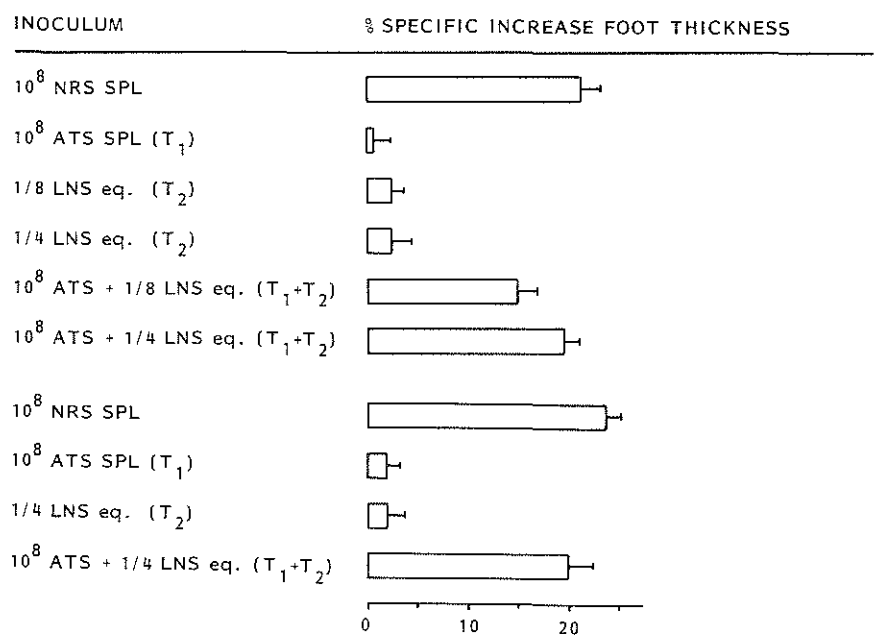

Fig. 2. Anti-DBA/2 DTH response by spleen cells from lethally irradiated (BALB/c x DBA/2)F1 (upper part) and DBA/2 (lower part) mice, reconstituted with spleen cells from NRS or ATS-treated BALB/c mice and/or 1/4 or 1/8 BALB/c LNS equivalent. Anti-DBA/2 DTH reactivity was determined 4 days after reconstitution. Horizontal bars represent 1 SEM (n=6).

that 10^8 ATS-treated spleen cells did not display anti-Mls DTH reactivity, in contrast to 10^8 NRS-treated spleen cells (Fig. 2, upper part). Inoculation of irradiated (BALB/c x DBA/2)F1 recipients with 1/8 or 1/4 LNS equivalent resulted in marginal anti-Mls DTH reactivitiy. However, inoculation of lethally irradiated (BALB/c x DBA/2)F1 mice with 10^8 ATS spleen cells as well as 1/8 or 1/4 LNS equivalent resulted in a much higher anti-Mls response than the sum of the responses generated by both cell populations separately.

To prevent any possible involvement of hybrid histocompatibility antigens (11,12) in the elicitation of the anti-host

DTH, we also used DBA/2 recipients. Also in this case inoculation of either ATS spleen cells or 1/4 LNS equivalent was found to cause only a minimal anti-Mls DTH reactivity, whereas injection of 10^8 ATS spleen cells as well as 1/4 LNS equivalent resulted in a clear synergistic anti-Mls DTH response (Fig. 2, lower part). A similar synergistic anti-Mls DTH response was observed after transplantation of 10^8 C3H/He ATS spleen cells and 1/2 C3H/He LNS equivalent (Fig. 3).

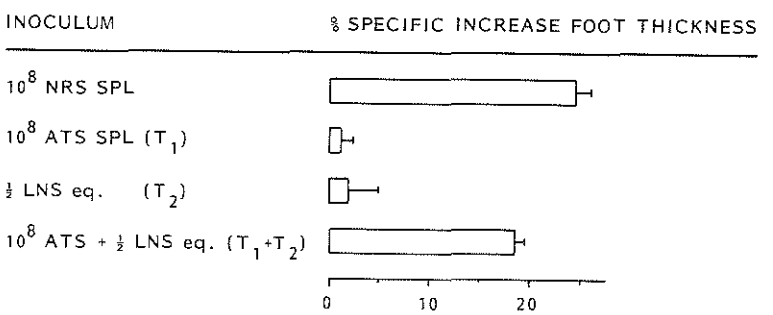

Fig. 3. Anti-AKR DTH response by spleen cells from lethally irradiated AKR mice, reconstituted with spleen cells from NRS or ATS-treated C3H/He mice and/or 1/2 C3H/He LNS equivalent. Anti-AKR DTH reactivity was determined 6 days after reconstitution. Horizontal bars represent 1 SEM (n=6).

Evidence that putative T1 and T2 cells are indeed T cells

To investigate whether the putative T1 and T2 cells are indeed T cells, anti-Thy-1.2 treatment *in vitro* was used for their selective elimination. This was done using the BALB/c-(BALB/c x DBA/2)F1 combination. Anti-Thy-1.2 treatment of spleen cells from NRS-treated or ATS-treated mice and anti-Thy-1.2 treatment of LNS cells completely prevented the anti-Mls DTH reactivity by these

cell populations. This shows that the responses normally mediated by these cell populations rely on T cells. Anti-Thy-1.2 treatment of either ATS spleen cells or LNS cells and subsequent transfer in combination with untreated LNS cells and ATS spleen cells, respectively, prevented a synergistic anti-Mls DTH response by the combined cell populations (Fig. 4). Therefore, both putative T1 and T2 cells are real T cells.

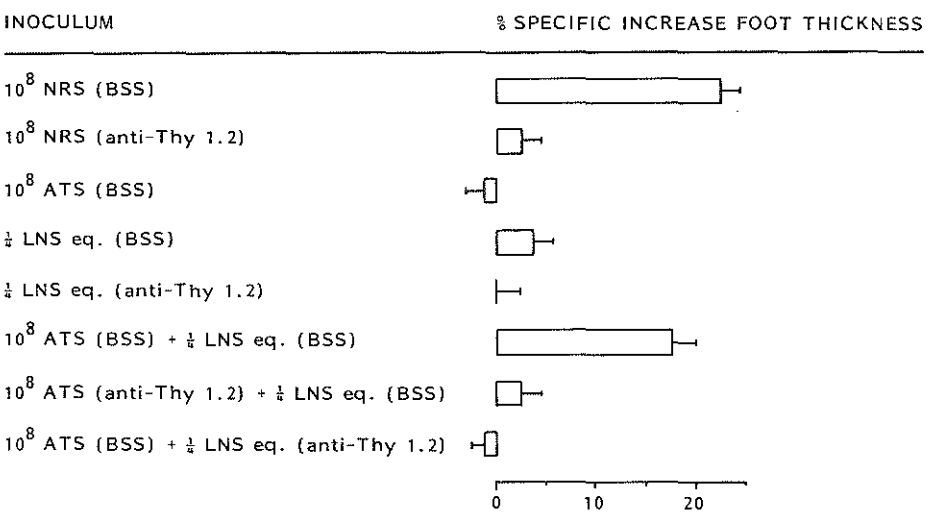

Fig. 4. Anti-DBA/2 DTH response by spleen cells from lethally irradiated (BALB/c x DBA/2)F1 mice, inoculated with spleen cells from NRS or ATS-treated BALB/c mice and/or 1/4 BALB/c LNS equivalent. Between brackets the in vitro pretreatment of the inocula. Anti-DBA/2 DTH reactivity was determined 4 days after irradiation and reconstitution. Horizontal bars represent 1 SEM (n=6).

Lyt-phenotype of T1 and T2 cells synergizing in the development of anti-Mls DTH

In order to investigate the Lyt phenotype of the T1 and T2 cells, anti-Lyt-1 and anti-Lyt-2 treatment in vitro was used for selective elimination of Lyt-1+ and Lyt-2+ cells. This was done in the C3H/He-AKR combination.

Pretreatment of spleen cells from ATS-treated mice with anti-Lyt-1.1 or anti-Lyt-2 antibodies in vitro before combining them with untreated LNS cells, prevented the synergistic anti-Mls DTH response by both cell populations in GvH (Fig. 5). On the other hand, when LNS cells were pretreated with monoclonal anti-Lyt-1.1 or anti-Lyt-2 antibodies in vitro before combining these cells with spleen cells from ATS-treated mice only the pretreatment with anti-Lyt-1.1 antibodies appeared to prevent the synergism. These results indicate that the T1 cells have the Lyt-1+2+ phenotype, while the T2 cells have the Lyt-1+2- phenotype.

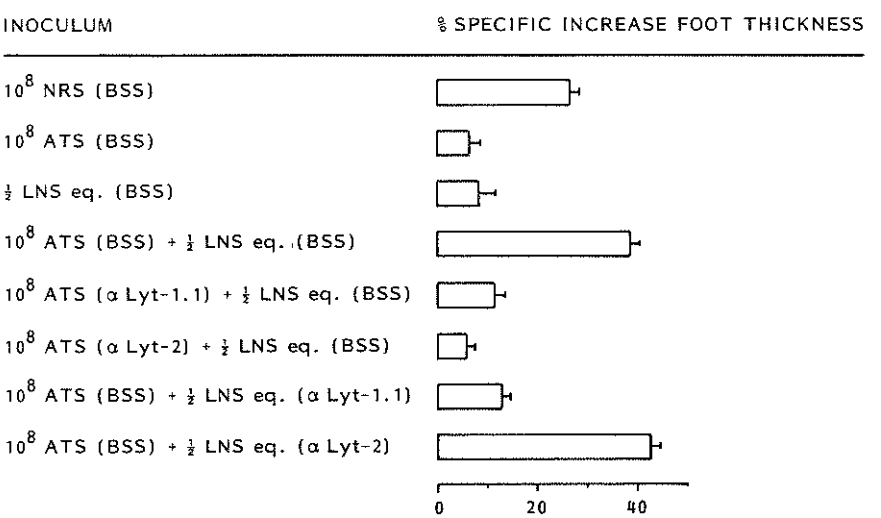

Fig. 5. Anti-AKR DTH response by spleen cells from lethally irradiated AKR mice, reconstituted with spleen cells from NRS or ATS-treated C3H/He mice and/or 1/2 C3H/He LNS equivalent. Between brackets the in vitro pretreatment of the inocula. Anti-AKR DTH reactivity was determined 6 days after irradiation and reconstitution. Horizontal bars represent 1 SEM (n=6).

Lyt-phenotype of T1 and T2 cells synergizing in the development of anti-H-2 DTH

In a previous paper we have shown that for maximal anti-host

DTH reactivity in H-2 incompatible donor-recipient combinations both T1 and T2 cells are required. It was demonstrated that the T1 cells were activated by the host H-2K/D alloantigens and the T2 cells by H-2I alloantigens (3). In order to compare the Lyt-phenotype of the T cells synergizing in the anti-Mls DTH response with that of the T cells synergizing in the GvH related anti-H-2 DTH response, we also determined the Lyt-phenotype of the T1 and T2 cells involved in the latter. Thus, spleen cells from ATS-treated DBA/2 mice and LNS cells of DBA/2 origin were incubated with monoclonal anti-Lyt-1.1 or anti-Lyt-2 antibodies in vitro before combining them with untreated LNS cells and untreated ATS spleen cells, respectively, to reconstitute lethally irradiated AKR recipient mice. Fig. 6 shows that treatment of the T1 cells with either anti-Lyt-1.1 or anti-Lyt-2 prevented the synergism which normally occurs after combining T1 and T2 cells.

Treatment of the T2 cells with anti-Lyt-1.1 similarly prevented the synergism, whereas treatment with anti-Lyt-2 did not. Thus, also in this instance T1 cells have the Lyt-1+2+ phenotype and T2 cells the Lyt-1+2- phenotype.

DISCUSSION

This study shows that optimal development of GvH-related anti-Mls reactivity requires short-lived, sessile Lyt-1+2+ T1 cells as well as long-lived, recirculating Lyt-1+2- T2 cells (Figs. 2, 3 and 5). This cellular cooperation between both T cell subpopulations results in a much stronger anti-Mls DTH response than the sum of the responses by both cell populations separately.

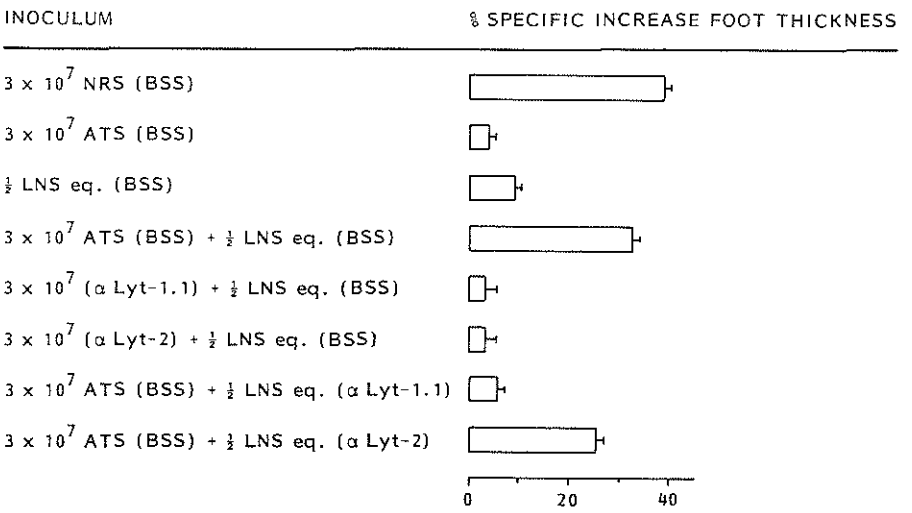

Fig. 6. Anti-AKR DTH response by spleen cells from lethally irradiated AKR mice, reconstituted with spleen cells from NRS or ATS-treated DBA/2 mice and/or 1/2 DBA/2 LNS equivalent. Between brackets the in vitro pretreatment of the inocula. Anti-AKR DTH reactivity was determined 5 days after irradiation and reconstitution. Horizontal bars represent 1 SEM (n=6).

Elimination of T2 cells by in vivo treatment with ATS completely prevented the development of anti-Mls DTH reactivity, even when large numbers of allogeneic T1 cells were used for transplantation. The same was found in acute and delayed GvH reactions across complete H-2 differences. Elimination of T2 cells by ATS treatment of donors of spleen or bone marrow cells also prevented anti-host DTH reactivity in lethally irradiated H-2 incompatible recipients (3,8). Under these conditions also synergism between T1 and T2 cells occurs (3). In H-2 incompatible donor-recipient combinations T2 cells are activated by antigens encoded by the H-2I region of the H-2 complex and differentiate into the anti-host DTH effector T cells (3). T1 cells, on the other hand, are activated by H-2K/D region coded antigens and amplify the response by the

T2 cells, but are by themselves incapable of mounting anti-host DTH reactivity. In a previous paper we have presented evidence that DTH effector T cells generated in GvH reactions due to H-2 compatible but Mls-locus incompatible donor-recipient combinations are directed towards the Mls antigens themselves (5). The present paper shows that these DTH effector T cells must be the progeny of T2 cells. The analogy with GvH-related DTH towards a complete H-2 haplotype, where T1 cells react to H-2K/D antigens (3), lead us to speculate that in the T1-T2 synergistic DTH response to Mls antigens the T1 cells are activated by other non-H-2 alloantigens than those encoded by the Mls-locus.

GvH reactions are not the only condition that T1 and T2 cells synergize. Muirhead and Cudkowicz (13) described a synergistic activity of T1 and T2 cells in the formation of anti-hapten antibodies. The helper effect was mediated by T2 cells. This response was enhanced by the T1 amplifier cells (13). Feldman and Erb (14) showed that mixtures of T1 and T2 cells were also synergistic in the anti-TNP-KLH antibody response in vitro (14).

The Lyt phenotypes of T lymphocytes involved in the in vitro generation of helper activity for the antibody response to TNP-KLH have been identified: the precursors of the helper T cells were found to be Lyt-$1^{+}2^{-}3^{-}$. The amplifier cells, on the other hand, appeared to have the Lyt-123^{+} phenotype (15). The Lyt phenotype of the T1 and T2 cells involved in the synergistic anti-host DTH response during GvH appeared to be the same as that of the T1 and T2 cells synergizing in the in vitro antibody response. In the H-2 and non-H-2 incompatible, but Mls compatible, DBA/2-AKR combination (Fig. 6) as

well as in the H-2 compatible, but Mls incompatible, C3H-AKR combination (Fig. 5) the T1 cells appeared to have the Lyt-1+2+ phenotype and the T2 cells the Lyt-1+2- phenotype. The apparently identical (Lyt-1+2-) phenotype for precursors of helper T cells and DTH reactive T cells is in harmony with our previous studies with cloned helper T cells suggesting that helper activity and DTH reactivity can be mediated by the same T cells (16).

Presumably there are two separate lines of intrathymic differentiation: one line which expresses Lyt-1, but not Lyt-23 antigens, and another which expresses all these three Lyt antigens during differentiation (17,18). A part of the population of Lyt-123$^+$ T cells is depleted by adult thymectomy and corresponds to the subpopulation of T1 cells (19), the remainder belonging to the T2 subpopulation (20). The Lyt-1+2- population of T cells is resistant to adult thymectomy (15) and thus corresponds to the subpopulation of T2 cells. These cells are capable of displaying helper activity (15,16) and DTH (21).

Röllinghof and Wagner (22) have shown that the generation of cytotoxic T lymphocytes against allogeneic fibroblasts and allogeneic UV-light irradiated spleen cells can be considerably amplified by the addition of third party cells, which are H-2 identical with the responder cells, but different with regard to Mls-locus coded lymphocyte activating determinants (LADs). They conclude that LADs encoded by the Mls locus can substitute for H-2I region incompatibility, thereby strengthening the 'two signal' hypothesis proposed by Bach et al. (23) and the analogy between GvH-related DTH towards H-2I (3) and Mls-locus coded

antigens. Furthermore, no cytotoxic lymphocytes are generated against Mls-locus coded antigens (24), which thus resemble the H-2I region. However, when using the congenic mouse strain BALB.D2.Ma ($H\text{-}2^d$; Mls^a) a strong cytotoxic T cell activity was detected after preimmunization with BALB/c (Mls^b) lymphocytes and subsequent restimulation in vitro. No cytotoxic activity could be detected in the reverse direction (i.e., Mls^b anti Mls^a) although it is in this direction that MLR-reactivity is induced (25).

Alleles of the Mls-locus can induce a variety of immune responses: MLR, cytotoxic responses, GvH, transplant rejection and helper and suppressor effects (26-29). Furthermore, synergy between T cell subpopulations, which previously has been shown in GvH-related DTH to H-2 alloantigens, now also appears to take place in the response to Mls-locus coded antigens. Taken together this data further supports the notion that the Mls-locus of the mouse codes for antigens that are able to induce similar activities as the major histocompatibility complex.

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REFERENCES

1. Cantor, H., and Asofsky, R., J. Exp. Med. 131, 235, 1970.
2. Raff, M.C., and Cantor, H., Progr. Immunol. 1, 83, 1971.
3. Wolters, E.A.J., and Benner, R., Cell. Immunol. 59, 115, 1981.
4. Wolters, E.A.J., and Benner, R., Nature 279, 642, 1979.
5. Wolters, E.A.J., Brons, N.H.C., van der Kwast, Th.H., and Benner, R., Cell. Immunol. 51, 215, 1980.
6. Festenstein, H., Transplant. Rev. 15, 62, 1973.
7. Wolters, E.A.J., and Benner, R., Transplantation 26, 40, 1978.
8. Wolters, E.A.J., Brons, N.H.C., Benner, R., and Vos, O., In 'Experimental Hematology Today' (S.J. Baum and G.D. Ledney, Eds.) pp. 163-170, Springer Verlag, New York, 1979.
9. Jooste, S.V., Lance, E.M., Levey, R.H., Medawar, P.B., Ruszkiewicz, M., Sharman, R., and Taub, R.N., Immunology 15, 697, 1968.
10. Tigelaar, R.E., and Asofsky, R., J. Exp. Med. 137, 239, 1973.
11. Fathman, C.G., and Nabholz, M., Eur. J. Immunol. 7, 370, 1977.
12. Fathman, C.G., Watanabe, T., and Augustin, A., J. Immunol. 121, 259, 1978.
13. Muirhead, D.Y., and Cudkowicz, G., J. Immunol. 120, 579, 1978.
14. Feldman, M., and Erb, P., Z. Immun.-Forsch. 153, 217, 1977.
15. Feldman, M., Beverly, P.C.L., Woody, J., and McKenzie, I.F.C., J. Exp. Med. 145, 793, 1977.
16. Bianchi, A.T.J., Hooijkaas, H., Benner, R., Tees, R., Nordin, A.A., and Schreier, M.H., Nature 290, 62, 1981.
17. Scollay, R., Kochen, M., Butcher, E., and Weissman, I., Nature 276, 79, 1978.
18. Mathieson, B.J., Sharrow, S.O., Campbell, P.S., and Asofsky, R., Nature 277, 478, 1979.
19. Cantor, H., and Boyse, E.A., Contemp. Topics Immunobiol. 7, 47, 1977.
20. Scollay, R.G., Nature 300, 529, 1982.
21. Vadas, M.A., Miller, J.F.A.P., McKenzie, I.F.C., Chism, S.E., Shen, F.W., Boyse, E.A., Gamble, J.R., and Whitelaw, A.M., J. Exp. Med. 144, 10, 1976.
22. Röllinghof, M., and Wagner, H., J. Immunogenetics 2, 301, 1975.
23. Bach, F.H., Bach, M.L., and Sondel, P., Nature 259, 273, 1976.
24. Peck, A.B., and Bach, F.H., Scand. J. Immunol. 4, 53, 1975.
25. Leben, L., and Festenstein, H., Transplant. Proc. 15, 189, 1983.
26. Berumen, L., Halle-Pannenko, O., and Festenstein, H., Transplant. Proc. 15, 213, 1983.
27. Bartova, J., and Ivanyi, D., J. Immunogenetics 2, 365, 1975.
28. Festenstein, H., In 'Immunobiology of bone marrow transplantation' (C.B. Dupond and R.A. Good, Eds.) pp. 13-16, Grune and Stratton, New York, 1976.
29. Röllinghof, M., and Wagner, H., J. Immunol. 114, 1329, 1975.

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 ($H\text{-}2^a$) and A.SW ($H\text{-}2^s$) 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.AL (H-2^{a1}) mice were purchased from YEDA Research and Development Co. Ltd. at the Weissman Institute of Science, Rehovot, Israel. B10.BYR (H-2^{by1}) mice were bred at our own department from breeding pairs kindly provided by Prof. J. Klein, Max Planck-Institut für Biologie, Tubingen, 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 X-irradiation, generated in a Philips Müller MG 300 X-ray machine. Radiation control mice died within 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 x 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 only received the challenge. The swelling of these control mice varied between 12 and 20%. For measuring GvH-related DTH reactivity 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 was transferred iv into a normal secondary recipient five days after reconstitution. The secondary recipients were syngeneic to the original spleen donors. The secondary recipient mice were challenged into the dorsum of the right hind foot with 2 x 10^7 spleen cells, syngeneic with the irradiated recipients. 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 always 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.

RESTRICTED RECOGNITION OF H-2 SUBREGION CODED ANTIGENS IN DTH

RESPONDER STRAIN	IMMUNIZING CELLS	IMMUNIZING H-2 SUBREGION	CHALLENGE	SPECIFIC INCREASE FOOT THICKNESS	SHARED REGIONS OF H-2 COMPLEX
B10.A	B10.AQR	K	B10.AQR		I D
B10.A	B10.AQR	K	B10.T(6R)		D
B10.A	B10.T(6R)	KI	B10.T(6R)		D
B10.A	B10.T(6R)	KI	B10.AQR		I D
B10.A	B10.AQR	K	B10.AQR B10.T(6R)		I D D

0 20 40

Fig. 1. H-2 restricted recognition of H-2K coded alloantigens in DTH. B10.A responder mice were sc immunized with 1×10^7 B10.AQR or B10.T(6R) spleen cells and challenged with 2×10^7 B10.AQR and/or 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.

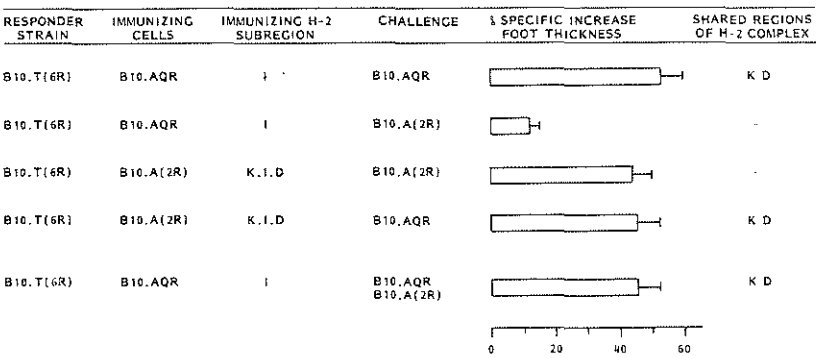

Fig. 2. H-2 restricted recognition of H-2I subregion coded alloantigens in DTH. B10.T(6R) responder mice were sc immunized with 1×10^7 B10.AQR or B10.A(2R) spleen cells and 5 days later challenged with 2×10^7 B10.AQR and/or B10.A(2R) spleen cells. See legend to Fig. 1 for the other details.

This assay, which was originally developed by Brent et al. (1962), is based upon local transfer of 5×10^6 immunized lymphoid cells to naive recipients, which are either identical or partially identical to the original spleen cell inoculum used for immunization. This transfer is done into the dorsum of a hind foot of naive recipients five days after sc immunization. The foot swelling of these secondary recipients was measured 24 h after transfer. Fig 3, 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, but not after transfer to K+I incompatible B10.T(6R) recipients (Fig. 3, line 2). The same was found for the A.TL-A.AL-A.TH combination (Fig. 3, 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. 3, lines 5 and 6). Moreover, lymphoid cells from A.SW mice immunized to D alloantigens from A.TH mice, transferred to D incompatible A.TH naive recipients, produced a significant ILT response, whereas after transfer to I+D incompatible A.TL naive recipients a much lower ILT response was found (Fig. 3, lines 7 and 8).

RESTRICTED RECOGNITION OF H-2 SUBREGION CODED ANTIGENS BY DTH REACTIVE T CELLS IN THE LYMPHOCYTE TRANSFER ASSAY

IMMUNIZING CELLS	IMMUNIZED DONOR STRAIN	IMMUNIZING H-2 SUBREGION	RECIPIENT STRAIN	RESPONSE ILT-NLT X 10^{-2} MM	SHARED REGIONS OF H-2 COMPLEX
B10.AQR	B10.A	K	B10.AQR		I D
B10.AQR	B10.A	K	B10.T(6R)		D
A.TL	A.AL	K	A.TL		I D
A.TL	A.AL	K	A.TH		D
B10.AQR	B10.T(6R)	I	B10.AQR		K D
B10.AQR	B10.T(6R)	I	B10.BR		-
A.TH	A.SW	D	A.TH		K I
A.TH	A.SW	D	A.TL		K

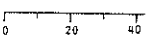

Fig. 3. H-2 restricted recognition of H-2K, I or D subregion coded alloantigens by DTH reactive T cells in the immune lymphocyte transfer (ILT) assay. B10.A, A.AL, B10.T(6R) and A.SW mice were immunized with 1 x 10^7 B10.AQR, A.TL, B10.AQR and A.TH spleen cells, respectively. Five days later the draining 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. As a control, the left hind foot was injected with a similar number of not activated lymph node cells (NLT). After 24 h the thickness was measured of both hind feet. 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).

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

Finally, 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 cell donors used to reconstitute the irradiated allogeneic recipients, and measurement of their DTH reactivity. Therefore, after transfer, the secondary recipients are challenged in the right hind foot with spleen cells syngeneic to the irradiated primary recipients. The subsequent foot swelling is determined 24 h after challenge. In this assay, only reactions to H-2I and Mls-coded alloantigens occur (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 from 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 good response occurred (Fig. 4, line 1). However, when the I alloantigens were presented in the context of foreign K+D, K or D molecules, much weaker DTH reactions were found (Fig. 4, lines 2, 3 and 4).

The same phenomenon occurred in the A.TH-A.TL-A.AL combination: when the I alloantigens were presented to GvH activated DTH effector T cells in the context of the original syngeneic K+D molecules, a good response was found (Fig. 3, line 5). However, when these I alloantigens were presented in combination with foreign K molecules, no reaction was found (Fig. 3, line 6). Thus, I antigens have to be presented with syngeneic K+D molecules to ensure optimal GvH-related DTH reactivity.

RESTRICTED RECOGNITION OF CLASS II ANTIGENS IN GVH RELATED DTH.

RESPONDER STRAIN	IRRADIATED RECIPIENT	IMMUNIZING H-2 SUBREGION	CHALLENGE	% SPECIFIC INCREASE FOOT THICKNESS	SHARED REGIONS OF H-2 COMPLEX
B10.T(6R)	B10.AQR	I	B10.AQR		K D
B10.T(6R)	B10.AQR	I	B10.BR		-
B10.T(6R)	B10.AQR	I	B10.BYR		K
B10.T(6R)	B10.AQR	I	B10.A		D
A.TH	A.TL	I	A.TL		K D
A.TH	A.TL	I	A.AL		D

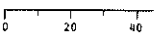

Fig. 4. H-2 restricted recognition of H-2I coded alloantigens by DTH reactive cells in a GvH-related DTH assay. Lethally irradiated B10.AQR and A.TL mice were reconstituted with 2 x 10^7 spleen cells from B10.T(6R) and A.TH donors, respectively. Five days later, spleen and lymph node cells were transferred 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 or A.AL spleen cells, which differ in the indicated H-2 subregions from the B10.T(6R) and A.TH responder mice. See legend to Fig. 1 for the other details.

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 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 by alloantigen processing and presentation by APC will be synrestricted.

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.

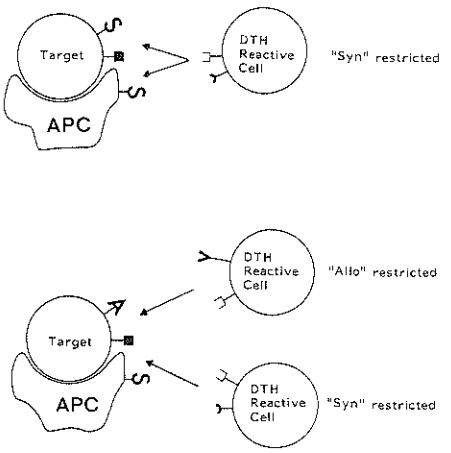

Fig. 5. Model for syngeneically and allogeneically H-2 restricted recognition of H-2 subregion coded alloantigens.

Thus, when H-2 subregion coded alloantigens are presented during the sc induction of the DTH reactivity on cells which are syngeneic to the 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 (Figs. 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 synrestricted (due to APC-processing) and one allorestricted. 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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REFERENCES

Bevan, M.J.: The major histocompatibility complex determines susceptibility to cytotoxic T cells directed against minor histocompatibility antigens. J. Exp. Med. 142: 1349-1364, 1975.

Bianchi, A.T.J., Hooijkaas, H., Benner, R., Tees, R., Nordin, A.A., and Schreier, M.H.: Clones of helper T cells mediate antigen-specific H-2 restricted DTH. Nature 290: 62-63, 1981.

Brent, L., Brown, J.B. and Medawar, P.B.: Quantitative studies on tissue transplantation immunity. VI. Hypersensitivity reactions associated with the rejection of homografts. Proc. Roy. Soc. Ser. B (London) 156: 187-209, 1962.

Doherty, P.C., Blanden, R.V., and Zinkernagel, R.M.: Specificity of virus-immune effector T cells for H-2K or H-2D compatible interactions: implication for H antigen diversity. Transplant. Rev. 29: 89-124, 1976.

Gardner, I., Bowern, N.A., and Blanden, R.V.: Cell-mediated cytotoxicity against ectromelia virus-infected target cells. I. Specificity and kinetics. Eur. J. Immunol. 4: 63-67, 1974.

Kindred, B.: H-2 restricted lethal graft-vs-host disease. Immunogenetics 4: 527-533, 1981.

Kindred, B.: H-2 restricted GvH reaction caused by T cells from normal donors of certain strains. Immunogenetics 17: 203-209, 1983a.

Kindred, B.: H-2 restricted GvHR: Foreign determinants and restriction elements. Immunogenetics in press, 1983b.

Klein, J., Chiang, C.L., and Hauptfeld, V.: Histocompatibility antigens controlled by the I region of the murine H-2 complex. II. K-D region compatibility is not required for I region cell-mediated lymphocytotoxicity. J. Exp. Med. 145: 450-454, 1977.

Korngold, R., and Sprent, J.: H-2 restriction of T cells causing lethal graft-vs-host disease across minor histocompatibility barriers in mice. Transplant. Proc. 13: 1271-1219, 1981.

Korngold, R., and Sprent, J.: Features of T cells causing H-2 restricted lethal graft-vs-host disease across minor histocompatibility barriers. J. Exp. Med. 155: 872-883, 1982.

Koszinowski, U., and Thomssen, R.: Target cell-dependent T cell-mediated lysis of vaccinia virus-infected cells. Eur. J. Immunol. 5: 245-251, 1975.

Minami, M., and Shreffler, D.C.: Ia-positive stimulator cells are required in primary, but not in secondary mixed leukocyte reactions against H-2K and H-2D differences. J. Immunol. 126: 1774-1779, 1981.

Miller, J.F.A.P., Vadas, M.A., Whitelaw, A., and Gamble, J.: H-2 gene complex restricts transfer of delayed type hypersensitivity in mice. Proc. Natl. Acad. Sci. USA 72: 5092-5098, 1975.

Rock, K.L., Barnes, M.C., Germain, R.N., and Benacerraf, B.: The role of Ia molecules in the activation of T lymphocytes. II. Ia-restricted recognition of allo K/D antigens for class I MHC-stimulated mixed lymphocyte responses. J. Immunol. 130: 457-462, 1983.

Shearer, G.M., Rehn, T.G., and Garbarino, C.A.: Cell-mediated lympholysis of trinitrophenyl-modified autologous lymphocytes. Effector cell specificity to modified cell suface components controlled by the H-2K and H-2D serological regions of the murine major histocompatibility complex. J. Exp. Med. 142: 1348-1364,1975.

Smith, F.I., and Miller, J.F.A.P.: Delayed type hypersensitivity to allogeneic cells in mice. III. Sensitivity to cell-surface antigens coded by the major histocompatibility complex and by other genes. J. Exp. Med. 150: 965-976, 1979.

Swain, S.L.: Significance of class 1 and class 2 major histocompatibility complex antigens: help to allogeneic K and D antigens does not involve I recognition. J. Immunol. 126: 2307-2309, 1981.

Vadas, M.A., Miller, J.F.A.P., Whitelaw, A.M., and Gamble, J.R.: Regulation by the H-2 gene complex of delayed type hypersensitivity. Immunogenetics 4: 137-153, 1977.

Vadas, M.A., and Greene, M.I.: Role of the MHC in delayed type hypersensitivity. in 'The role of the major histocompatibility complex in immunobiology', ed. M.E. Dorf, J. Wiley & Sons, 271-302, 1981.

Van der Kwast, Th.H., and Benner, R.: T1 and T2 lymphocytes in primary and secondary delayed type hypersensitivity of mice. I. Contribution in the response to sheep red blood cells and to allogeneic spleen cells. Cell. Immunol. 39: 194-203, 1978.

Van der Kwast, Th.H.: H-2 restricted recognition of minor histocompatibility antigens in delayed type hypersensitivity. J. Immunogenetics 7: 315-324, 1980.

Weiss, S., and Dennert, G.: T cell lines active in the delayed type hypersensitivity reaction (DTH). J. Immunol. 126: 2031-2035, 1981.

Wolters, E.A.J., and Benner, R.: Immunobiology of the Graft-versus-Host reaction. I. Symptoms of Graft-versus-Host disease in mice are preceded by delayed-type hypersensitivity to host histocompatibility antigens. Transplantation 26: 40-45, 1978.

Wolters, E.A.J., and Benner, R.: Functional separation in vivo of both antigens encoded by H-2 subregion and non-H-2 loci. Nature 279: 642-643, 1979.

Wolters, E.A.J., van der Kwast, Th.H., Odijk, L.M., and Benner, R.: Differential responsiveness to H-2-subregion-coded antigens in Graft-versus-Host and Host-versus-Graft reactions. Cell. Immunol. 57: 389-399, 1981.

Zinkernagel, R.M., and Doherty, P.C.: Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. Nature 248: 701-702, 1974.

Zinkernagel, R.M., and Doherty, P.C.: H-2 compatibility requirements for T cell mediated lysis of target cells infected with lymphocytic choriomeningitis virus. Different cytotoxic T cell specificities are associated with structures coded for H-2K or H-2D. J. Exp. Med. 141: 1427-1436, 1975.

Zinkernagel, R.M.: H-2 restriction of virus-specific T cell mediated effector functions in vivo. II. Adoptive transfer of delayed-type hypersensitivity to murine lymphocytic choriomeningitis virus is restricted by the K and D region of H-2. J. Exp. Med. 144: 776-787, 1976.

Zinkernagel, R.M., Althage, A., Adler, B., Blanden, R.V., Davidson, W.F., Kees, U., Dunlop, M.C.B., and Shreffler, D.C.: H-2 restriction of cell-mediated immunity to an intracellular bacterium. Effector T cells are specific for Listeria antigen in association with H-2I region coded self-markers. J. Exp. Med. 145: 1353-1367, 1977.

SUPPRESSION OF ANTIGRAFT IMMUNITY BY PREIMMUNIZATION

I. Kinetic Aspects and Specificity[1]

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SUMMARY

Intravenous injection of 2,000 rad of 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.

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 × CBA/Rij)F_1 (H-$2^{b/a}$), 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, Füllingsdorf, Switzerland. The age of the responder mice varied between 12 and 24 weeks. All mice used were females.

Preparation of cell 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 1×10^7 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 previously (*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

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s.c. immunization with 1×10^7 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 8×10^5 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 immune mice minus 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 × 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 × CBA)F_1 mice were given s.c. injections of 1×10^7 irradiated BALB/c or (C57BL × 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).

TABLE 1. Preimmunization with allogeneic cells

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

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

[b] Mean responses ± 1 SE ($n = 5$).

The determination of the optimal dose of i.v. injected spleen cells was done with different groups of (C57BL × CBA)F_1 mice that were injected with 10^4, 10^5, 10^6, or 10^7 irradiated BALB/c or (C57BL × 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 cells 7 days later, and tested for anti-A.SW DTH reactivity again 5

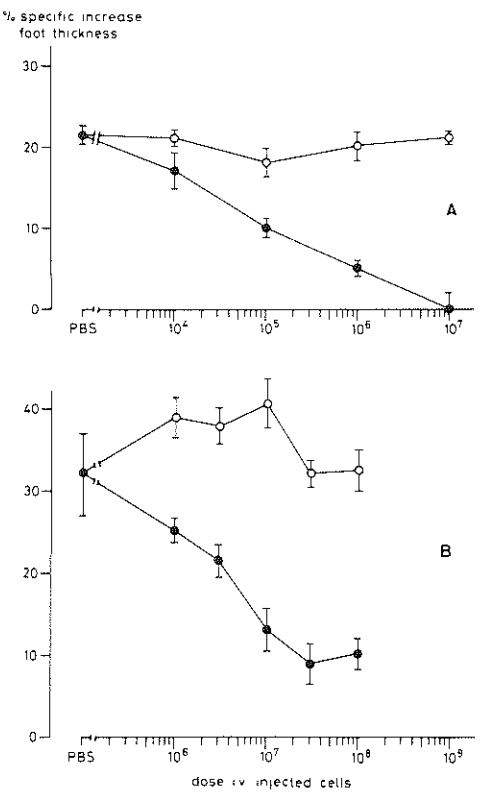

FIGURE 1. Determination of the optimal dose of i.v. injected spleen cells for suppression of DTH reactivity to allogeneic spleen cells. A: (C57BL × CBA)F_1 mice were given injections of 1×10^4, 1×10^5, 1×10^6, or 1×10^7 irradiated BALB/c (●) or (C57BL × CBA)F_1 spleen cells (○). Seven days later all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

days later. Also, in this combination i.v. preimmunization by 1 $\times 10^7$ 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 × CBA)F_1 mice were given i.v. injections of either 1 × 10^7 irradiated BALB/c or 1 × 10^7 irradiated (C57BL × CBA)F_1 spleen cells. Seven days later all mice were s.c. immunized with 1 × 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 ($H\text{-}2^k$) and AKR ($H\text{-}2^k$) mice, which only differ for minor H antigens.

Groups of BALB/c mice were given i.v. injections of 1 × 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 × 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 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 × 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 ($H\text{-}2^d$) mice were given i.v. injections of either 1 × 10^7 irradiated H-2-incompatible BALB.K ($H\text{-}2^k$), 1 × 10^7 minor H-incompatible DBA/2 ($H\text{-}2^d$), or 1 × 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 DTH reactivity only oc-

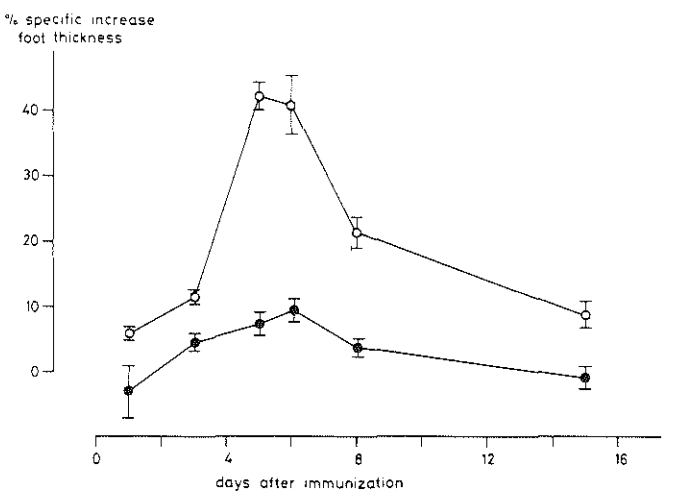

FIGURE 2. Effect of i.v. preimmunization upon the kinetics of the DTH reactivity to allogeneic spleen cells. (C57BL × CBA)F_1 mice were given i.v. injections of 1 × 10^7 irradiated BALB/c (●) or (C57BL × CBA)F_1 spleen cells (○). Seven days later all mice were s.c. immunized with 1 × 10^7 BALB/c spleen 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).

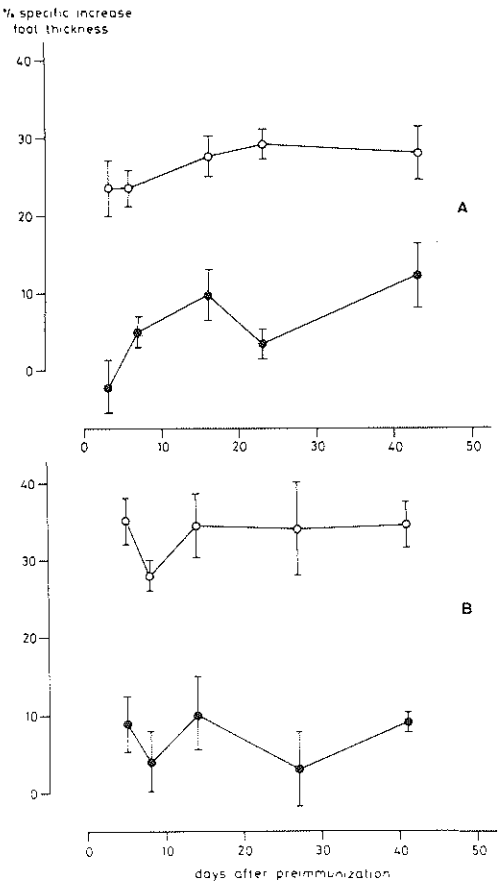

FIGURE 3. Time course of the immunosuppression induced by i.v. preimmunization. A: Groups of BALB/c mice were given i.v. injections of 1×10^7 irradiated BALB.K (●) or BALB/c spleen cells (○) on days −43, −23, −16, −7, and −3. On day 0, groups of these mice were s.c. immunized with BALB.K spleen cells, and on day 6 all mice were challenged. B: Groups of C3H/Lw mice were given i.v. injections of 1×10^7 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 ($n = 5$).

curred when the i.v. and s.c. injections were done with identical spleen cells (Fig. 5).

Similar experiments were done with B10.ScSn ($H\text{-}2^b$) responder mice given i.v. and s.c. injections of either H-2-incompatible B10.G ($H\text{-}2^q$) 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 obtained in a similar protocol with C3H/Lw ($H\text{-}2^k$) responder mice, CWB ($H\text{-}2^b$) spleen cells, and AKR ($H\text{-}2^k$) 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 ($H\text{-}2^d$) mice were given i.v. injections of either 1×10^7 irradiated H-2-incompatible BALB.K ($H\text{-}2^k$), 1×10^7 H-2-incompatible BALB/B ($H\text{-}2^b$), or 1×10^7 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 1×10^7 BALB.K or 1×10^7 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 ($H\text{-}2^b$) mice were given i.v. injections of either B10.A ($H\text{-}2^u$), B10.G ($H\text{-}2^q$), or B10.ScSn ($H\text{-}2^b$) 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

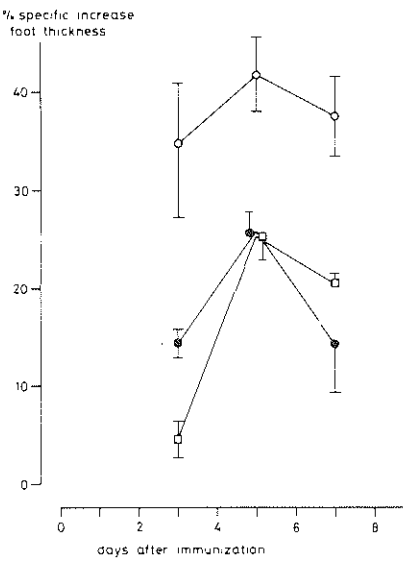

FIGURE 4. Effect of i.v. preimmunization upon the secondary DTH response to non-H-2 alloantigens. Groups of DBA/2 mice were given i.v. injections of 1×10^7 irradiated BALB/c (●) or DBA/2 spleen cells (○). Seven days later all mice were s.c. immunized with 1×10^7 BALB/c spleen cells, and another 6 weeks 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 ($n = 5$).

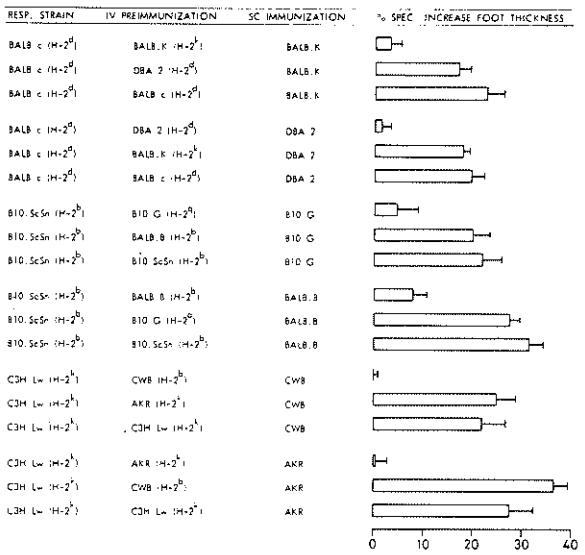

FIGURE 5. Specificity of i.v. induced suppression for H-2 and non-H-2 alloantigens. Responder mice were given i.v. injections of 1×10^7 irradiated allogeneic or syngeneic spleen cells and s.c. immunized with 1×10^7 allogeneic spleen cells 7 days later. Challenge for DTH was performed on day 6 after s.c.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

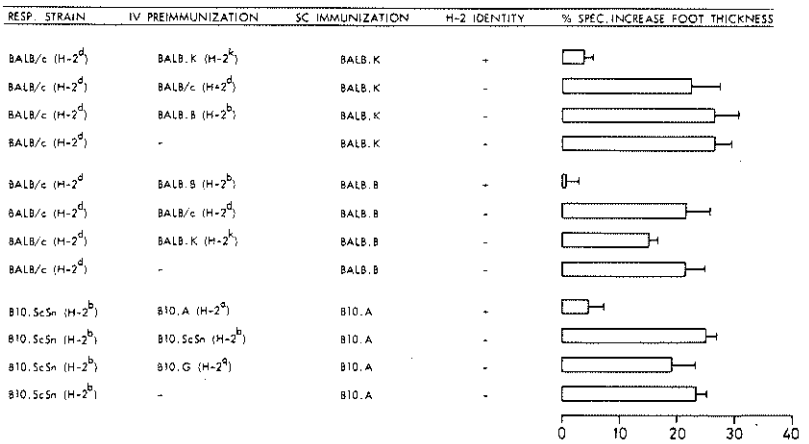

FIGURE 6. Specificity of i.v. induced suppression for different *H-2* haplotypes. Responder mice were given i.v. injections of 1×10^7 irradiated allogeneic or syngeneic spleen cells and s.c. immunized with 1×10^7 allogeneic spleen cells 7 days later. Challenge for DTH was performed on day 5 after s.c.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

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 × CBA)F_1 mice ($H\text{-}2^{b/q}$) were given i.v. injections of either 1×10^7 irradiated H-2- and minor H-incompatible BALB/c ($H\text{-}2^d$) or syngeneic (C57BL × 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 3, 4, 6, and 8 the proliferative activity in the draining inguinal lymph nodes of the different groups of mice was determined. (C57BL × 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.

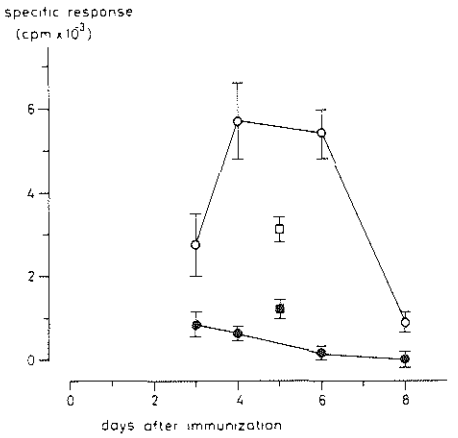

FIGURE 7. Proliferative activity in the draining lymph nodes of immunosuppressed mice after s.c. immunization. Groups of (C57BL × CBA)F_1 mice were given i.v. injections of 1×10^7 irradiated BALB/c (●) or (C57BL × CBA)F_1 (○) spleen cells on day −7. On day 0, the mice of both groups were s.c. immunized 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 1×10^7 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 were 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 ($n = 5$).

preimmunized with minor H-incompatible DBA/2 or syngeneic BALB/c spleen cells, and s.c. immunized with allogeneic H-2-compatible DBA/2 ($H\text{-}2^d$) 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*). Further 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 antilymphocyte 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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LITERATURE CITED

1. Feldmann M, Beverley PCL, Woody J, et al: 1977 J Exp Med 145: 793
2. Lagrange PH, Makaness GB: 1978 J Exp Med 148: 235
3. Zembala M, Asherson GL: 1973 Nature 244: 227
4. Pinto M, Brent L, Thomas AV: 1974 Transplantation 17: 477
5. Nagiro H, Namoto K, Kuroiwa A, et al: 1978 Int Arch Allergy Appl Immunol 56: 48
6. McMaster R, Levy JG: 1975 J Immunol 115: 1400
7. Rich SS, Rich RR: 1974 J Exp Med 140: 1588
8. Folch H, Waksmann B: 1974 J Immunol 113: 140
9. Peavy DL, Pierce CW: 1974 J Exp Med 140: 356
10. Hodes RJ, Hathcock KS: 1976 J Immunol 116: 167
11. Asherson GL, Zembala M: 1975 Curr Top Microbiol Immunol 72: 55
12. Kirchner H, Holden HT, Herberman RB: 1976 J Immunol 115: 1212
13. Kirchner H, Chused TM, Herberman RB, et al: 1974 J Exp Med 139: 1473
14. Schwartz A, Askenase PW, Geshon RK: 1978 J Immunol 121: 1573
15. Smith F, Miller JFAP: 1979 Int Arch Allergy Appl Immunol 58: 285
16. Van der Kwast ThH, Olthof JG, Benner R: 1979 Cell Immunol 47: 192
17. White E, Hildemann WH: 1969 Transplant Proc 1: 395
18. Brent L, Opara SC: 1979 Transplantation 27: 120
19. Kulkarni SS, Kulkarni AD, Gallagher MT, et al: 1979 Cell Immunol 47: 192
20. Miller SD, Claman HN: 1976 J Immunol 117: 1519
21. Bach BA, Sherman L, Benacerraf B, et al: 1978 J Immunol 121: 1460
22. Van der Kwast ThH, Olthof JG, Benner R: 1977 Cell Immunol 34: 85
23. North RJ, Mackaness GB, Elliott RW: 1972 Cell Immunol 3: 680
24. Sprent J, Miller JFAP, Mitchell GF: 1971 Cell Immunol 2: 171
25. Nadler LM, Hodes RJ: 1977 J Immunol 118: 1886
26. Chiu KM, Faanes RB, Choi YS: 1980 Cell Immunol 49: 283
27. Miller SD, Sy MS, Claman HB: 1977 Eur J Immunol 7: 165
28. Miller SD, Sy MS, Claman HN: 1978 Eur J Immunol 121: 274
29. Bloom BR, Hamilton LD, Chase MW: 1964 Nature 201: 689
30. Rouse BT, Wagner H: 1972 J Immunol 109: 1282
31. Cantor H, Boyse EA: 1975 J Exp Med 141: 1376
32. Okada M, Klimpel GR, Kuppers RC: 1979 J Immunol 122: 2527

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SUPPRESSION OF ANTIGRAFT IMMUNITY BY PREIMMUNIZATION.

II. CHARACTERIZATION OF THE SUPPRESSOR CELLS

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Summary

Intravenous immunization of mice with irradiated (20 Gy) or non-irradiated allogeneic spleen cells 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 subcutaneous (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 mainly 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 be 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 (ATx) and can be depleted by anti thymocyte serum (ATS) treatment.

Introduction

Induction of immune reactivity and tolerance *in vivo* has been shown to depend on the dose 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 either 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, while 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 hart (16,17) transplant survival. In most of these studies additional immunosuppression (e.g. by anti lymphocyte serum or cyclophosphamide) appeared to be required in order to reveal the suppression by the suppressor T cells (15,18).

In previous studies we have shown that subcutaneous (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).

Iv preimmunization 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).

This paper deals with 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.

Materials and Methods

Mice. (C57BL/Rij x CBA/Rij)F1 (H-$2^{b/q}$), BALB/c (H-2^{d}) and A.SW

($H\text{-}2^s$) mice were purchased from the Laboratory Animals Centre of the Erasmus University, Rotterdam. DBA/2 ($H\text{-}2^d$) mice were purchased from the Radiobiological Institute TNO, Rijswijk, The Netherlands. Swiss ($H\text{-}2^s$) mice were purchased from the Central Institute for the Breeding of Laboratory Animals TNO, Zeist, The Netherlands. C3H/Tif ($H\text{-}2^k$) mice were purchased from Bomholtgard, Ry, Denmark. B10.D2 ($H\text{-}2^d$) and B10.BR ($H\text{-}2^k$) 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 5×10^7 irradiated (20 Gy) allogeneic spleen cells, unless indicated otherwise. In general, DTH was induced by sc immunization with 1×10^7 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 Table 1.

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)A '+' means that that particular donor-recipient combination is compatible, whereas a '-' means that that combination is incompatible with regard to the H-2 or non-H-2 histocompatibility antigens.

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 mentioned 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 mth before iv suppression. Mice were anaesthetized 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 made in the left upper abdomen. For splenectomy, the splenic blood vessels were tied in a single suture, then cut and the spleen 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 surgery was performed as described by Miller (26). Avertin was used for anaesthesia. 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 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. Before

use, the ATS and NRS was absorbed with a pool of washed mouse blood cells to remove the cytotoxic activity. We have previously shown that the above procedure depletes the long-lived recirculating T cells (T2 cells)(cf.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, USA. 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 injections

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 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. 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. Subcutaneous immunization of mice with allogeneic spleen cells induces a state of DTH reactivity (30). In order to investigate whether 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 nonirradiated 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

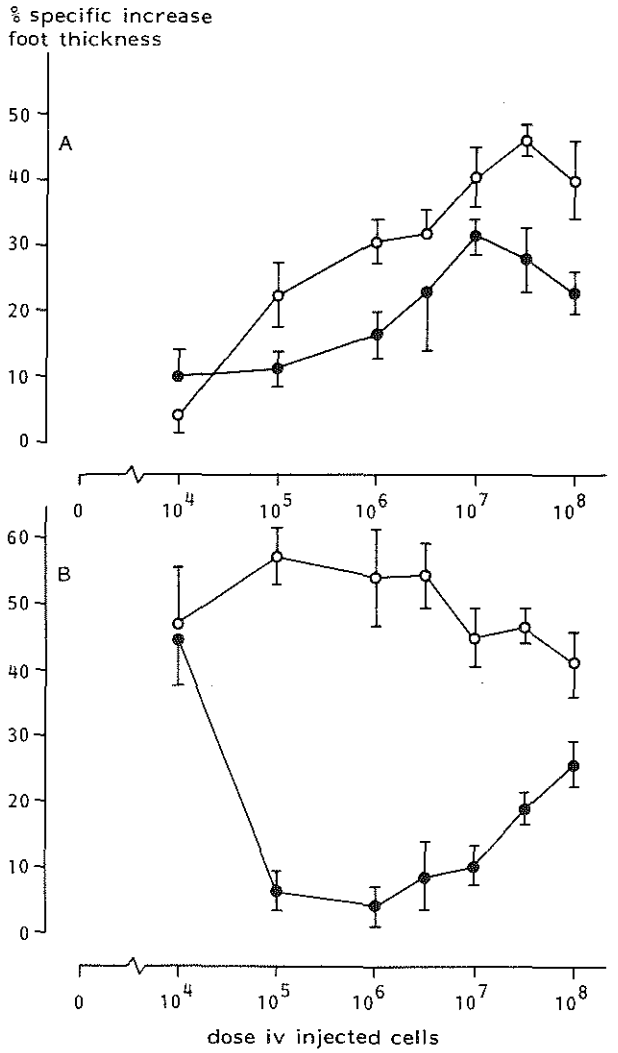

Fig. 1. (A) Induction of DTH reactivity by iv immunization with allogeneic cells. Swiss mice were iv injected with either 1 x 10^4, 1 x 10^5, 1 x 10^6, 3 x 10^6, 1 x 10^7, 3 x 10^7 or 1 x 10^8 irradiated (●) or nonirradiated (○) A.SW spleen cells. Five days later all mice were challenged for DTH. (B)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

induced by about 1 x 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.

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, while 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 data from these experiments, depicted in Fig. 2, 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 x 10^7 irradiated BALB/c spleen cells 4 days before transfer. Transfer of the

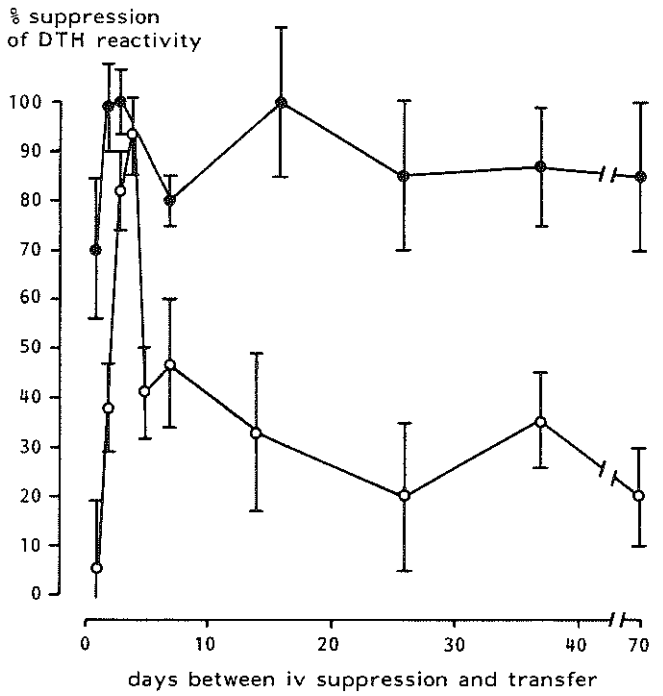

Fig. 2. Systemic transfer of iv induced suppression. Groups of (C57BL x CBA)F1 mice were iv preimmunized with 5 x 10^7 irradiated BALB/c spleen cells. At each of the indicated intervals after iv preimmunization, one group of mice was sc immunized with 1 x 10^7 BALB/c spleen cells (●), while spleen and 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 x 10^7 BALB/c spleen cells. At all intervals a group of (C57BL x CBA)F1 mice was included, which 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 represents 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%.

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 proportional 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.

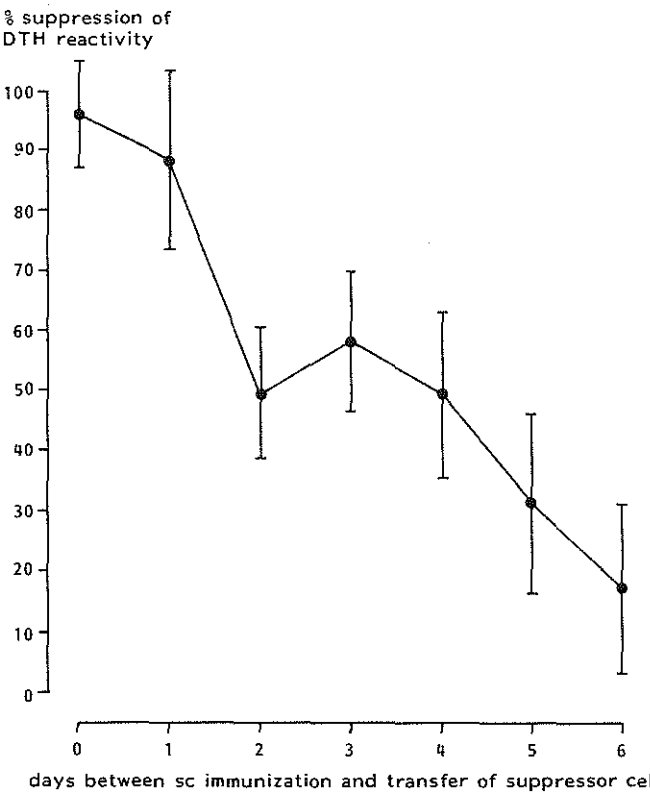

Fig. 3. Effect of infusion of activated suppressor cells at different intervals after sc immunization of the recipient mice. Spleen and lymph node cells from (C57BL x CBA)F1 mice, which were iv preimmunized with 5 x 10^7 irradiated BALB/c spleen cells, were systemically transferred at the same day or 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.

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 x 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 x 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 which were immunized with H-2 incompatible B10.D2 spleen cells, and with splenectomized and sham-splenectomized BALB/c responder mice which 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 x 10^8 spleen cells, 1 x 10^8 lymph node cells or 2 x 10^8 of a mixture of both cell types from suppressed (C57BL x CBA)F1 donor mice to naive recipients.
A few hours later the recipient mice were sc immunized with 1 x 10^7 H-2 and non-H-2 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 to transfer suppression. Even lymph node cells from mice, which 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).

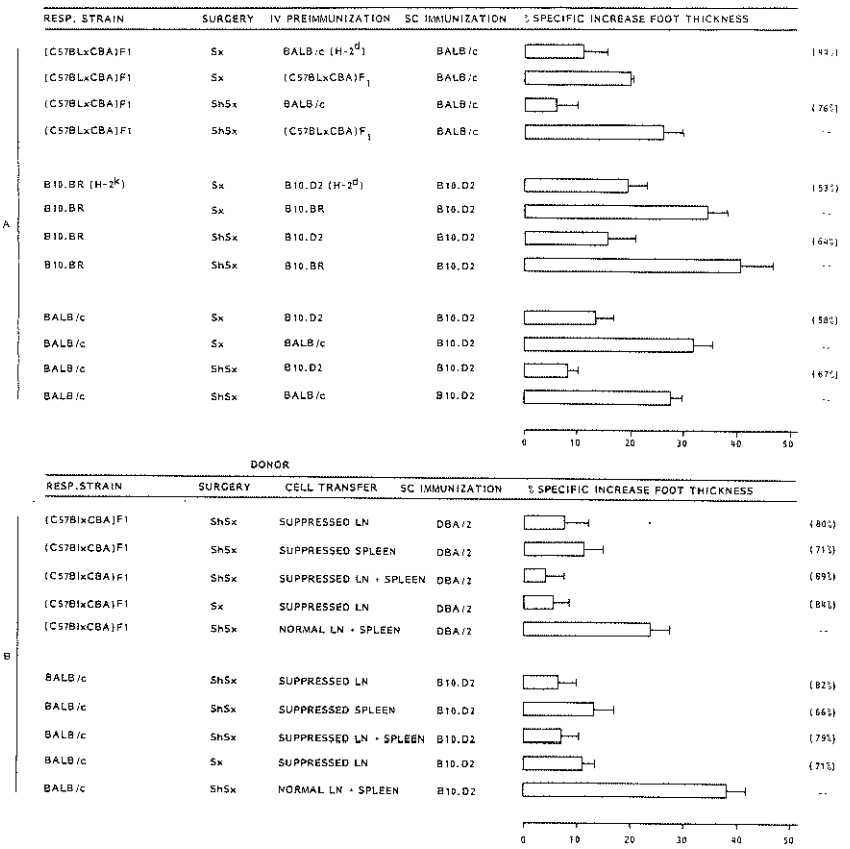

Fig. 4. (A) Effect of splenectomy on the iv induction of suppression. Responder mice were splenectomized (Sx) or sham-splenectomized (ShSx) four weeks before iv preimmunization with 5 x 10^7 irradiated allogeneic or syngeneic spleen cells. Seven days later all mice were sc immunized with 1 x 10^7 allogeneic 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 percentage specific suppression is 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 5×10^7 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 1×10^7 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 (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

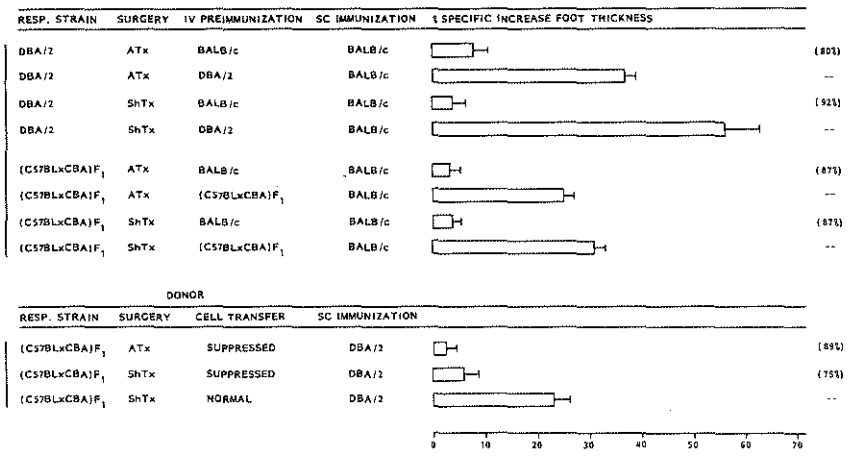

Fig. 5. (A) Effect of adult thymectomy on the iv induction of suppression Responder mice were thymectomized (ATx) or sham-thymectomized (ShTx) ten weeks before iv preimmunization with 5×10^7 irradiated allogeneic or syngeneic spleen cells. Seven days later all mice were sc immunized with 1×10^7 allogeneic spleen cells. Challenge for DTH was performed on day 6 after sc immunization. Each column represents the mean response ± SE (n=6). (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 5×10^7 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 1×10^7 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 is shown in parentheses.

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).

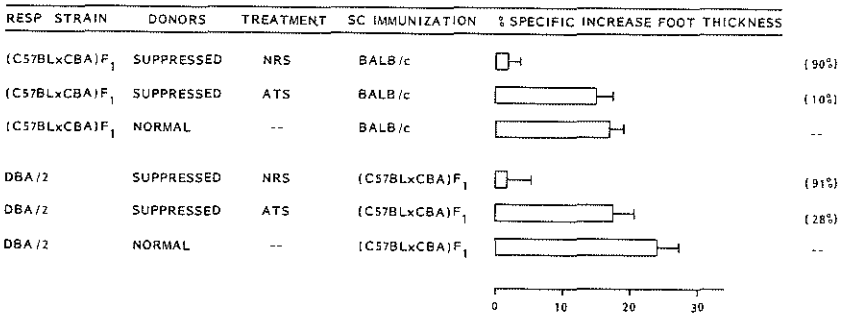

Fig. 6. Effect of anti-thymocyte serum (ATS) treatment on the transfer of suppression. Donor mice were treated with ATS or NRS on day -5 and day -2 and iv preimmunized with 5 x 10^7 allogeneic spleen cells on day 0. Four days later the spleen and lymph node cells were systemically transferred to syngeneic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

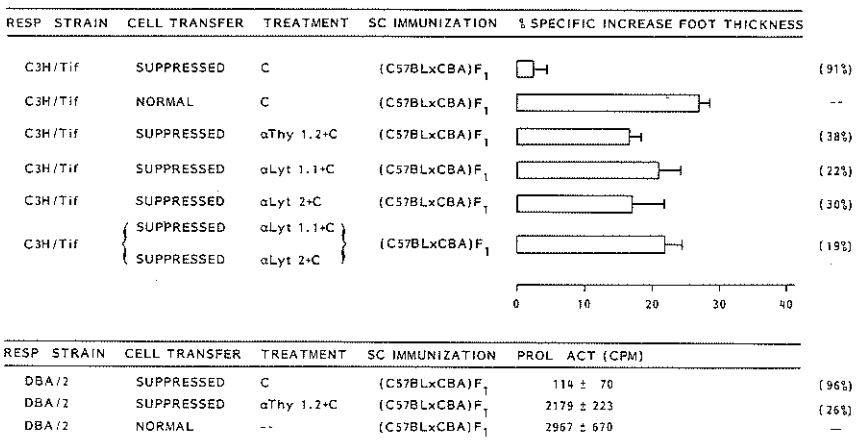

Fig. 7. (A) Surface markers of the suppressor cells involved in suppression of DTH to histocompatibility antigens. By means of systemic transfer, groups of responder mice received spleen and lymph node cells from normal, syngeneic mice or from syngeneic mice which had been iv injected with 5×10^7 irradiated 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 1×10^7 allogeneic spleen cells and tested for DTH another 6 days later. Each column represents the mean response ± SE (n=6). The percentage specific suppression is 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 5×10^7 irradiated allogeneic spleen cells 4 days previously ('suppressed cells'). On the day of transfer all mice were sc immunized with 1×10^7 allogeneic spleen cells and the proliferative activity in the draining lymph nodes was measured 5 days later.

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, while 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, that the suppressor T cells influenced the induction phase of the DTH response.

Discussion

The data presented in this paper 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 due to the iv immunization becomes manifest only after secondary sc immunization (cf. reference 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), while 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 can 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 suppressor T cells. Apparently, the suppressor T cells are unable to suppress the induction and functional activity of simultaneously activated DTH reactive T cells, while 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 on suppression of DTH against haptens (32) and heterologous erythrocytes (33,34) and in studies on 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 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 show 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 by 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 pre-immunization 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 (5) phase and efferent (40) phase 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 lifespan 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, while 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 occurrence 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).

Acknowledgments

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References

1. Lagrange, P.H., Mackaness, G.B., and Miller, T.E. Influence of dose and route of antigen injection on the immunological induction of T cells. J. Exp. Med. 1974;139:528.
2. Greene, M.I., and Benacerraf, B. Studies on hapten specific T cell immunity and suppression. Immunol. Rev. 1980;50:163.
3. Van der Kwast, Th.H., Bianchi, A.T.J., Bril, H. and Benner, R. Suppression of antigraft immunity by preimmunization. I. Kinetic

aspects and specificity. Transplantation 1981;31:79.
4. Miller, S.D., and Claman, H.N. The induction of T cell tolerance by using hapten-modified lymphoid cells. I. Characteristics of tolerance induction. J. Immunol. 1976;117:1519.
5. Claman, H.N., Miller, S.D., Sy, M.S., and Moorhead, J.W. Suppressive mechanisms involving sensitization and tolerance in contact allergy. Immunol. Rev. 1980;50:105.
6. Claman, H.N., Miller, S.D., Conlon, P.J., and Moorhead, J.W. Control of experimental contact sensitivity. Adv. Immunol. 1980;30:121.
7. Asherson, G.L., and Zembala, M. T suppressor cells and suppressor factor which act at the efferent stage of contact sensitivity skin reaction: their production by mice injected with water soluble, chemically reactive derivates of oxazolone and picryl chloride. Immunology 1980;42:1005.
8. Germain, R.N., and Benacerraf, B. A single major pathway of T lymphocyte interactions in antigen-specific immune suppression. Scand. J. Immunol. 1981;13:1.
9. Zembala, M., Asherson, G.L., and Colizzi, V. Hapten-specific T suppressor factor recognizes both hapten and I-J region products on haptenized spleen cells. Nature 1982;297:411.
10. Moorhead, J.W. Tolerance and contact sensitivity to DNFB in mice. VI. Inhibition of afferent sensitivity by suppressor T cells in adoptive tolerance. J. Immunol. 1976;117:807.
11. Miller, S.D., Sy, M.-S., and Claman, H.N. Suppressor T cell mechanisms in contact sensitivity. II. Afferent blockade by alloinduced suppressor T cells. J. Immunol. 1978;121:274.
12. Asherson, G.L., and Zembala, M. Suppression of contact sensitivity by T cells in the mouse. I. Demonstration that suppressor T cells act on the effector stage of contact sensitivity. and their induction following in vitro exposure. Proc. R. Soc. Lond. B. 1974;187:329.
13. Miller, S.D., Sy, M.S., and Claman, H.N. Suppressor T cell mechanisms in contact sensitivity. I. Efferent blockade by syn-induced suppressor T cells. J. Immunol. 1978;121:265.
14. Pinto, M., Brent, L., and Thomas, A.V. Specific unresponsiveness to skin allografts in mice. III. Synergistic effect of tissue extracts, Bordetella pertussis and antilymphocyte serum. Transplantation 1974;17:477.
15. Brent, L., and Opara, S.C. Specific unresponsiveness to skin allografts in mice. V. Synergy between donor tissue extract, procarbazine hydrochloride, and antilymphocyte serum in creating long-lasting unresponsiveness mediated by suppressor T cells. Transplantation 1979;27:120.
16. Kulkarni, S.S., Kulkarni, A.D., Gallagher, M.D., and Trentin, J.J. Prolongation of cardiac allograft survival by pretreatment of recipient mice with donor blood or spleen cells plus cyclophosphamide. Cell.Immunol. 1979;47:192.
17. Kelley, S.E., and Corry R.J. Prolongation of mouse heart allograft survival by prior administration of nonspecific blood. Transplant.Proc. 1981;13:517.

18. Kilshaw P.J., Brent, L., and Pinto, M. Suppressor T cells in mice made unresponsive to skin allografts. Nature 1975;255:489.
19. Bianchi, A.T.J., Bril, H., and Benner, R. Alloantigen-specific suppressor T cells can also suppress the in vivo immune response to unrelated alloantigens. Nature 1983;301:614.
20. Bianchi, A.T.J., Hussaarts-Odijk, L.M., and Benner, R. Antigen-specific suppressor T cells suppress in vivo the cellular immune response to unrelated 'bystander' antigens. Transplant. Proc. 1983;15:760.
21. Bril, H., and Benner, R. Specific suppression of anti-host immune reactivity in graft-versus-host reaction. Adv. Exp. Med. Biol. 1982;149:577.
22. Bianchi, A.T.J., Hussaarts-Odijk, L.M., and Benner, R. Nonspecific suppression of antigraft immunity by antigen-specific T suppressor cells. Adv. Exp. Med. Biol. 1982;149:651.
23. Liew, F.Y. Regulation of delayed type hypersensitivity. VII. The role of I-J subregion gene products in the inhibition of delayed-type hypersensitivity to major histocompatibility antigen specific suppressor T cells. Eur. J. Immunol. 1981;11:883.
24. Bianchi, A.T.J., van der Kwast, Th.W., and Benner, R. Allo-I-J-antigens in suppression of DTH to H-2 subregion antigens. Immunol. Today. 1982;3:123.
25. Koch, G., Lok, B.D., van Oudenaren, A., and Benner, R. The capacity and mechanism of bone marrow antibody formation by thymus-independent antigens. J. Immunol. 1982;128:1497.
26. Miller, J.F.A.P. Studies on mouse leukaemia. The role of the thymus in leukaemogenesis by cell-free leukaemic filtrates. Brit. J. Cancer 1960;14:93.
27. Jooste, S.V., Lance, E.M., Levey, R.H., Medawar, P.B., Ruszkiewicz, M., Sharman, R., and Taub, R.M. Notes on the preparation and assay of anti-lymphocyte serum for use in mice. Immunology 1968;15:697.
28. Van der Kwast, Th.H., and Benner, R. T1 and T2 lymphocytes in primary and secondary delayed type hypersensitivity of mice. I. Contribution in the response to sheep red blood cells and to allogeneic spleen cells. Cell.Immunol. 1978;39:194.
29. North, R.J., Mackaness, G.B., and Elliot, R.W. The histogenesis of immunologically committed lymphocytes. Cell. Immunol. 1971; 3:680.
30. Van der Kwast, Th.H., Olthof, J.G., and Benner, R. Primary and secondary delayed type hypersensitivity to minor histocompatibility antigens in the mouse. Cell. Immunol. 1979;43:94.
31. Sy, M.-S., Miller, S.D., Moorhead, J.W., and Claman, H.N. Active suppression of 1 fluoro 2,4-dinitrobenzene immune T cells. Requirement of an auxillary T cell induced by antigen. J. Exp. Med. 1979;149:1197.
32. Dietz, M.H., Sy, M.-S., Benacerraf, B., Nisonoff, A., Greene, M.I., and Germain, R.N. Antigen- and receptor-driven regulatory mechanisms. VII. H-2 restricted anti-idiotypic suppressor factor from efferent suppressor T cells. J. Exp. Med. 1981;153:450.

33. Ramshaw, I.A., Bretscher, P.A., and Parish, C.R. Regulation of the immune response. I. Suppression of delayed-type hypersensitivity by T cells from mice expressing humoral immunity. Eur. J. Immunol. 1976;6:674.
34. Kaufmann, S.H.E., Ahmed, J.S., and Hahn, H. Transferable suppression and intrinsic unresponsiveness in delayed type hypersensitivity in sheep red blood cells of mice: two distinct mechanisms? Immunobiol. 1980;157:331.
35. Liew, F.Y. Regulation of delayed type hypersensitivity. VI. Antigen-specific suppressor T cells and suppressor factor for delayed type hypersensitivity to histocompatibility antigens. Transplantation 1982;33:69.
36. Claman, H.N. Hypothesis:T-cell tolerance - One signal? Cell. Immunol. 1978;48:201.
37. Ptak, W., Rozycka, D., Askenase, P.W., and Gershon, R.K. Role of antigen-presenting cells in the development and persistence of contact sensitivity. J. Exp. Med. 1980;151:362.
38. Lagrange, P.H., and Mackaness, G.B. Site of action of serum factors that block delayed-type hypersensitivity in mice. J. Exp. Med. 1978;148:235.
39. Sy, M.-S., Miller, S.D., Kowach, H.B., and Claman, H.N. A splenic requirement for the generation of suppressor T cells. J. Immunol. 1977;119:2095.
40. Asherson, G.L., Zembala, M., Mayhew, B., and Goldstein, A. Adult thymectomy prevention of the appearance of suppressor T cells which depress contact sensitivity to picrylchloride and reversal of adult thymectomy effect by thymus extract. Eur. J. Immunol. 1976;6:699.
41. Cantor, H., and Boyse, E.A. Functional subclasses of T lymphocytes bearing different Ly antigens. I. The generation of functionally distinct T cell subclasses is differentiative process independent of antigen. J. Exp. Med. 1975;141:1376.
42. Feldmann, M., Beverley, P.C.L., Woody, J., and McKenzie, I.F.C. T-T interactions in the induction of suppressor and helper T cells: Analysis of membrane phenotype of precursor and amplifier cells. J. Exp. Med. 1977;145:793.
43. Cantor, H., and Asofsky, R. Synergy among lymphoid cells mediating the graft-versus-host response. II. Synergy in graft-versus-host reactions produced by BALB/c lymphoid cells of differing anatomic origin. J. Exp. Med. 1970;131:235.
44. Scollay, R. Adult thymectomy does not alter the proportion of T cells of the Lyt123 subclass. Nature 1982;300:529.
45. Thompson, C.H., Potter, T.A., McKenzie, I.F.C., and Parish, C.R. The surface phenotype of a suppressor cell of delayed-type hypersensitivity in the mouse. Immunology 1980;40:87.
46. Cantor, H., and Gershon, R.K. Immunological circuits: cellular compositon. Fed. Proc. 1979;38:2058.
47. McKenzie, I.F.C., and Potter, T.A. Murine lymphocyte surface antigens. Adv. Immunol. 1979;27:179.

CHARACTERIZATION OF SUPPRESSOR T CELLS IN GRAFT-VERSUS-HOST REACTIONS

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SUMMARY

Intravenous (iv) immunization of mice with irradiated (2000 rads) allogeneic lymphoid cells induces the generation of suppressor T cells. Such suppressor T cells can suppress the anti-host delayed type hypersensitivity by other T cells during acute Graft-versus-Host reactions in irradiated recipient mice. Suppression of anti-host immune reactivity is detectable at least 50 days after iv induction of the state of suppression in the donors. The generation of suppressor T cells in the donors is associated with proliferation. Furthermore, the suppressor T cells need to proliferate further in the irradiated allogeneic hosts in order to display a maximal suppressive effect. The suppressor T cells were found to bear the Lyt-$1^{+}2^{+}$ phenotype.

INTRODUCTION

In a previous paper we have shown that delayed type hypersensitivity (DTH) reactions to H-2 and non-H-2 alloantigens, which normally arise after subcutaneous (sc) immunization of mice with allogeneic spleen cells, can be suppressed by intravenous (iv) preimmunization with similar irradiated allogeneic spleen cells (1). The extent of suppression depends on the dose of allogeneic spleen cells and the route of administration and is mediated by T cells (1,2).

Not only in DTH reactions iv induced suppression can be demonstrated. It has been shown that iv inoculation of TNP-modified or unmodified X-irradiated X5563 tumor cells does not prime mice for anti-tumor cytotoxic responses, but instead abolishes the ability of these mice to generate anti-tumor cytotoxic effector cells (3). Furthermore, suppressive activity has been demonstrated in animals during Graft-versus-Host (GvH) reactions (4-6) and in stable radiation chimeras (7,8). In the latter studies it was demonstrated that chimeras possess T lymphocytes in the spleen that specifically suppress donor anti-host mixed lymphocyte reactions and that suppression of GvH can be adoptively transferred to secondary hosts.

In the past, we have developed a DTH assay which is appropriate to measure the development of anti-host effector T cells during GvH (9). This assay is based upon secondary transfer of the recipients' spleen and lymph node cells into naive mice, challenge

of these mice with lymphoid cells syngeneic with the irradiated recipient, and measurement of the subsequent DTH response.
We have recently shown, using the protocol for induction of suppressor T cells originally devised by our laboratory (1,2), that iv preimmunization of donor mice with irradiated allogeneic lymphoid cells can also suppress anti-host DTH responses during acute GvH reactions (10). The suppressor T cells involved in this suppression are antigen-specific with regard to their antigen-recognition, but non-specific with regard to their suppressive effect (11,12). Thus, suppressor T cells induced by and specific for Class I alloantigens could suppress the anti-host DTH response to third party Class II alloantigens as well, provided the latter were presented in combination with the specific Class I alloantigens that originally had induced the suppressor T cells (11,12). In anti-allograft DTH this so-called 'bystander-suppression' could be demonstrated in all possible combinations of alloantigens (11,13).

So far there is a lack of data concerning the kinetics of this suppressive effect and the nature of the suppressor T cells involved. In the present study we investigaed these aspects. The data show that the suppression is a long-lasting phenomenon that is dependent on Lyt-1^+2^+ T cells that need to proliferate in order to display a maximal suppressive effect.

MATERIALS AND METHODS

Animals. (C57BL/Rij x CBA/Rij)F1 ($H\text{-}2^{b/q}$) female mice, 10 to 20-week-old, were bred at the Laboratory Animals Centre of the

Erasmus University, Rotterdam, The Netherlands. DBA/2 ($H\text{-}2^d$) female mice, 8 to 20 week old, were purchased from the Radiobiological Institute TNO, Rijswijk, The Netherlands, from OLAC Ltd., Bicester, United Kingdom and from Bomholtgard, Ry, Denmark. A.TL ($H\text{-}2^{t1}$) and A.TH ($H\text{-}2^{t2}$) female mice, 10 to 16 weeks old, were purchased from OLAC Ltd.

Preparation of cell suspensions. Mice were killed with carbon dioxide. Immediately after killing, the organs to be used (spleen and inguinal, axillary and mesenteric 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 750 rads (7.5 Gy) of whole body X-irradiation. Irradiation was performed in a Philips Müller MG 300 X-ray machine. The physical constants of the irradiation have been described elsewhere (9). Radiation control mice died within 21 days after irradiation. Spleen cell suspensions were irradiated with 2000 rads (20 Gy) in the same machine.

GvH-reactions. Acute GvH reactions were elicited by iv injection of either 1×10^7 or 2×10^7 nucleated spleen cells into lethally irradiated allogeneic recipients within four hours after irradiation.

Suppression. Suppressor T cells were induced by one iv injection

of 5 x 10^7 irradiated (2000 rads) allogeneic spleen cells 4 days before the use of these mice as donors of spleen cells. Non-suppressed control mice had been injected with the same number of irradiated syngeneic cells. In one experiment, induction of suppressor T cells was done at various intervals before use of the mice as donors of spleen cells to evoke GvH reactions.

Selective elimination of T cells. Monoclonal IgM anti-Thy-1.2 antibodies (clone F7D5) was purchased from OLAC Ltd., Bicester, United Kingdom. Cell suspensions were treated with anti-Thy-1.2 for 30 min at 4°C. After incubation, the cells were centrifuged, resuspended in BSS and incubated with guinea pig complement (Behringwerke AG, Marburg, FRG) for 15 min at 37°C. Thereafter the cells were washed three times and resuspended in BSS.

Anti-Lyt treatment. For elimination of Lyt-1 and Lyt-2 positive cells in vivo, monoclonal IgG2a anti-Lyt-1 and anti-Lyt-2 were used of rat origin. The hybridoma cells producing the anti-Lyt-1 (subclone 53-7-313) and the anti-Lyt-2 (subclone 53-6.72) antibodies were kindly provided by Drs. J. Ledbetter and L.A. Herzenberg (14). These antibodies were harvested from hybridoma supernatants. Suppressed donor mice were injected with 1 ml anti-Lyt-1 or 0.5 ml anti-Lyt-2 antibodies iv for 4 days, starting on the day of the iv injection of the irradiated allogeneic spleen cells.

Mitomycin C treatment. Treatment of spleen cells with mitomycin C (Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan) was done according to the

method of Blomgren and Svedmyr (15). A number of 2 to 5 x 10^8 spleen cells was incubated in 20 ml BSS containing 25 µg mitomycin C per ml during 30 min at 37°C. Thereafter, the cells were washed twice and resuspended in BSS.

Drug treatment. Vinblastin was purchased from Eli Lilly & Co., Indianapolis, Indiana, USA. Experimental mice received two ip injections of vinblastin (100 µg/recipient). The dosis used was previously found to be sufficient to abolish passive transfer of DTH reactivity (16). Control mice received similar injections of BSS only. Hydroxyurea (HU) was purchased from Calbiochem-Behring Corp., La Jolla, California, USA. Experimental mice received three ip injections of HU (1 gr/kg BW) 6 h apart. See Hodgson et al. for details (17). Control mice received similar injections of BSS only.

Assay for DTH. At various intervals after iv injection of spleen cells into lethally irradiated allogeneic mice, the recipient spleens and mesenteric and peripheral lymph nodes were removed, pooled and prepared for single cell suspensions. These cells were transferred into naive secondary recipients syngeneic to the donors of the spleen cells that induced the GvH reaction. Thirty minutes before transfer, the secondary recipient mice were ip injected with 15 U heparin (Liquémine, Hoffmann-La Roche & Co., Basel, Switzerland) to prevent embolism. The secondary recipients were challenged into the dorsum of the right hind foot with 2 x 10^7 spleen cells syngeneic with the irradiated recipients. The DTH response to this challenge was

measured as the difference in thickness of the hind feet 24, 48 and 72 h later.

In two experiments foot thickness was also measured at other time points. The specific increase in foot thickness was calculated as the relative increase in foot thickness of the secondary recipients minus the relative increase in foot thickness of control mice which had only received the challenge. The swelling of the control mice ranged from 15 to 25%.

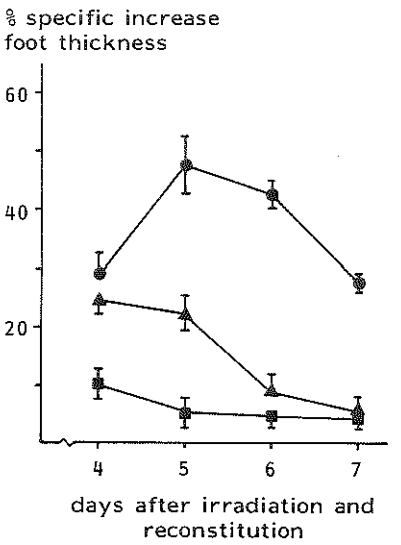

Fig. 1. Development of anti-host DTH reactivity in lethally irradiated (C57BL x CBA)F1 mice inoculated with either 1 x 10^7 suppressed (■), 1 x 10^7 non-suppressed (●) or 1 x 10^7 suppressed plus 1 x 10^7 non-suppressed (▲) DBA/2 spleen cells. Suppression of the DBA/2 donors was induced by iv preimmunization with 5 x 10^7 irradiated (C57BL x CBA)F1 spleen cells. DTH responses were measured 24 h after challenge. Each experimental point represents the arithmetic mean ± 1 SEM of at least 6 mice.

RESULTS

Suppression of anti-host immune reactivity in GvH by T cells

In previous papers we have shown that anti-host DTH reactivity in GvH can be suppressed by iv preimmunization of the donors with the relevant alloantigens (10-12). In the present experiments we injected the lymphoid cell donors iv with 5×10^7 irradiated syngeneic or allogeneic spleen cells. Donors pretreated in this way are referred to as 'non-suppressed' and 'suppressed', respectively. Four days after induction of suppression, 1×10^7 spleen cells from non-suppressed or suppressed mice were used to reconstitute lethally irradiated allogeneic recipients. At various days after reconstitution,

the anti-host DTH reactivity was determined in a passive transfer system as described in the Materials and Methods section. Fig. 1 shows that spleen cells from non-suppressed donors cause a sizable anti-host DTH response. Spleen cells from suppressed donors, on the other hand, display a reduced anti-host immune reactivity. The response by the non-suppressed spleen cells could be reduced by simultaneously transferred suppressed spleen cells. This effect was marginal when measured on day 4 after irradiation and reconstitution, but highly significant on day 5 and later (Fig. 1). This shows that the suppression is a dominant phenomenon. Suppression of anti-host DTH in case of simultaneously transferred non-suppressed and suppressed spleen cells was not only found in donor-recipient

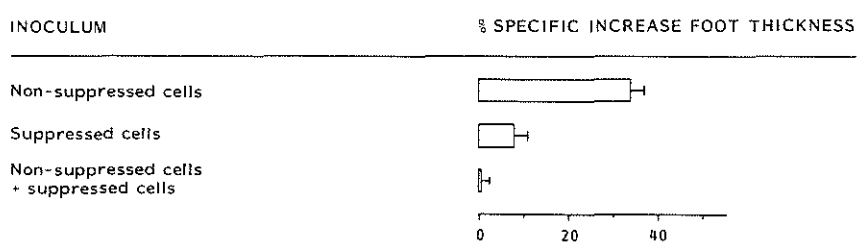

Fig. 2. Anti-host DTH reactivity in lethally irradiated A.TH mice inoculated with either 2 x 10^7 suppressed, 2 x 10^7 non-suppressed or 2 x 10^7 suppressed plus 2 x 10^7 non-suppressed A.TL spleen cells. Suppression of the A.TL donors was induced by iv preimmunization with 5 x 10^7 irradiated A.TH spleen cells. Anti-host DTH reactivity was determined 6 days after irradiation and reconstitution. For other details see legend to Fig. 1.

combinations differing for H-2 as well as non-H-2 alloantigens, but also in combinations differing for H-2I only (Fig. 2).

We used the same experimental set up to investigate whether the observed suppression is mediated by T cells. Therefore, the suppressed spleen cells were treated with monoclonal anti-Thy-1.2 antibodies and complement in vitro before combining these cells with non-suppressed cells and transfer into irradiated allogeneic recipients. It was found that this treatment of the suppressed cells abolished the state of suppression (Fig. 3).

Time course of the immunosuppression induced by iv preimmunization

The duration of the suppressive effect induced by iv injection was investigated by transfer of DBA/2 spleen cells into lethally irradiated (C57BL x CBA)F1 mice either 3, 4, 5, 7, 10 or 50 days after induction of suppression. As can be seen from Fig. 4, the induced state of suppression can be demonstrated still at 50 days after the iv preimmunization. However, the period that suppressed spleen

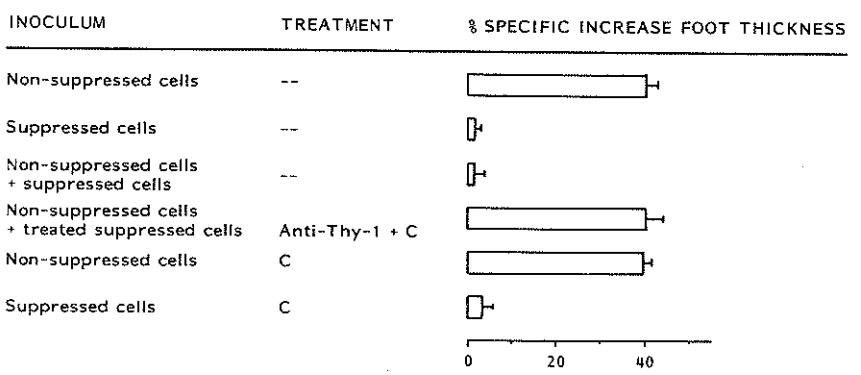

Fig. 3. Anti-host DTH reactivity in lethally irradiated (C57BL x CBA)F1 mice inoculated with either 1 x 10^7 non-suppressed, 1 x 10^7 suppressed, 1 x 10^7 non-suppressed plus 1 x 10^7 suppressed or 1 x 10^7 non-suppressed plus 1 x 10^7 anti-Thy-1.2 and complement treated suppressed DBA/2 spleen cells. Recipients were tested for anti-host DTH reactivity 6 days after irradiation and reconstitution. For other details see legend to Fig. 1.

cells can suppress the response by simultaneously transferred non-suppressed spleen cells is much shorter. Already 10 days after the iv preimmunization the suppressor T cells can no longer suppress the reactivity mediated by the non-suppressed cells.

Role of proliferation in the development of suppressor T cell activity

The role of proliferation in the development of suppressor T cell activity was studied by treatment of the iv preimmunized mice with the antimitotic drugs vinblastin and hydroxyurea (HU). Suppressed mice were injected with 100 µg vinblastin ip or 1 mg/kg HU ip either 3 or 30 days after induction of suppression. At 15 h after injection of vinblastin or the last injection of HU, 1 x 10^7 spleen cells were used to reconstitute irradiated allogeneic recipients. Fig. 5 shows that treatment of iv preimmunized donor mice with these antimitotic drugs prevented the suppression at both inter-

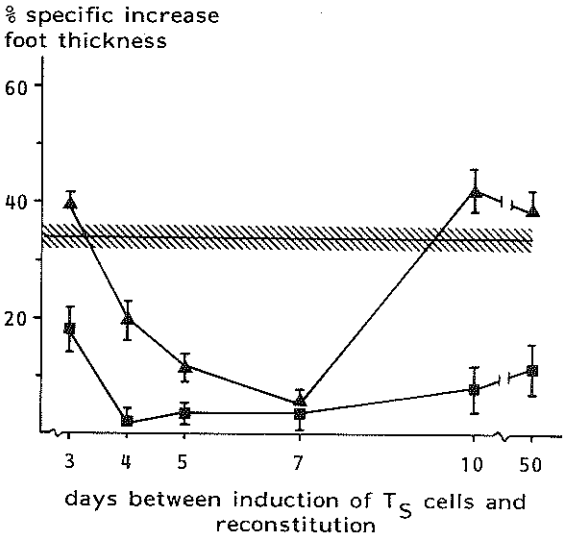

Fig. 4. Time course of the suppression induced by iv pre-immunization. Irradiated allogeneic (C57BL x CBA)F1 mice were inoculated with 1 x 10^7 suppressed (■), 1 x 10^7 non-suppressed plus 1 x 10^7 suppressed (▲) or 1 x 10^7 non-suppressed (hatched area) DBA/2 spleen cells 3, 4, 5, 7, 10 and 50 days after iv pre-immunization. Anti-host DTH reactivity was determined 6 days after irradiation and reconstitution. For other details see legend to Fig. 1.

vals tested. So the induction of suppressor T cells by iv preimmunization and their maintenance are associated with proliferation. Furthermore, the observation that pretreatment of non-suppressed mice with vinblastin and HU does not affect the anti-host DTH response confirms our previous conclusion (18) that the T cells mediating the anti-host DTH mainly belong to the pool of long-lived non-proliferating T cells.

Whether or not suppressor T cells need to proliferate after their transfer into the irradiated allogeneic recipients was studied by pretreatment of spleen cells from suppressed donors with 25 µg/ml mitomycin C in vitro before their transfer into the irradiated recipients. This dose of mitomycin C blocks DNA

synthesis and thereby prevents proliferation. Incubation of the suppressed spleen cells with 25 µg/ml mitomycin C before combining these cells with non-suppressed cells abolished the suppression (Fig. 6), indicating that the suppressor T cells need to proliferate further in the irradiated recipients in order to display a maximal suppressive effect.

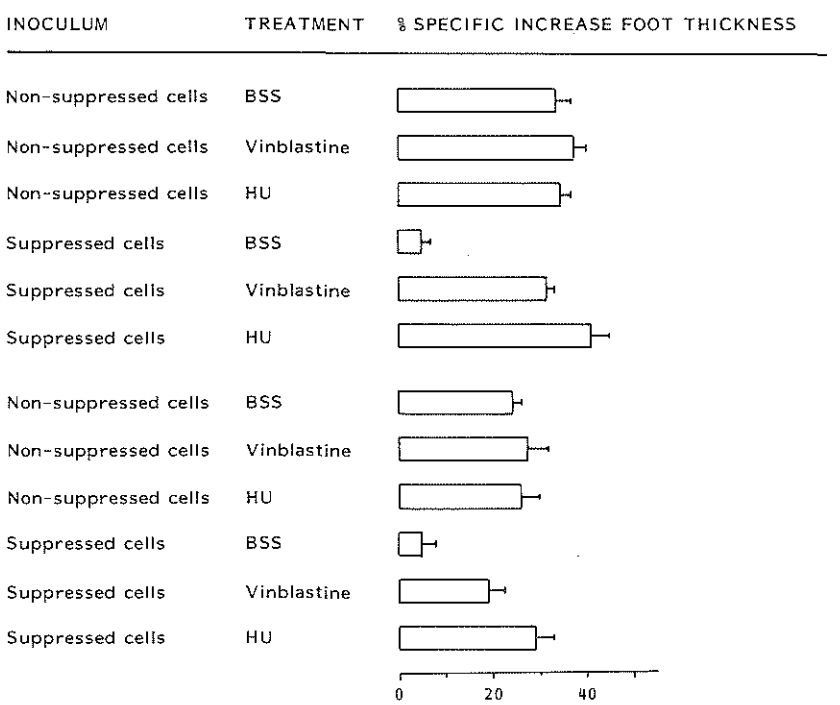

Fig. 5. Anti-host DTH reactivity in lethally irradiated (C57BL x CBA)F1 mice inoculated with 1 x 10^7 spleen cells from BSS, vinblastin or hydroxyurea treated non-suppressed or similarly treated suppressed DBA/2 mice. Drug treatment was performed either 3 (upper part) or 30 days (lower part) after induction of suppression, i.e. 15 h before use of these donor mice to reconstitute the irradiated allogeneic recipients. For the drug doses used, see section 'Materials and Methods'. For other details see legend to Fig. 1.

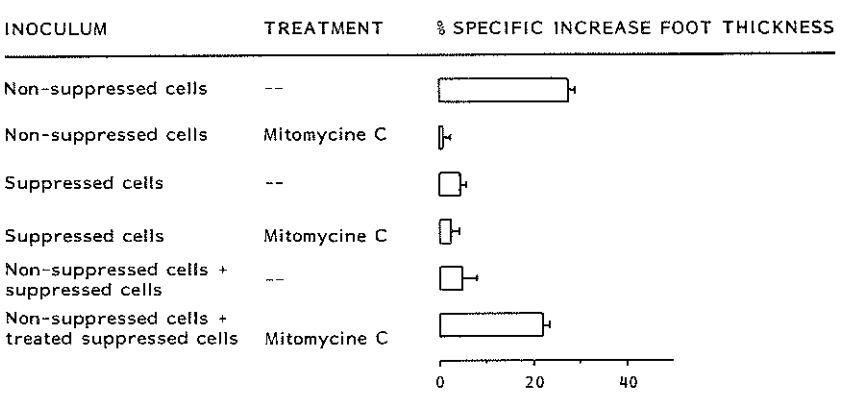

Fig. 6. Anti-host DTH reactivity in lethally irradiated (C57BL x CBA)F1 mice inoculated with either 1 x 10^7 non-suppressed, 1 x 10^7 suppressed, 1 x 10^7 non-suppressed plus 1 x 10^7 suppressed, 1 x 10^7 non-suppressed plus 1 x 10^7 mitomycin C-treated suppressed, or 1 x 10^7 mitomycin C treated non-suppressed DBA/2 spleen cells. Recipients were tested for anti-host DTH reactivity 6 days after irradiation and reconstitution. DTH responses were measured 40 h after challenge. For other details see legend to Fig. 1.

Lyt surface markers of suppressor T cells

To investigate the Lyt phenotype of the suppressor T cells involved in suppression of anti-host DTH during acute GvH reactions we injected suppressed donors with anti-Lyt-1 or anti-Lyt-2 antibodies during the first four days after the iv preimmunization. This was done in the DBA/2 - (C57BL x CBA)F1 and the A.TL - A.TH combination. Fig. 7 shows that in both combinations tested the suppression was abolished by both anti-Lyt-1 and anti-Lyt-2 antibodies. Experiments in which the suppressed spleen cells were treated with anti-Lyt-1 or anti-Lyt-2 antibodies and complement *in vitro* before transfer into the irradiated allogeneic recipients displayed the same results (data not shown).

When irradiated mice, reconstituted with allogeneic spleen cells from non-suppressed donors, were daily injected iv with mono-

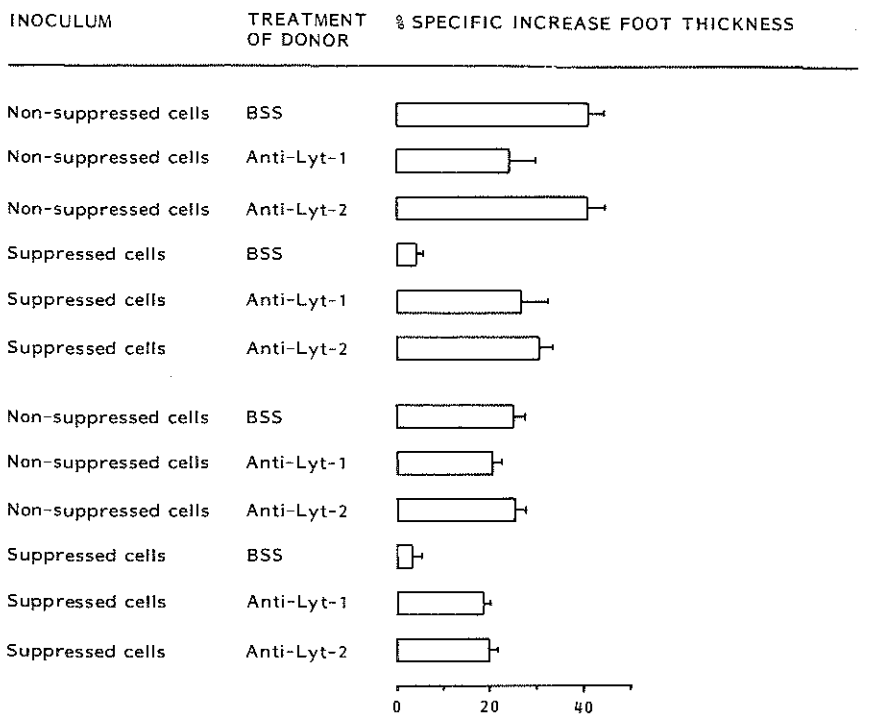

Fig. 7. Effect of anti-Lyt-1 and anti-Lyt-2 treatment in vivo upon the generation of suppressor T cells subsequent to iv preimmunization. Suppressed and non-suppressed DBA/2 and A.TL donor mice were daily iv injected with either 1 ml anti-Lyt-1, 0.5 ml anti-Lyt-2 or 1 ml BSS. This was done for a period of 4 days starting on the day of the suppressive injection of irradiated (C57BL x CBA)F1 and A.TH spleen cells, respectively and ending on the day before spleen cell transplantation. Suppressed and non-suppressed DBA/2 spleen cells were transferred into irradiated (C57BL x CBA)F1 mice (upper part), while suppressed and non-suppressed A.TL spleen cells were transferred into irradiated A.TH mice (lower part). Anti-host DTH reactivity was determined 6 days after irradiation and reconstitution. For other details see legend to Fig. 1.

clonal anti-Lyt-1 or anti-Lyt-2 antibodies, a curtailment of the DTH reaction was found after anti-Lyt-1 treatment, most likely due to inactivation of the DTH reactive T cells. Anti-Lyt-2 treatment on the other hand, prolonged the DTH reaction. In the latter case, foot swelling still occurred 144 h after challenge (Fig. 8).

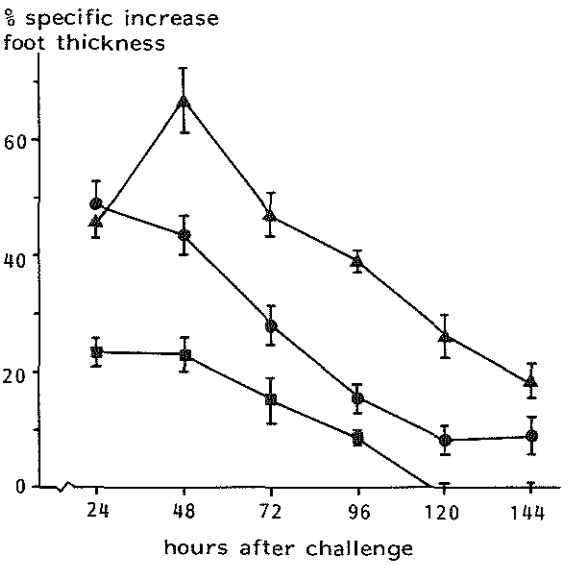

Fig. 8. Influence of treatment with anti-Lyt-1 and anti-Lyt-2 antibodies upon the anti-host DTH response of mice subjected to GvH. GvH was induced in lethally irradiated (C57BL x CBA)F1 mice by inoculation of 1 x 10^7 spleen cells from naive DBA/2 mice. Irradiated reconstituted mice were treated with either 1 ml anti-Lyt-1 (■), 0.5 ml anti-Lyt-2 (▲) or 1 ml BSS (●) iv during the first 5 days after reconstitution. On the 6th day the mice were tested for anti-host DTH reactivity. DTH responses were measured 24, 48, 72, 96, 120 and 144 h after challenge of the secondary recipients. For other details see legend to Fig. 1.

DISCUSSION

Intravenous administration of irradiated allogeneic lymphoid cells to mice results in the generation of suppressor T cells which can suppress the anti-host DTH response in GvH. This has been demonstrated in a variety of donor-recipient combinations (10,12). The suppressor T cells are antigen-specific with regard to their activation, but non-specific with regard to their suppressive effect (11,12).

The present study adds to this data a.o. that these T cells require proliferation in order to display maximal suppressive activity. This proliferation occurs during the activation of the suppressor T cells subsequent to the iv injection of irradiated allogeneic spleen cells (Fig. 5) as well as during the induction of GvH (Fig. 6). Blomgren et al. (19), studying cellular cooperation during GvH have also provided evidence that cells mediating suppressive effects require proliferation in order to function optimally. These authors found that mixtures of thymocytes and lymph node cells yield synergistic or antagonistic Graft-versus-Host reactions when inoculated into newborn F1 hybrids, depending on the cell dosages of thymocytes used. High ratios of the number of thymocytes and lymph node cells favoured synergism, whereas low ratios favoured antagonism. Mitomycin C treatment of the thymocytes in vitro before transfer into allogeneic recipients abolished the suppressor activity indicating that the cells mediating the suppressive effect require proliferation (19).

The mode of action of the suppressor T cells might well be that they inhibit DNA synthesis and thereby proliferation of the alloreactive DTH effector T cells. Several investigators have shown that for maximal GvH reactions proliferation is needed. Meuwissen and Good (20) have demonstrated that donor spleen cells, incubated with mitomycin C did not exhibit GvH reactivity as measured in the splenomegaly assay. Furthermore, Wolters and Benner (21) demonstrated that full development of specific anti-host DTH responsiveness during the acute GvH reaction is dependent on proliferation of the reactive

T cells. In contact sensitivity, Asherson et al. (22) have shown that immune cells can specifically depress the DNA synthetic response to antigen. They immunized mice with the contact sensitizing agent picryl chloride and transferred these spleen cells 3-18 days later into naive syngeneic recipients. These recipients were immunized with picryl chloride, whereafter the DNA synthesis was assessed in the regional lymph nodes. Injection of 3×10^7 immune cells depressed the DNA synthesis at 4 days after immunization with a mean depression of about 60 per cent. Furthermore, our own group has shown that the suppressor T cells activated by iv immunization with irradiated allogeneic spleen cells suppress the proliferation in the regional lymph nodes after sc immunization with allogeneic lymphoid cells (1).

Activated suppressor T cells generated by our protocol can also suppress already activated DTH effector T cells when tested in the immune lymphocyte transfer assay (Bianchi et al., to be published). Also for in vitro antibody formation it has been shown that suppressor T cells might affect not only the proliferation of helper T cells, but also their functional activity (23).

In a previous study on anti-graft DTH we have shown that suppression persists for at least 40 days after the iv suppressive immunization (1). The present study on GvH corroborates this finding and, furthermore, shows that this suppression is based upon suppressor T cells with a long-lasting proliferative activity (Fig. 5).

Liew and Howard have demonstrated profound antigen-specific

suppression of DTH to sheep red blood cells (SRBC) in primed mice for at least one year. This long-term impairment of DTH is maintained by memory suppressor T cells with low proliferative activity (24). This also holds for DTH to histocompatibility antigens (25). In the latter study it was shown that suppression of DTH to alloantigens induced by a single iv injection of a high dose of irradiated allogeneic cells lasts for at least 200 days (25).

It is remarkable that the ability of suppressed spleen cells to inhibit the response by nonsuppressed cells can be demonstrated only during a relatively short period, namely from 4 to 10 days after the suppressive immunization (Fig. 4). Other authors have encountered similar phenomena (6,24). It might be that the state of suppression actually consists of two phases. The first phase of 4 to 10 days might be due to suppressor T cells that can suppress the response by the alloreactive T cells mediating DTH. The second phase might be due to clonal inactivation or clonal deletion of those alloreactive T cells that are specific for the alloantigens used for iv immunization.

Evidence for such an underlying mechanism of the long-lasting suppression has been presented by others, but has been obtained under different conditions (23,26).

Alternatively different types of suppressor T cells or suppressor T cells in a different state of activation or different numbers of suppressor T cells might be required for the reduced GvH-related DTH response after transfer of suppressed spleen cells only and after simultaneous transfer of suppressed and non-suppressed

spleen cells. Studies aimed to discriminate between these possibilities are in progress.

Our finding that the cells accounting for the suppressive effect are sensitive to anti-Lyt-1 as well as anti-Lyt-2 treatment suggests that either the suppressor T cells involved bear the Lyt-1^+2^+ phenotype, or that different suppressor T cell subpopulations with a different Lyt-phenotype are involved. Both possibilities might well be correct. Germain and Benacerraf (27) have integrated the proposals of several authors about the pathway of the suppressor T cell cascade (27). The cascade they advocate is initiated by a Lyt-1^+ or Lyt-123^+, cyclophosphamide sensitive antigen-specific T cell (Ts1) which produces an idiotypic suppressor factor (TsF1) that acts across H-2 and V_H differences. This TsF1 induces a second population of suppressor cells (Ts2). This cell population bears Lyt-1 antigens (28,29) and may also produce a factor (TsF2) which is restricted by H-2 and V_H linked genes. The target of this factor is an antigen-primed Lyt-2^+ cell population (Ts3). The Ts3 population or a factor (TsF3) produced by this population may interact directly or indirectly with the T cells that mediate e.g. contact sensitivity or helper activity (29,30).

The prolonged kinetics of the DTH response in the secondary recipients after <u>in vivo</u> administration of anti-Lyt-2 antibodies to the mice subjected to GvH, indicates that GvH-related DTH reactivity is the result of a delicate balance between DTH reactive T cells and suppressor T cells. Similar data have been provided by Cantor et al. (31) for the anti-SRBC antibody response. These authors

showed that the net helper activity after immunization with SRBC is determined by the relative proportions of SRBC-specific helper T cell activity and suppressor T cell activity. Removal of Lyt-2^+ T cells from the spleen of SRBC primed mice augmented the anti-SRBC response in vitro.

ACKNOWLEDGMENTS

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REFERENCES

1. Van der Kwast, Th.H., Bianchi, A.T.J., Bril, H., and Benner, R., Transplantation 31, 79, 1981.
2. Bianchi, A.T.J., Hussaarts-Odijk, L.M., and Benner, R., Transplant.Proc. 15, 7, 1983.
3. Fujiwara, H., and Shearer, G.M., Cell. Immunol. 59, 181, 1981.
4. Hardin, J.A., Chused, T.M., and Steinberg, A.D., J.Immunol. 111, 651, 1973.
5. Rouse, B.T., and Warner, N.L., J.Immunol. 113, 904, 1974.
6. Shand, F.L., Immunology 29, 953, 1975.
7. Tutschka, P.J., Hess, A.D, Beschorner, W.E., Santos, G.W., Transplantation 32, 203, 1981.
8. Tutschka, P.J., Ki, P.F., Beschorner, W.E., Hess, A.D., and Santos, G.W., Transplantation 32, 321, 1981.
9. Wolters, E.A.J., and Benner, R., Transplantation 26, 40, 1978.
10. Bril, H., and Benner, R., Adv. Exp. Med. Biol. 149, 577, 1982.
11. Bianchi, A.T.J., Bril, H., and Benner, R., Nature 301, 614, 1983.
12. Bril, H., Molendijk-Lok, B.D., and Benner, R., Transplantation 36, 323, 1983.
13. Bianchi, A.T.J., Hussaarts-Odijk, L.M., and Benner, R., Cell. Immunol., in press.
14. Ledbetter, J.A., and Herzenberg, L.A., Immunol. Rev. 47, 63, 1979.
15. Blomgren, H., and Svedmyr, E., Cell.Immunol. 2, 285, 1971.
16. Van der Kwast, Th.H., Olthof, J.G., de Ruiter, H., and Benner, R., Cell.Immunol., 43, 94, 1979.

17. Hodgson, G.S., Bradley, T.R., Martin, R.F., Sumner, M., and Fry, P., Cell Tissue Kinetics 8, 51, 1975.
18. Wolters, E.A.J., and Benner, R., Cell.Immunol. 59, 115, 1981.
19. Blomgren, H., and Jacobsson, H., Cell.Immunol. 12, 296, 1974.
20. Meuwissen, H.J., and Good, R.A., Nature 215, 634, 1967.
21. Wolters, E.A.J., and Benner, R., Transplantation 27, 39, 1979.
22. Asherson, G.L., Wood, P.J., and Mayhew, B., Immunology 29, 1057, 1975.
23. Green, D.R., Gershon, R.K., and Eardley, D.D., Proc.Natl.Acad.Sci. 78, 3819, 1981.
24. Liew, F.Y., and Howard, J.G., Eur. J. Immunol. 10, 937, 1980.
25. Liew, F.Y., Transplantation 33, 69, 1982.
26. Sy, M.-S., Miller, S.D., Kowach, H.B., and Claman, H.N., J.Immunol. 119, 2095, 1977.
27. Germain, R.N., and Benacerraf, B., Scand.J.Immunol., 13, 1, 1981.
28. Weinberger, J.Z., Germain, R.N., Benacerraf, B., and Dorf, M.E., J.Exp.Med. 152, 161, 1980.
29. Sunday, M.E., Benacerraf, B., and Dorf, M.E., J.Exp.Med. 153, 1445, 1981.
30. Minami, M., Okuda, K., Furusawa, S., Benacerraf, B., and Dorf, M.E., J.Exp.Med. 154, 1390, 1981.
31. Cantor, H., Shen, F.W., and Boyse, E.A., J.Exp.Med. 143, 1391, 1976.

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 Mls 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

Table 1 Specificity of antigen recognition by suppressor T cells

System	Expt	Responding strain	Preimmunization i.v.	Immunization s.c.	% Specific increase foot thickness
HvG	A	BALB/c (H-2^d)	BALB.B (H-2^b)	BALB.B (H-2^b)	
		BALB/c	BALB/c	BALB.B (H-2^b)	
		BALB/c	BALB.K (H-2^k)	BALB.B (H-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)	

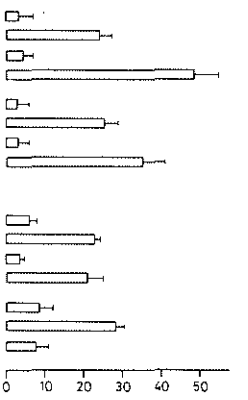
System	Expt	Responding cells	Preimmunization i.v.	Irradiated host	
GvH	E	BALB/c (H-2^d)	BALB.B (H-2^b)	BALB.B (H-2^b)	
		BALB/c	BALB/c	BALB.B (H-2^b)	
		BALB/c	BALB.K (H-2^k)	BALB.B (H-2^b)	

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 haplotypes (group A), the H-2 subregion differences (groups 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 dddd d*, BALB.K *k kkkk*, B10.BR *k kkkk*, B10.A *k kkkd*, B10.AKM *k kkkq*, B10.A(2R) *q kkkb*, B10.AQR *q kkkd*, B10.T(6R) *q qqqd*.

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

System	Expt	Responding strain	Preimmunization i.v.	Immunization s.c.	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) (K I)	B10.T(6R) (K I)	
		B10.A	B10.A	B10.T(6R) (K I)	B10.T(6R) (K I)	
		B10.AQR	B10.A (K)	B10.A x.T(6R) F_1 (K I)	B10.T(6R) (I)	
		B10.AQR	B10.AQR	B10.A x.T(6R) F_1 (K I)	B10.T(6R) (I)	
System	Expt	Responding cells	Preimmunization i.v.	Irradiated 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 (K I)	A.TH (K I)	A.TH (K I)	
		A.AL	A.AL	A.TH (K I)	A.TH (K I)	
		A.AL	A.TL (K)	A.TH (K I)	A.TH (K I)	



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×.KF1 means F1 hybrid of BALB/c×BALB.K, B10.A×T(6R) means F1 hybrid of B10.A×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.

response to a challenge by 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.

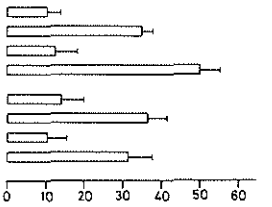
Nonspecific suppressive effects have been demonstrated previously *in vivo*[7,8] and *in vitro*[9-13]. Contact sensitizing agents[14,15] as well as hapten-derivatized syngeneic[16,17] and allogeneic[18] cells readily induce suppressor T cell activity. The generation of this activity is dependent on complex 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 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

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

System	Expt	Responding strain	Preimmunization i.v.	Immunization s.c.	Challenge	% Specific increase foot thickness
HvG	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.

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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1. Van der Kwast, Th. H., Bianchi, A. T. J., Bril, H. & Benner, R. *Transplantation* **31**, 79–85 (1981).
2. Bril, H. & Benner, R. *Adv. exp. Med. Biol.* **149**, 577–583 (1982).
3. Bianchi, A. T. J., Hussaarts-Odijk, L. M. & Benner, R. *Transplantn Proc.* (in the press).
4. Wolters, E. A. J. & Benner, R. *Nature* **279**, 642–643 (1979).
5. Wolters, E. A. J. & Benner, R. *Transplantation* **26**, 40–45 (1978).
6. Wolters, E. A. J. & Benner, R. *Transplantation* **27**, 39–42 (1979).
7. Liew, F. Y. *Eur. J. Immun.* **11**, 883–888 (1981).
8. Zembala, M., Asherson, G. L. & Colizzi, V. *Nature* **297**, 411–413 (1982).
9. Rich, S. S. & Rich, R. R. *J. exp. Med.* **140**, 1588–1602 (1974).
10. Rich, S. S. & Rich, R. R. *J. exp. Med.* **142**, 1391–1402 (1975).
11. Fresno, M., Nabel, G., McVay-Boudreau, L., Furthmayer, H. & Cantor, H. *J. exp. Med.* **153**, 1246–1259 (1981).
12. Fresno, M., McVay-Boudreau, L., Nabel, G. & Cantor, H. *J. exp. Med.* **153**, 1260–1274 (1981).
13. Fresno, M., McVay-Boudreau, L. & Cantor, H. *J. exp. Med.* **155**, 981–993 (1982).
14. Asherson, G. L. & Zembala, M. *Immunology* **42**, 1005–1013 (1980).
15. Claman, H. N., Miller, S. D., Conlon, P. J. & Moorhead, J. W. *Adv. Immun.* **30**, 121–157 (1980).
16. Miller, S. D., Sy, M.-S. & Claman, H. N. *J. Immun.* **121**, 265–273 (1978).
17. Bach, B. A., Sherman, L., Benacerraf, B. & Greene, M. I. *J. Immun.* **121**, 1461–1468 (1978).
18. Miller, S. D., Sy, M.-S. & Claman, H. N. *J. Immun.* **121**, 274–280 (1978).
19. Dietz, M. H. *et al. J. exp. Med.* **153**, 450–463 (1981).
20. Sy, M.-S. Nisonoff, A., Germain, R. N., Benacerraf, B. & Greene, M. I. *J. exp. Med.* **153**, 1415–1425 (1981).
21. Bianchi, A. T. J., Van der Kwast, Th. H. & Benner, R. *Immun. Today* **3**, 123–124 (1982).
22. Opelz, G., Sengar, D. P. S., Mickey, W. R. & Terasaki, P. I. *Transplantn Proc.* **5**, 253–259 (1973).
23. Williams, K. A. & Morris, P. J. in *Organ Transplantation—Present State, Future Goals* (ed. Slavin, S.) (Elsevier, Amsterdam, 1980).

SPECIFIC AND NONSPECIFIC T-CELL-MEDIATED SUPPRESSION OF ANTIHOST IMMUNE REACTIVITY IN GRAFT-VERSUS-HOST REACTION[1]

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Intravenous immunization of mice with irradiated (2000 rads) allogeneic lymphoid cells induces the generation of suppressor T cells. Such suppressor T cells are capable of suppressing the antihost immune reactivity during acute and delayed graft-versus-host reactions. These suppressor T cells are strictly antigen-specific as far as their activation is concerned, but also suppress the reaction against unrelated antigens presented by the irradiated host.

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Since the original report of Gershon et al. (*1*) about suppressor T cells, many investigators have reported T-cell-mediated suppression of immune function, for example, in delayed-type hypersensitivity (2, 3), graft-versus-host (GVH) reactivity (*4, 5*), helper T cell function (*6*), allograft rejection (*7*), cell-mediated lympholysis (*8, 9*) and mixed lymphocyte reactivity (*10, 11*). Antigen-specific as well as nonspecific suppressor T cell effects have been described (*11–17*).

Elsewhere (*18*) we have shown that delayed-type hypersensitivity (DTH) to major and minor histocompatibility antigens, which normally arises after s.c. immunization of mice, can be suppressed by i.v. preimmunization with irradiated allogeneic spleen cells. Others have shown, using a similar approach, that preimmunization with haptenated syngeneic lymphoid cells (*19, 20*), irradiated allogeneic lymphoid cells (*21*), or a high

dose of heterologous erythrocytes (*22, 23*) also leads to a state of suppression of DTH. Furthermore, i.v. preimmunization with irradiated allogeneic lymphoid cells can prolong skin allograft survival (*24*).

Previously, we developed a DTH assay that can be used to measure the development of antihost effector T cells during GVH reactions (*25*). This assay is based upon transfer of lymphoid cells from irradiated mice with GVH disease at different intervals after the irradiation and the transplantation of allogeneic lymphoid cells. The secondary recipients used are syngeneic to the original donor. These secondary recipients, which thus had received GVH-activated lymphoid cells, were challenged in the dorsum of the right hind feet with spleen cells syngeneic to the mice subjected to GVH. The subsequent DTH response was calculated by measuring the difference in thickness of the hind feet of the challenged secondary recipients. In the present study we investigated whether suppressor T cells induced by i.v. preimmunization of donor mice with irradiated allogeneic lymphoid cells can also suppress the antihost DTH response during acute and delayed GVH reactions.

MATERIALS AND METHODS

Animals. (C57BL/RijxCBA/Rij)F_1 (H-$2^{b/q}$) female mice, 10–40 weeks old, and BALB/c (H-2^d) female mice, 10–16 weeks old, were bred at the Laboratory Animals Centre of the Erasmus University, Rotterdam, the Netherlands. DBA/2 (H-2^d) male and female mice, and (BALB/cxDBA/2)F_1 (H-2^d) female mice, all 8–25 weeks old, were purchased from the Radiobiological Institute TNO, Rijswijk, The Netherlands, and from OLAC Ltd., Bicester, United Kingdom. B10.ScSn (H-2^b), B10.BR (H-2^k), B10.D2 (H-2^d), BALB.B (H-2^b), BALB.K (H-2^k), (BALB/cxBALB.K)F_1 (H-$2^{d/k}$), A.TL (H-2^{t1}), A.TH (H-2^{t2}), B10.A (H-2^a), B10.AQR (H-2^{v1}) and B10.T(6R) (H-2^{v2}) female mice, 8–14 weeks old, were purchased from OLAC Ltd. AKR (H-2^k) and C3H/Tif (H-2^k) female mice, 10–20 weeks old, were purchased from Bomholtgard, Ry, Denmark. A.AL (H-2^{a1}) female mice, 20 weeks old, were obtained from Yeda, Rehovot, Israel. (B10.D2×B10.BR)F_1 (H-$2^{d/k}$) and (B10.D2×B10.ScSn)F_1 (H-$2^{d/b}$) female mice were bred at our own laboratory. When mice of both sexes were used within one experiment, the combinations were always chosen in such a way that anti H-Y responses were impossible.

Preparation of cell suspensions. Mice were killed with carbon dioxide. Immediately after killing, the organs to be used (spleens and inguinal, axillary, and mesenteric lymph nodes) were removed, placed in a balanced salt solution (BSS) and squeezed through a nylon-gauze filter to provide a single-cell suspension. Bone marrow cells were collected by flushing femurs and tibias with BSS. Nucleated cells were counted with a Coulter counter, Model B. The cell suspensions obtained consisted of at least 90% viable cells.

Irradiation. The recipient mice received 750 rads of whole-body X-irradiation, except BALB/c and DBA/2 mice, which received 600 rads. Irradiation was performed in a Philips Müller MG 300 X-ray machine. The physical constants of the irradiation have been described elsewhere (*25*). All radiation control mice died from 14–21 days after irradiation. Spleen cell suspensions were irradiated with 2000 rads in the same machine.

GVH reactions. Acute GVH reactions were elicited by i.v. injection of 1×10^7, 2×10^7 or 5×10^7 nucleated allogeneic spleen cells into lethally irradiated recipients within 4 hr of irradiation. Delayed GVH reactions were induced by 1×10^7 nucleated allogeneic bone marrow cells.

Suppression. Suppressor cells were induced by one i.v. injection of 5×10^7 irradiated (2000 rads) allogeneic spleen cells 4 days before these mice were used as donors of spleen or bone marrow cells. Nonsuppressed mice had been injected with the same number of irradiated syngeneic cells.

Assay for DTH. An outline of the set-up used to determine the antihost DTH reactivity in GVH is given in Figure 1. At various intervals after i.v. injection of spleen or bone marrow cells into lethally irradiated allogeneic mice, the recipient spleens and mesenteric and peripheral lymph nodes were removed, pooled, and prepared for a single-cell suspension. These cells were transferred into naive secondary recipients syngeneic to the donors of the spleen or bone marrow cells that induced the GVH reaction. At 30 min before transfer, the secondary recipient mice had been injected i.p. with 15 U heparin (Liquémine, Hoffmann-La Roche & Co., Ltd.) to prevent embolism. The secondary recipients were challenged in the dorsum of the right hind foot with 2×10^7 spleen cells syngeneic to the irradiated recipients. The DTH response to this challenge was measured as the difference in thickness of the hind feet 24, 48, and 72 hr later. The specific increase in foot thickness was calculated as the relative increase in foot thickness of the secondary recipients minus the relative increase in foot thickness of control mice that only received the challenge. The swelling of the control mice ranged from 15 to 25%.

RESULTS

Suppression of antihost immune reactivity during acute GVH reactions. To investigate whether GVH reactions can be suppressed by suppressor T cells, we used the same protocol for induction of suppressor T cells that has proved to be effective in inducing suppression of host-versus-graft immune responses (*18*). Thus, the donor mice were pretreated by i.v. injection of syngeneic or allogeneic irradiated spleen cells. Donors pretreated in this way are referred to as *nonsuppressed* and *suppressed* mice, respectively. Acute GVH reactions were evoked by i.v. injection of nonsuppressed or suppressed spleen cells into lethally irradiated allogeneic mice. The antihost immune response was measured in the DTH assay at various intervals after irradiation and reconstitution.

GVH reactions evoked by transfer fo nonsuppressed DBA/2 spleen cells into (C57BL×CBA)F_1 mice showed a peak antihost DTH response at 5 days after irradiation and reconstitution. After reconstitution with suppressed spleen cells, however, a marginal response was found (Fig. 2). This difference was not only observed at 24 hr after challenge, but also at 48 and at 72 hr (data not shown), indicating that the suppression was not due to an altered kinetics of the DTH response in the secondary recipients.

We have shown elsewhere that during acute GVH reactions

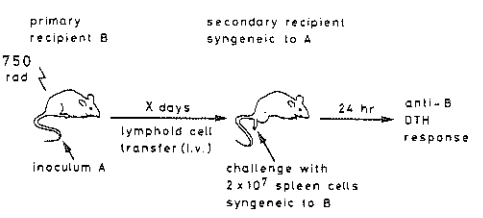

FIGURE 1. Scheme of the experimental system used to demonstrate GVH-related DTH reactivity against host histocompatability antigens.

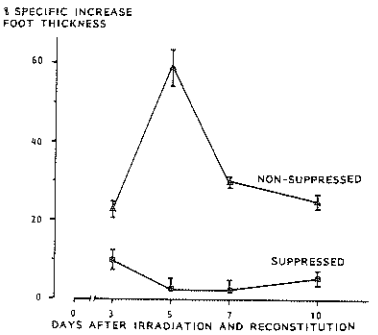

FIGURE 2. Development of antihost DTH reactivity in lethally irradiated (C57BL×CBA)F_1 mice inoculated with 1×10^7 spleen cells from suppressed or nonsuppressed DBA/2 mice. DTH responses were measured 24 hr after challenge. Each experimental point represents the arithmetic mean ± 1 SEM of 6–18 mice.

only H-2I and Mls-locus-coded antigens can evoke antihost DTH responses, and that other histocompatibility antigens can induce an amplification of this response (*26, 27*). Different classes of histocompatibility antigens can induce different immunological activities, so we investigated whether H-2I and Mls-locus-coded antigens were also able to induce suppression of GVH-related DTH reactivity. For H-2I this was done in donor-recipient combinations of A.TH and A.TL mice. Figure 3 (upper part) shows that preimmunization of the donors with H-2I incompatible irradiated spleen cells indeed effectively suppressed the antihost DTH response to these antigens. Similarly, preimmunization of BALB/c and C3H mice with irradiated spleen cells incompatible for the Mls locus (and several other non-H-2 loci) led to a severely suppressed GVH-related DTH response to the relevant Mls-locus-coded antigens (Fig. 3, lower part).

Suppression of antihost immune reactivity during delayed GVH reactions. Antihost DTH reactivity also occurs after bone marrow transplantation into irradiated allogeneic recipient mice (*28*). Intravenous preimmunization of the donors with the relevant allogeneic irradiated spleen cells reduced the capacity of their bone marrow cells to induce a delayed GVH-related DTH response. Thus, irradiated (C57BL×CBA)F_1 mice reconstituted with 1×10^7 bone marrow cells from suppressed DBA/2 mice showed a lower antihost DTH response than mice reconstituted with nonsuppressed bone marrow cells (Fig. 4).

Specificity of suppression of antihost immune reactivity. Elsewhere we have shown that the suppression of antihost DTH reactivity resulting from i.v. preimmunization of the donors is mediated by suppressor T cells (*29*). The specificity of this T-cell-dependent suppression was studied at two different levels: (A) the activation of the suppressor T cells, and (B) the suppressive effect by the suppressor T cells once they are activated.

The specificity of activation of the suppressor T cells was investigated by i.v. preimmunization of the donors with irradiated allogeneic spleen cells of a particular H-2 haplotype, and subsequent investigation of the GVH-related DTH reactivity in recipients of another H-2 haplotype. Thus, BALB/c mice were pretreated with irradiated BALB/c, BALB.B, or BALB.K spleen cells i.v., and were subsequently used as donors of spleen cells to induce GVH in irradiated BALB.B and BALB.K recipients. Five days after irradiation and reconstitution the antihost immune reactivity was determined. It was found that spleen cells from BALB/c mice suppressed to BALB.B or BALB.K do not react against BALB.B and BALB.K recipients, respectively (Fig. 5, upper part). However, BALB/c mice suppressed to BALB.K did respond to BALB.B. Similar data were obtained in experiments with B10.D2 donor mice and B10.ScSn and B10.BR recipients (Fig. 5, lower part). So, H-2 identity is required between cells used to induce the suppressor T cells

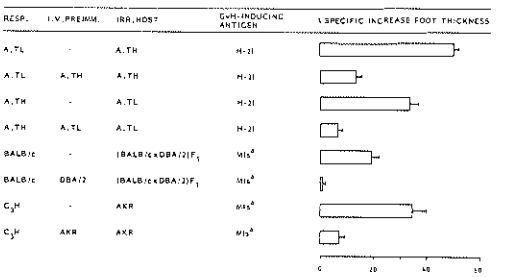

FIGURE 3. Antihost DTH reactivity in lethally irradiated A.TL and A.TH mice inoculated with 2×10^7 spleen cells from suppressed or nonsuppressed A.TH and A.TL mice, respectively (upper part), and antihost DTH reactivity in lethally irradiated (BALB/c×DBA/2)F_1 and AKR mice inoculated with 5×10^7 spleen cells from suppressed or nonsuppressed BALB/c and C3H/Tif mice, respectively (lower part). DTH responses were determined in a transfer system 5 days after reconstitution, and measured 24 hr after challenge. Each horizontal bar represents the arithmetic mean ± 1 SEM of 6–12 mice.

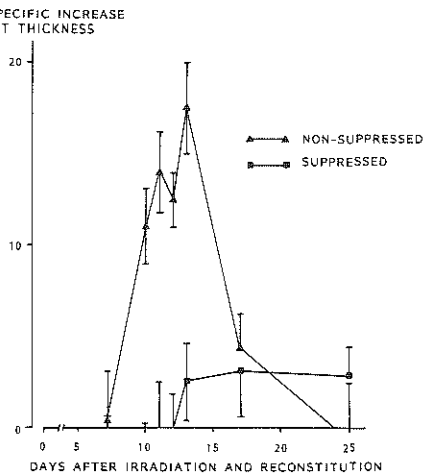

FIGURE 4. Development of antihost DTH reactivity in lethally irradiated (C57BL×CBA)F_1 recipients inoculated with 1×10^7 bone marrow cells from suppressed or from nonsuppressed DBA/2 mice. DTH reactivity was determined in a transfer system at various intervals after reconstitution, and measured 24 hr after challenge. Each experimental point represents the arithmetic mean ± 1 SEM of 6 mice.

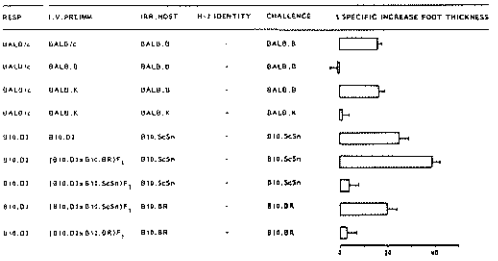

FIGURE 5. Specificity of suppressor T cells activated by i.v. injection of irradiated spleen cells of different H-2 haplotypes. BALB/c mice were preimmunized with BALB/c, BALB.B, or BALB.K spleen cells. Four days later 2×10^7 spleen cells from these pretreated BALB/c mice were used to reconstitute irradiated BALB.B and BALB.K mice. According to the same schedule, B10.D2 mice were injected i.v. with B10.D2, (B10.D2×B10.BR)F_1 or (B10.D2×B10.ScSn)F_1 irradiated spleen cells. Four days later 5×10^7 spleen cells from these pretreated B10.D2 mice were used to reconstitute lethally irradiated B10.ScSn and B10.BR recipients. In all groups of mice the antihost DTH reactivity was determined in a transfer system 5 days after reconstitution of the recipients. DTH responses were measured 24 hr after challenge. Each horizontal bar represents the arithmetic mean ± 1 SEM of 6 mice.

and the irradiated recipients in order to ensure optimal suppression (i.e., the suppressor T cells are antigen-specific).

The specificity of the suppressive effect by activated suppressor T cells was studied by using spleen cells from BALB.B mice suppressed to BALB/c to reconstitute irradiated (BALB/c×BALB.K)F_1 mice. These recipients have, in addition to the H-2^d antigens of BALB/c that induced the suppressor T cells, third party H-2^k antigens of BALB.K origin. It was found that the reaction against the bystander H-2^k alloantigens was effectively suppressed (Fig. 6). The same result was obtained in a set-up with A.TH, A.TL, and A.AL mice that were used to investigate the influence of suppressor T cells induced by H-2K upon the GVH-related anti-H-2I DTH response (Fig. 7). Thus, antigen-specific (Fig. 5) suppressor T cells are capable of nonspecific suppression of GVH-related DTH to a complete H-2 haplotype (Fig. 6) as well as to H-2I alloantigens (Fig. 7), provided that these antigens are presented as bystanders to the alloantigens that had induced the suppressor T cells.

Preimmunization i.v. with particular H-2 alloantigens could also suppress the GVH-related DTH response to a challenge of completely different H-2 alloantigens, provided that both sets of alloantigens were inherited by the irradiated recipient mice (Fig. 6, third bar). This shows that the suppressor T cells inhibit the GVH-related DTH response by suppression of the induction of the antihost DTH reactivity.

DISCUSSION

This study shows that i.v. injection of mice with irradiated allogeneic lymphoid cells induces the generation of antigen-specific suppressor T cells that are capable of suppressing the antihost DTH response in GVH disease. This is in agreement with the results obtained by other laboratories: e.g., DTH to hapten-modified syngeneic (*19*, *20*) and allogeneic (*21*) lymphoid cells and heterologous erythrocytes (*22*, *23*) can be suppressed by preimmunization using the i.v. route. Similar suppressive effects of i.v. preimmunization have been shown in contact sensitivity (*30*, *31*) and transplant rejection (*24*, *32*). Furthermore, Halle-Pannenko et al. (*33*, *34*) have shown that mortality attributable to GVH can be delayed and reduced by treating the donors with soluble recipient H-2 alloantigens. Both the delay of mortality and the cumulative mortality were found to depend on the dose of soluble H-2 antigens used to preimmunize the donors (*33*). Furthermore, their studies showed (*34*) that lethal GVH reactions to minor histocompatibility antigens could be decreased by simultaneous immunization of the donors with H-2 and non-H-2 alloantigens. This decrease varied as a function of the H-2 haplotype used for preimmunization. It is not clear from the studies of Halle-Pannenko et al., however, whether suppressor T cells are involved in the delay and reduction of mortality that they observed.

Van Bekkum and Knaan-Shanzer (*35*) described suppression of GVH disease in mice by T lymphocytes. They grafted lethally irradiated mice with semiallogeneic bone marrow and spleen cells, and observed acute GVH disease with 100% mortality within 12 days. However, when thymus cells of 4–6-day-old mice were added to the graft, the mortality was reduced and delayed. This suppressive effect was proved to be mediated by T cells.

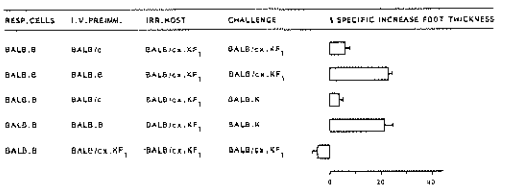

FIGURE 6. Capacity of spleen cells from suppressed donors to suppress the GVH-related DTH reactivity to bystander H-2 alloantigens. BALB.B donor mice were preimmunized with BALB/c, BALB.B, or (BALB/c×BALB.K)F_1 irradiated spleen cells. Four days later 2×10^7 spleen cells from these suppressed donors were used to reconstitute lethally irradiated (BALB/c×BALB.K)F_1 mice. Five days after irradiation and reconstitution the antihost DTH reactivity was determined by challenging secondary recipients with (BALB/c×BALB.K)F_1 or BALB.K spleen cells. DTH responses were measured 24 hr after challenge. Each horizontal bar represents the arithmetic mean ± 1 SEM of 6 mice.

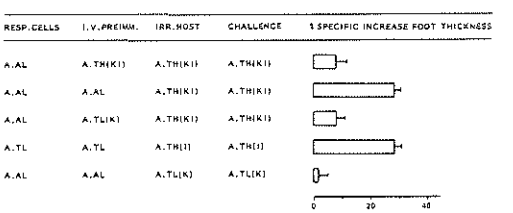

FIGURE 7. Specificity of the suppressive effect mediated by suppressor T cells. A.AL donors were i.v. preimmunized with A.TH, A.AL, or A.TL irradiated spleen cells. Four days later, 2×10^7 spleen cells from these donors were transferred into lethally irradiated A.TH mice. Five days after reconstitution the antihost DTH reactivity was determined. The H-2 subregion differences are shown in parentheses. See the legend to Fig. 6 for further experimental details.

We have previously shown that the i.v.-induced state of suppression that regulates host-versus-graft and GVH DTH responses is due to suppressor T cells (*29, 36*). Here we show that these suppressor T cells are antigen-specific as far as their activation is concerned (Fig. 5), but that they can also suppress the reaction against unrelated bystander antigens (Figs. 6 and 7). In the literature, nonspecific T-cell-dependent suppressive effects have been demonstrated in vivo (*37*) and in vitro (*11, 14–17*). The generation of this activity depends upon complex interactions of three sets of suppressor T cells in a regulatory circuit under control of IgH and H-2 genes (*38, 39*). Fresno et al. (*15–17*) 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 mol. wt. The former unit suppresses both antigen-specific and other Lyt-1+ T cells.

Liew, using high antigen doses and cyclophosphamide to induce suppressor T cell activity, has shown that suppression of DTH to H-2 subregion products can only be induced if the H-2 incompatibility includes the I-J subregion. The suppressor T cells induced in that way are antigen-specific, recognize the allo-I-J molecules, and suppress the DTH response to other H-2 subregion products if these are presented on the same cells as the allo-I-J determinants (*40*). The suppressor T cells activated by our protocol do not need to recognize allo-I-J determinants (this is in contrast to the data presented in Fig. 3, lower part).

The observation that bone marrow cells from suppressed mice mediate only a marginal delayed antihost DTH response—in contrast to bone marrow cells from nonsuppressed mice—suggests that activated suppressor T cells can also localize in the bone marrow. This property is not unique for activated suppressor T cells. It has been reported by others that long-lived T cells (*41, 42*) and effector T cells for DTH (*43, 44*) and contact sensitivity (*45*)—as well as T cells involved in the rejection of xenogeneic tumor transplants (*46*)—have this tendency to localize in the marrow.

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LITERATURE CITED

1. Gershon RK, Cohen P, Hencin R, Liebhaber SA. Suppressor T cells. J Immunol 1972; 108: 586.
2. Zembala M, Asherson GL. T cell suppression of the T cell phenomenon of contact sensitivity. Nature 1973; 244: 227.
3. Lagrange PH, Mackaness GB. Site of action of serum factors that block delayed-type hypersensitivity in mice. J Exp Med 1978; 148: 235.
4. McMaster R, Levy JG. Immunosuppression of normal lymphoid cells by serum from mice undergoing chronic graft-vs-host disease. J Immunol 1975; 115: 1400.
5. Miyazaki S, Nomoto K, Kuroiwa A, Goya N, Takeya K. Experimental models for prevention of graft-versus-host reaction in bone marrow transfusion: III. Reversible and irreversible differentiation of lymphocytes destined for cytotoxicity to effector cells for splenomegaly. Int Arch Allery Appl Immunol 1978; 56: 57.
6. Feldmann M, Beverly PCL, Woody J, McKenzie IFC. T-T interaction in the induction of suppressor and helper T cells: analysis of membrane phenotype of precursor and amplifier cells. J Exp Med 1977; 145: 793.
7. Pinto M, Brent L; Thomas AV. Specific unresponsiveness to skin allografts in mice: III. Synergistic effect of tissue extracts, *Bordetella pertussis* and antilymphocyte serum. Transplantation 1974; 17: 477.
8. Peavy DL, Pierce CW. Cell-mediated immune responses in vitro: I. Suppression of the generation of cytotoxic lymphocytes by Concanavalin A and Concanavalin A-activated spleen cells. J Exp Med 1974; 140: 356.
9. Hodes RJ, Hathcock KS. In vitro generation of suppressor cell activity: suppression of in vitro induction of cell-mediated cytotoxicity. J Immunol 1976; 116: 167.
10. Folch H, Waksman B. The splenic suppressor cell: II. Suppression of the mixed lymphocyte reaction by thymus-dependent adherent cells. J Immunol 1974; 113: 140.
11. Rich SS, Rich RR. Regulatory mechanisms in cell-mediated immune responses: I. Regulation of mixed lymphocyte reactions by alloantigen-activated thymus-derived lymphocytes. J Exp Med 1974; 140: 1588.
12. Rich SS, Rich RR. Regulatory mechanisms in cell-mediated immune responses: II. A genetically restricted suppressor of mixed lymphocyte reactions released by alloantigen-activated spleen cells. J Exp Med 1975; 142: 1391.
13. Tada T, Okamura T. The role of antigen-specific T cell factors in the immune response. Adv Immunol 1979; 28: 1.
14. Germain RN, Benacerraf B. Helper and suppressor T cell factors. Springer Semin Immunopathol 1980; 3: 93.
15. Fresno M, Nabel G, McVay-Boudreau L, Furthmayer H, Cantor H. Antigen-specific T lymphocyte clones: I. Characterization of a T lymphocyte clone expressing antigen-specific suppressive activity. J Exp Med 1981; 153: 1246.
16. Fresno M, McVay-Boudreau L, Nabel G, Cantor H. Antigen-specific T lymphocyte clones: II. Purification and biological characterization of an antigen-specific suppressive protein synthesized by cloned T cells. J Exp Med 1981; 153: 1260.
17. Fresno M, McVay-Boudreau L, Cantor H. Antigen-specific T lymphocyte clones: III. Papain splits purified T suppressor molecules into two functional domains. J Exp Med 1982; 155: 981.
18. Van der Kwast ThH, Bianchi ATJ, Bril H, Benner R. Suppression of antigraft immunity by preimmunization: I. Kinetic aspects and specificity. Transplantation 1981; 31: 79.
19. Bach BA, Sherman L, Benacerraf B, Greene MI. Mechanisms of regulation of cell-mediated immunity: II. Induction and suppression of delayed-type hypersensitivity to azobenzene-arsonate-coupled syngeneic cells. J Immunol 1978; 121: 1460.
20. Weinberger JZ, Germain RN, Benacerraf B, Dorf ME. Hapten-specific T cell responses to 4-hydroxy-3-nitrophenyl acetyl: V. Role of idiotypes in the suppressor pathway. J Exp Med 1980; 152: 161.
21. Miller SD, Sy MS, Claman HN. Suppressor T cell mechanisms in contact sensitivity: II. Afferent blockade by allo-induced suppressor T cells. J Immunol 1978; 121: 274.
22. Liew FY. Regulation of delayed-type hypersensitivity: I. T suppressor cells for delayed-type hypersensitivity to sheep erythrocytes in mice. Eur J Immunol 1977; 7: 714.
23. Thompson CH, Potter TA, McKenzie IFC, Parish CR. The surface phenotype of a suppressor cell of delayed-type hypersensitivity in the mouse. Immunology 1980; 40: 87.
24. Okazaki H, Maki T, Wood M, Monaco AP. Effect of a single transfusion of donor-specific and nonspecific blood on skin allograft survival in mice. Transplantation 1980; 30: 421.
25. Wolters EAJ, Benner R. Immunobiology of the graft-versus-host reaction: I. Symptoms of graft-versus-host disease in mice are preceded by delayed-type hypersensitivity to host histocompatibility antigens. Transplantation 1978; 26: 40.
26. Wolters EAJ, Benner R. Functional separation in vivo of both antigens encoded by H-2 subregion and non-H-2 loci. Nature 1979; 279: 642.
27. Wolters EAJ, Benner R. Different target antigens for T cell subsets acting synergistically in vivo. Nature 1980; 286: 895.
28. Wolters EAJ, Brons NHC, Benner R, Vos O. Anti-host immune

reactivity after allogeneic bone marrow transplantation. In: Baum SJ, Ledney GD, eds. Experimental hematology today, 1979. Berlin: Springer, 1979: 163.
29. Bril H, Benner R. Specific suppression of anti-host immune reactivity in graft-versus-host reaction. Adv Exp Med Biol 1982; 149: 577.
30. Asherson GL, Zembala M. T suppressor cells and suppressor factor which act at the efferent stage of the contact sensitivity skin reaction: their production by mice injected with water-soluble, chemically reactive derivatives of oxazolone and picryl chloride. Immunology 1980; 42: 1005.
31. Sunday ME, Benacerraf B, Dorf ME. Hapten-specific T cell responses to 4-hydroxy-3-nitrophenyl acetyl: VIII. Suppressor cell pathways in cutaneous sensitivity responses. J Exp Med 1981; 153: 811.
32. Wood P, Horsburgh T, Brent L. Specific unresponsiveness to skin allografts in mice: VI. Graft survival in mice pretreated with blood. Transplantation 1981; 31: 8.
33. Halle-Pannenko O, Martyre MC, Mathé G. Prevention of graft-versus-host reaction by donor pretreatment with soluble H-2 antigens. Transplantation 1971; 11: 414.
34. Halle-Pannenko O, Pritchard LL, Motta R, Mathé G. Lethal graft-versus-host reactions to minor histocompatibility antigens is decreased by donor immunization against H-2 and varies as a function of the H-2 haplotype. Transplant Proc 1979; 11: 652.
35. Van Bekkum DW, Knaan-Shanzer S. Characterization of the thymocyte involved in the suppression of graft-versus-host disease in mice. Exp Hematol 1981; 9 (Suppl. 9): 160.
36. Bianchi ATJ, Hussaarts-Odijk LM, Benner R. Antigen-specific suppressor T cells suppress *in vivo* the cellular immune response to unrelated 'bystander' antigens. Transplant Proc 1983; 15: 760.
37. Zembala M, Asherson GL, Colizzi W. Hapten-specific T suppressor factor recognizes both hapten and I-J region products on hapteinized spleen cells. Nature 1982; 297: 411.
38. Dietz MH, Sy MS, Benacerraf B, Nisonoff A, Greene MI, Germain RN. Antigen- and receptor-driven regulatory mechanisms: VII. H-2 restricted anti-idiotypic suppressor factor from efferent suppressor T cells. J Exp Med 1981; 153: 450.
39. Sy MS, Nisonoff A, Germain RN, Benacerraf B, Greene MI. Antigen- and receptor-driven regulatory mechanisms: VIII. Suppression of idiotype-negative, p-azobenzenearsonate-specific T cells results from the interaction of an anti-idiotypic second-order T suppressor cell with a cross-reactive–idiotype-positive, p-azobenzenearsonate-primed T cell target. J Exp Med 1981; 153: 1415.
40. Liew FY. Regulation of delayed-type hypersensitivity: VII. The role of I-J subregion gene products in the inhibition of delayed-type hypersensitivity to major histocompatibility antigens by specific suppressor T cells. Eur J Immunol 1981; 11: 883.
41. Röpke C. Hougen HP, Everett NB. Long-lived T and B lymphocytes in the bone marrow and thoracic duct lymph of the mouse. Cell Immunol 1975; 15: 82.
42. Press OW, Rosse C. Clagett J. Phytohemagglutinin-induced differentiation and blastogenesis of precursor T cells from mouse bone marrow. J Exp Med 1977; 146: 735.
43. Youdim S, Stutman O, Good RA. Thymus dependency of cells involved in transfer of delayed hypersensitivity to Listeria monocytogenes in mice. Cell Immunol 1973; 8: 395.
44. Phillips-Quagliata JM, Wertenbacker C, Bensinger DO, Quagliata R. Cellular events in tolerance: II. Thymus-bone marrow cell cooperation in the immune response to BSA in Wistar Furth rats. Cell Immunol 1972; 4: 134.
45. Asherson GL, Zembala M. Anatomical location of cells which mediate contact sensitivity in the lymph nodes and bone marrow. Nature New Biol 1973; 244: 176.
46. Marusić M. Transfer of immunity by transfer of bone marrow cells: T cell dependency. Cell Immunol 1978; 38: 440.

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INFLUENCE OF 2'-DEOXYGUANOSINE UPON THE DEVELOPMENT OF DTH EFFECTOR T CELLS AND SUPPRESSOR T CELLS IN VIVO

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SUMMARY

Subcutaneous (sc) immunization of mice with allogeneic spleen cells can induce delayed type hypersensitivity (DTH) to both major and minor histocompatibility antigens. Intravenous (iv) immunization with allogeneic spleen cells, on the other hand, induces a poor state of DTH. Furthermore, iv immunization with allogeneic spleen cells, especially if they have been irradiated, induces suppressor T lymphocytes. These suppressor T cells are capable of suppressing the Host-versus-Graft (HvG) DTH reactivity which normally arises after sc immunization. Moreover, they can suppress the development of anti-host DTH effector T cells during Graft-versus-Host (GvH) reactions. These models for HvG and GvH DTH reactivity were used to study the influence of 2'-deoxyguanosine (dGuo) and guanosine (Guo) on the generation of DTH reactive T cells and suppressor T cells *in vivo*. It was found that daily intraperitoneal (ip) administration of 0.01 mg dGuo to iv immunized mice partially prevented the generation of suppressor T cell activity, while daily administration of 0.1 or 1 mg dGuo resulted in a complete abolition. Administration of dGuo has no effect on the anti-host DTH reactivity by spleen cells from nonsuppressed donors except a daily dose of 10 mg. This dose proved to be toxic for precursors of DTH effector T cells. Daily ip injection of Guo had neither an effect on the generation of suppressor T cells, nor on the generation of DTH effector T cells. The effect of dGuo was found to be due to a direct effect on suppressor T cells and not to the induction of contrasuppressor

cells. These data suggest a differential sensitivity of DTH reactive T cells and suppressor T cells for dGuo.

Since suppressor T cells and DTH reactive T cells require proliferation for expressing maximal functional activity in the systems employed, both cell types probably have different enzyme activities involved in the purine metabolism. Probably, both cell types have similar deoxycytidine kinase activities but different nucleotidase (5'NT) activities, that in suppressor T cells being the lowest. If so, suppressor T cells will accumulate dGTP, which causes an inhibition of the ribonucleotide reductase activity and thus of the DNA synthesis by these cells.

INTRODUCTION

Since the original observation of Giblett and coworkers (1,2), it has been firmly established that deficiencies for the enzymes adenosine deaminase (ADA) and purine nucleoside phosphorylase (PNP) are the underlying causes of certain immunodeficiency diseases. A number of hypotheses have been put forward to relate the lymphocyte dysfunction to the biochemical abnormalities. One hypothesis states that deoxyadenosine and deoxyguanosine in ADA and PNP deficient patients, respectively, are not metabolized but are excreted instead into the plasma by dividing non-lymphoid cells. The deoxynucleosides are selectively trapped by lymphocytes with high levels of deoxycytidine kinase and low levels of intracellular deoxynucleotidase. Because nucleotides do not traverse the plasma

membrane, deoxyadenosinetriphosphate (dATP) and deoxyguanosine-triphosphate (dGTP) progressively accumulate intracellularly and consequently inhibit the enzyme ribonucleotide reductase and therefore DNA synthesis (3).

Gelfand et al. (4) have described that antigen-induced human suppressor T cell activity in vitro was abrogated by micromolar concentrations of dGuo. Helper T cells and precursor B lymphocytes were found to have a more than 1,000-fold higher resistance to dGuo. Also in vivo generated murine suppressor T cells, capable of abrogating the antibody forming cell (PFC) response to TNP-ovalbumin, were selectively inhibited by dGuo, in contrast to the helper T cells (5). However, from these studies it is not clear whether dGuo inhibits the proliferation, the differentiation and/or the activity of suppressor T cells.
Also the question has not been answered whether the proliferation of helper T cells is sensitive to dGuo.

The purpose of the present study was to investigate whether dGuo was capable of abrogating the proliferation-dependent induction of murine DTH effector T cell activity and suppressor T cell activity in vivo under both Host-versus-Graft (HvG) and Graft-versus-Host (GvH) conditions. Under these conditions, the activation of T cells by histocompatibility antigens can be measured in a delayed type hypersensitivity (DTH) assay (6,7). The responses measured in this assay are determined by the activities of both DTH effector T cells, that are probably identical to helper T cells (8), and suppressor T cells.

MATERIALS AND METHODS

Mice. (C57BL/Rij x CBA/Rij)F1 (H-$2^{b/q}$), BALB/c (H-2^{d}) and B10.A (H-2^{a}) female mice, 10 to 20-week-old, were bred at the Laboratory Animals Center of the Erasmus University, Rotterdam, The Netherlands. DBA/2 (H-2^{d}) female mice, 10 to 16-week-old, were purchased from the Radiobiological Institute TNO, Rijswijk, The Netherlands, the Medical Biological Laboratory TNO, Rijswijk, and OLAC Ltd., Bicester, United Kingdom. A/J (H-2^{a}) female mice, 10 to 16-week-old, were purchased from OLAC Ltd.

Preparation of cell suspensions. Mice were killed using carbon dioxide. Immediately after killing, the lymphoid organs (spleen and inguinal, axillary and mesenteric lymph nodes) were prepared for single-cell suspensions in a balanced salt solution (BSS) as described previously (7). Nucleated cells were counted with a Coulter counter Model B.

Irradiation. For lethal irradiation of mice, a dose of 7.5 Gy whole body X-irradiation was applied, generated in a Philips Müller MG 300 X-ray machine as described previously (7). Radiation control mice died within 16 days. Spleen cell suspensions were irradiated with 20 Gy in the same machine as used for irradiation of the mice.

Host-versus-Graft reaction. Responder mice were subcutaneously (sc) immunized with 1 x 10^{7} unirradiated nucleated allogeneic spleen cells, suspended in a volume of 0.1 ml BSS.

These cells were equally distributed over both inguinal areas. In previous papers (6,9) we have shown that immunization with H-2 and non-H-2 alloantigens according to this procedure induces maximal DTH responses.

Acute Graft-versus-Host reaction. Acute GvH were elicited by intravenous (iv) injection of 1×10^7 nucleated spleen cells into lethally irradiated allogeneic mice 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 assay for measuring HvG and GvH immune reactivity has been described in detail in previous papers (6,7). HvG DTH responses were elicited in previously immunized mice (see above) by sc injection of a challenge dose of 2×10^7 unirradiated allogeneic spleen cells into the dorsum of the right hind foot. The DTH response to this challenge was measured as the difference in thickness of the hind feet 24 h, 48 h and 72 h later. In the figures, the 24 h values are presented. The DTH responses at 48 and 72 h were in harmony with those at 24 h, but lower. The specific increase in foot thickness was calculated as the percentage increase in foot thickness of the immune mice minus the percentage increase in foot thickness of control mice which only received the challenge. The increase in foot thickness of these challenged control mice varied between 15 and 25%.

For measuring the anti-host DTH reactivity under GvH conditions a number of cells equivalent to the total cell yield obtained from

spleen, inguinal, axillary and mesenteric lymph nodes from an irradiated and reconstituted recipient mouse was transferred iv into a normal secondary recipient syngeneic to the original spleen donor mouse 5 days after reconstitution. Thirty minutes before transfer, the secondary recipient mice were intraperitoneally (ip) injected with 15 U heparin (Liquémine, Hoffman-La Roche & Co. Ltd., Basel, Switzerland) to prevent embolism. The secondary recipient mice were challenged into the dorsum of the right hind foot with 2×10^7 unirradiated spleen cells, syngeneic with the irradiated recipients. The subsequent DTH response was measured and calculated as described above for HvG DTH responses. Fig. 1 shows the set up of the GvH DTH assay.

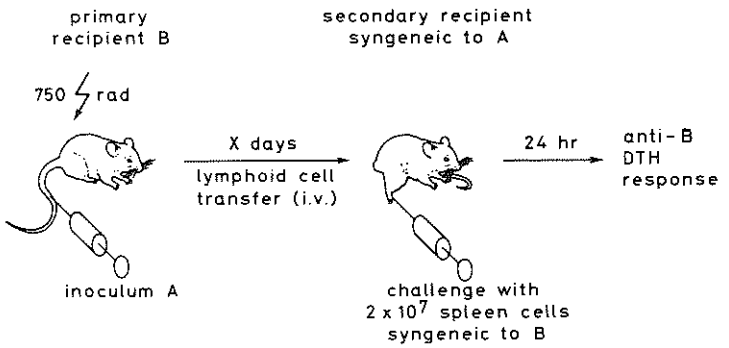


Fig. 1. Scheme of the experimental set up used to determine the GvH-related DTH reactivity against host histocompatibility antigens.

Induction of suppression. Alloantigen-specific suppressor T cells were induced by a single iv injection of 5×10^7 irradiated (20 Gy) spleen cells of the appropriate allogeneic mouse strain, as described previously (10,11). Control mice received a dose of 5×10^7 irradiated syngeneic spleen cells.

Drug treatment. Deoxyguanosine (dGuo), no. D-9125, grade II, was purchased from Sigma Chemical Company, Saint Louis, Missouri, USA. Experimental mice received ip injections of 0.001, 0.01, 0.1, 1 or 10 mg dGuo in 0.5 ml BSS. Control mice received BSS only. One half to 10 h following a single injection of 1 mg dGuo, serum levels of 0.2-0.3 μM dGuo were measured by high performance liquid chromatography (HPLC).

Guanosine (Guo), 2965H, C-136, was purchased from Koch-Light Laboratories Ltd., Colnbrook, Berkshire, United Kingdom. Experimental mice received ip injections of 0.001, 0.01, 0.1, 1 or 10 mg Guo in 0.5 ml BSS. Control mice received BSS only.

The schedules of drug treatments in relation to the immunizations and DTH assay are indicated in the legends to the figures.

RESULTS

The influence of dGuo on the development of murine DTH effector T cells and suppressor T cells was studied under HvG and GvH conditions. Suppressor T cells were induced by the protocol that previously was shown to be effective in suppression of HvG and GvH immune responses (10,11). Thus mice were iv immunized with the relevant alloantigens ('suppressed') several days before induction of HvG DTH or before using the mice as donors of spleen cells for induction of GvH in lethally irradiated allogeneic recipients.

Influence of different doses of dGuo and Guo upon the induction of DTH effector T cells and suppressor T cells

The effect of different doses of dGuo and Guo upon the generation of DTH effector T cells and suppressor T cells was studied under GvH conditions. Therefore, B10.A donor mice were suppressed by means of an iv injection of irradiated (C57BL x CBA)F1 spleen cells. Control B10.A mice received an iv injection of irradiated syngeneic spleen cells. Subsequently, both groups of mice received four daily injections of different doses of dGuo or Guo (0.001, 0.01, 0.1, 1 or 10 mg). On the fifth day, the spleen cells of these mice were used to elicit GvH reactions in lethally irradiated (C57BL x CBA)F1 recipients. Five days after irradiation and reconstitution the anti-host DTH reactivity was determined as described in the section 'Materials and Methods'. Fig. 2 (upper part) shows that dGuo treatment of the spleen cell donors did not affect the development of anti-host DTH unless a dose as high as 10 mg dGuo was given per day (upper line).

The iv induced suppression, on the other hand, was abrogated by much lower doses. This abrogation could be detected partially when 0.01 mg dGuo was used and was maximal when the donors were treated with 0.1 or 1 mg dGuo per day (lower line).

Fig. 2 (lower part) shows the results when Guo instead of dGuo was administered to the non-suppressed and suppressed donor mice. Guo did neither affect the generation of anti-host DTH reactivity (upper line) nor the iv induced suppression (lower line), at least not within the dose range tested.

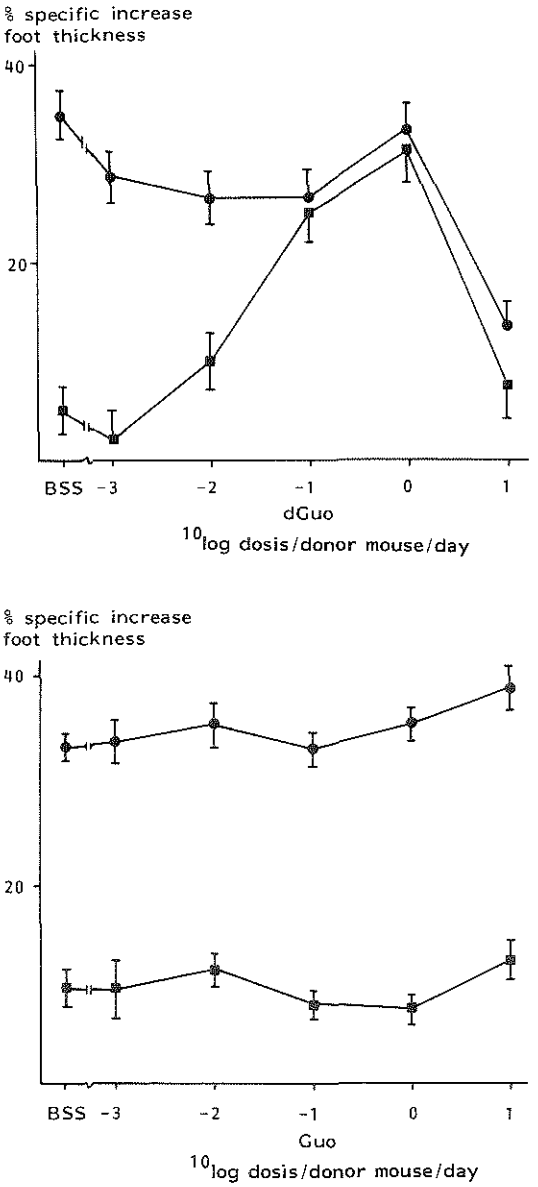


Fig. 2. Effect of different doses of dGuo (upper part) and Guo (lower part) upon the generation of suppressor T cells and precursors of DTH reactive T cells as determined in the GvH-related anti-host DTH response. B10.A donor mice were used to elicit GvH reactions in lethally irradiated (C57BL x CBA)F1 mice. Four days before their use as donors, the B10.A mice were either suppressed by means of an iv injection of 5 x 10^7 irradiated (20 Gy) (C57BL x CBA)F1 spleen cells, or injected with the same dose of irradiated syngeneic spleen cells. These suppressed and non-suppressed donors were subsequently treated with either 0.001, 0.01, 0.1, 1 or 10 mg dGuo, Guo or BSS ip for four days. The anti-host DTH reactivity was determined on the fifth day after irradiation and reconstitution. Values represent the arithmetic mean of the DTH response ± 1 SEM (n=6).

The GvH related DTH response by non-suppressed spleen cells can be reduced by simultaneously transferred suppressed spleen cells (11). We used this set up to substantiate that dGuo indeed affects the development of suppressor T cell activity. Fig. 3 shows that suppressed spleen cells from mice treated with dGuo (1 mg per mouse) for 4 days, are no longer capable of inhibiting the response by non-suppressed spleen cells. This result was found in both combinations tested, namely BALB/c- B10.A and DBA/2-(C57BL x CBA)F1.

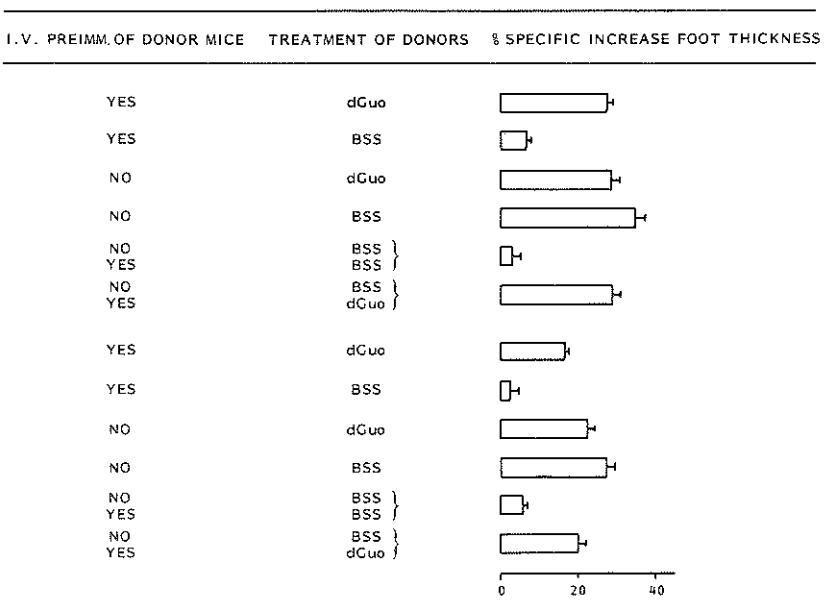


Fig. 3. Influence of dGuo treatment of the allogeneic spleen cell donors upon the anti-host DTH reactivity in lethally irradiated B10.A (upper part) and (C57BL x CBA)F1 mice (lower part). The recipient B10.A and (C57BL x CBA)F1 mice were inoculated with 1×10^7 suppressed, 1×10^7 non-suppressed or 1×10^7 suppressed plus 1×10^7 non-suppressed BALB/c or DBA/2 spleen cells, respectively. Anti-host DTH reactiivity was determined six days after irradiation and reconstitution. The interval between induction of suppression in the donors and their use for reconstitution was four days.
The dGuo treatment consisted of 1 mg per mouse per day. Anti-host DTH reactivity was determined on the sixth day after irradiation and reconstitution. Values represent the arithmetic mean of the DTH response ± 1 SEM (n=6).

The data presented thus far suggest that dGuo directly affects suppressor T cells. However, other possibilities may exist, e.g., the induction of contrasuppressor cells (12). To investigate this possibility, several combinations of spleen cells from dGuo or BSS-treated non-suppressed and suppressed mice were used to reconstitute lethally irradiated allogeneic mice. Fig. 4 shows that suppressed cells from BSS-treated mice can suppress the reactivity of simultaneously transferred non-suppressed spleen cells (third bar). When suppressed cells from BSS-treated mice were combined with suppressed cells from dGuo treated mice a clear suppression was found (fifth bar). The reverse should have been the case when contrasuppressor cells had been induced. In this experiment the dGuo was very potent in abrogating the suppressive effect (fourth and six bar).

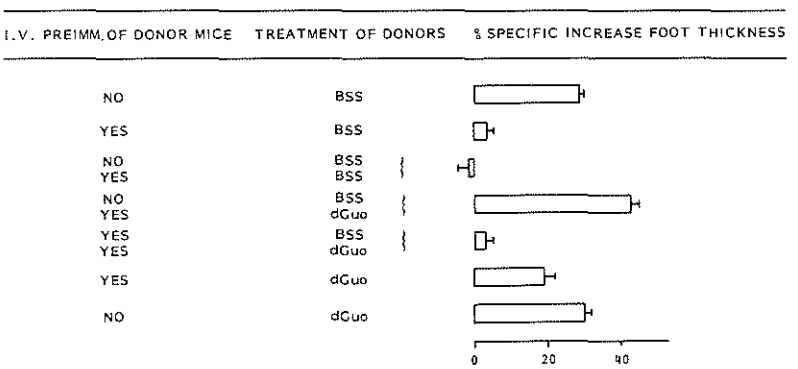
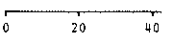


Fig. 4. Direct influence of dGuo on suppressor T cells. Suppressed and non-suppressed spleen cells were obtained from B10.A mice iv injected with 5 x 10^7 irradiated (C57BL x CBA)F1 spleen cells. During 4 days after the iv injection the mice were treated with either dGuo or BSS. Various combinations of spleen cells from such B10.A mice were used to induce GvH reactions in lethally irradiated (C57BL x CBA)F1 recipients. Anti-host DTH reactivity was determined six days after irradiation and reconstitution. The interval between induction of suppression in the donors and their use for reconstitution was four days. The dGuo treatment consisted of 1 mg per mouse per day. For other experimental details see Fig. 3.

Influence of dGuo upon the induction of DTH effector T cells and suppressor T cells regulating HvG DTH responses

To study the effect of dGuo and, as a control, Guo upon the generation of DTH effector T cells, (C57BL x CBA)F1 mice were sc immunized with H-2 and non-H-2 incompatible DBA/2 spleen cells, daily injected with dGuo or Guo, and challenged with DBA/2 spleen cells 6 days after the sc immunization. It appeared that neither Guo nor dGuo inhibited the primary DTH response to these allogeneic spleen cells (Fig. 5, upper part).

RESP. STRAIN	S.C. IMMUNIZATION	TREATMENT	% SPECIFIC INCREASE FOOT THICKNESS
$(C_{57}BLxCBA)F_1$	DBA/2	Guo	
$(C_{57}BLxCBA)F_1$	DBA/2	dGuo	
$(C_{57}BLxCBA)F_1$	DBA/2	BSS	



RESP. STRAIN	I.V. PREIMM	TREATMENT	S.C. IMMUNIZATION	% SPECIFIC INCREASE FOOT THICKNESS
$(C_{57}BLxCBA)F_1$	$(C_{57}BLxCBA)F_1$	Guo	DBA/2	
$(C_{57}BLxCBA)F_1$	$(C_{57}BLxCBA)F_1$	dGuo	DBA/2	
$(C_{57}BLxCBA)F_1$	$(C_{57}BLxCBA)F_1$	BSS	DBA/2	
$(C_{57}BLxCBA)F_1$	DBA/2	Guo	DBA/2	
$(C_{57}BLxCBA)F_1$	DBA/2	dGuo	DBA/2	
$(C_{57}BLxCBA)F_1$	DBA/2	BSS	DBA/2	

Fig. 5. Influence of dGuo and Guo upon the generation of DTH effector T cells and suppressor T cells as determined in the HvG DTH response. The effect upon the generation of DTH effector T cells (upper part) was studied in (C57BL x CBA)F1 mice, sc immunized with 1 x 10^7 DBA/2 spleen cells. One mg dGuo in BSS, 1 mg Guo in BSS or BSS only was administered daily from the day of sc immunization till the day before challenge. The effect of dGuo and Guo upon the generation of suppressor T cells (lower part) was studied in (C57BL x CBA)F1 responder mice, iv preimmunized with irradiated DBA/2 spleen cells. Seven days later all mice were sc immunized with 1 x 10^7 DBA/2 spleen cells.
In the latter experiments, all mice were treated with either 1 mg dGuo in BSS, 1 mg Guo in BSS or BSS only from the day of iv preimmunization till the day of sc immunization. All mice were challenged with DBA/2 spleen cells 6 days after sc immunization.
All values represent the arithmetic mean of the DTH response ± 1 SEM(n=6).

Fig. 5, lower part, shows the effect of dGuo and Guo treatment on the induction of suppressor T cells by iv administered irradiated allogeneic spleen cells. Thus, (C57BL x CBA)F1 mice were iv injected with irradiated H-2 and non-H-2 incompatible DBA/2 spleen cells and treated with Guo or dGuo for 7 days. Subsequently, all mice were sc immunized with DBA/2 spleen cells and 6 days later the mice were challenged. Suppression of the DTH response of (C57BL x CBA)F1 responder mice against DBA/2 spleen cells was observed in the BSS-treated control group. The dGuo treatment, but not the Guo treatment, during the period between iv and sc immunization, inhibited the development of suppressor activity. However, the dGuo treatment did not interfere with the generation of DTH effector T cells due to the sc immunization. Apparently, the precursors of the DTH effector T cells are insensitive to treatment with 1 mg dGuo per mouse per day.

Fig. 6 shows the influence of dGuo treatment upon the induction of suppressor T cells by iv administered irradiated allogeneic spleen cells using transfer of the suppressor cells to prevent any possible interference of dGuo with DTH effector cells. Therefore, (C57BL x CBA)F1 mice were iv injected with irradiated H-2 and non-H-2 incompatible DBA/2 spleen cells, and treated with dGuo for 4 days. Subsequently, the spleen and lymph node cells were transferred to syngeneic (i.e., (C57BL x CBA)F1) recipients. These secondary recipients were sc immunized with DBA/2 spleen cells immediately after the cell transfer, and challenged 6 days later. Suppression of the DTH response of (C57BL x CBA)F1 mice against DBA/2 spleen cells was observed in the Guo and BSS-treated control groups. The dGuo

treatment inhibited the development of suppressor activity to a large extent. The same result was found in the H-2 compatible, but non-H-2 incompatible, B10.A-A/J combination (Fig. 6). Guo treatment did not inhibit the development of suppressor activity.

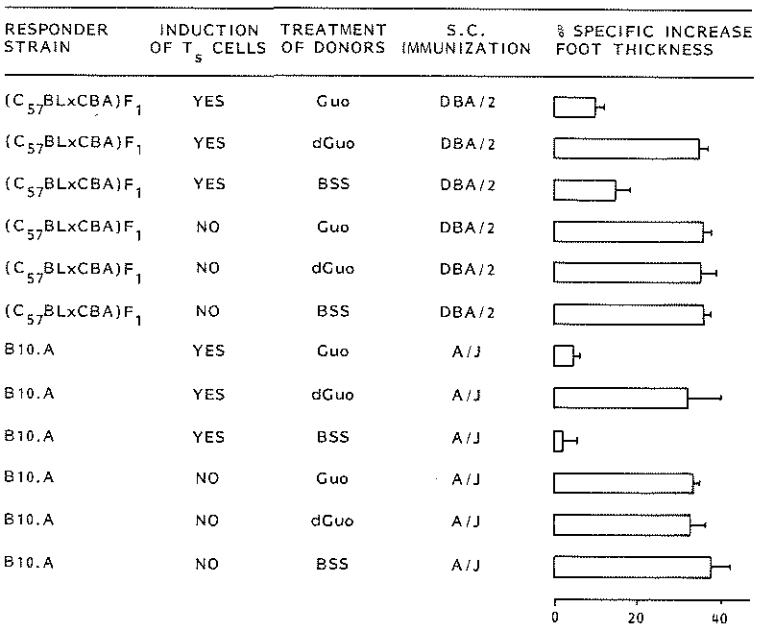


Fig. 6. Influence of dGuo upon the generation of suppressor T cell activity as determined on the HvG DTH response. (C57BL x CBA)F1 and B10.A mice were 'suppressed', i.e., iv injected with 5×10^7 irradiated DBA/2 and A/J spleen cells, respectively. During the following 4 days all mice were treated with either 1 mg dGuo in BSS, 1 mg Guo in BSS or BSS only. After this period, the spleen and lymph node cells were transferred to syngeneic recipient mice, which were sc immunized with DBA/2 or A/J spleen cells immediately after transfer. Six days after immunization the mice were challenged with DBA/2 and A/J spleen cells, respectively. Values represent the arithmetic mean of the DTH response ± 1 SEM (n=6).

Influence of dGuo treatment of mice subjected to GvH upon the suppressor T cell activity regulating the anti-host DTH response

The effect of dGuo upon the proliferation and activity of DTH

effector T cells and suppressor T cells was studied by dGuo treatment of mice subjected to acute GvH. Non-suppressed as well as suppressed donors were used to reconstitute the irradiated allogeneic recipients. Four hours after reconstitution, the recipient mice received their first ip injection of dGuo, followed by a daily injection during 4 days.

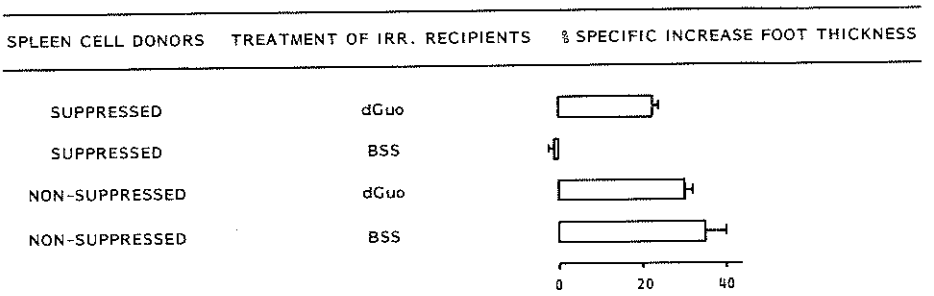


Fig. 7. Influence of dGuo treatment of mice subjected to GvH upon the anti-host DTH response. GvH was induced in lethally irradiated (C57BL x CBA)F1 mice by inoculation of 1 x 10^7 spleen cells from either suppressed or non-suppressed DBA/2 mice. Irradiated, reconstituted mice were treated with 1 mg dGuo in BSS or BSS only on day 0, 1, 2, 3 and 4 after reconstitution. On day 5 the mice were tested for anti-host DTH reactivity. For other experimental details see Fig. 3.

It was found that irradiated mice, reconstituted with spleen cells from suppressed donors and treated with dGuo, displayed substantial anti-host DTH reactivity, in contrast to the BSS-treated control group (Fig. 7). Irradiated mice, reconstituted with spleen cells from non-suppressed donors and treated with dGuo, responded as the control group. So, dGuo does not inhibit the anti-host DTH effector T cells, but does inhibit the further development and/or functional activity of suppressor T cells in the irradiated recipients.

DISCUSSION

In this paper we show that administration of dGuo, but not Guo, inhibits the generation of murine suppressor T cell activity but not the generation of DTH. The effect of dGuo seems to be a direct effect on suppressor T cells and not an effect via contrasuppressor cells since irradiated recipients inoculated with spleen cells from iv preimmunized allogeneic mice treated with dGuo in combination with spleen cells from iv preimmunized mice not treated with dGuo displayed a suppressed response (Fig. 4). The reverse should have been the case when contrasuppressor T cells had been induced.

Dose response studies showed that 0.001 up to 10 mg Guo does not have any influence on the suppressed as well as the nonsuppressed anti-host DTH (Fig. 2). In contrast, 10 mg dGuo, but not lower doses, inhibited the nonsuppressed anti-host DTH and thus the generation of DTH reactive T cells. The iv induced suppression, on the other hand, could be abrogated by much lower doses: 0.01 mg dGuo already inhibited the iv induced suppression. This 1,000-fold higher resistance to dGuo for DTH effector activity as compared with suppressor T cell activity was also reported by Gelfand et al. for helper and suppressor T cells (4). It was concluded in that study that all T cell proliferative events are susceptible to dGuo toxicity. Apparently helper and suppressor T cells did not necessarily differ in the activities of enzymes involved in the purine metabolism, particularly deoxycytidine kinase and 5'NT.

These authors explained the differential effect of dGuo on helper T cells and suppressor T cells by supposing that helper T cells, in contrast to suppressor T cells, do not need to proliferate in order to become functionally active. In our models, however, both suppressor T cells and DTH reactive T cells require proliferation for expressing maximal functional activity (13,14, Bril et al., submitted). Since administration of dGuo only affected the suppressor T cell activity, our studies suggest a different enzymatic make-up with regard to the purine metabolism.

In agreement with the hypothesis mentioned in the 'Introduction' and the suggestion of Dosch (5) that precursors of suppressor T cells have a purine enzyme pattern similar to thymocytes, our results can be explained by assuming an accumulation of dGTP with a resultant inhibition of ribonucleotide reductase and DNA synthesis in suppressor T cells but not in DTH effector T cells. This explanation assumes high deoxycytidine kinase and low intracellular deoxynucleotidase activities in suppressor T cells. We suggest that DTH effector T cells, on the other hand, have high deoxycytidine kinase and deoxynucleotidase activities which prevent accumulation of dGTP. In support of this view are the observations of Boss et al. (15) and van Laarhoven et al. (16) that human suppressor T cells have lower 5'NT activities than helper T cells. Zachowski et al. (17) reported that murine thymomas of helper T phenotype exhibited distinct 5'NT activity, whereas thymomas of suppressor T phenotype lack 5'NT activity. However, Massaia et al. (18) showed higher 5'NT activities in human suppressor T cells than in helper T cells.

This apparent discrepancy was ascribed to the fact that in the latter studies non-proliferating lymphocytes were investigated, while it was assumed that the 5'NT activity is related to the proliferative activation of the different subsets of mature T lymphocytes. It must be stressed, however, that Carson et al. (19) recently described a soluble intracellular deoxynucleotidase activity which may be more important than ecto-5'NT in protection from deoxynucleoside toxicity. This intracellular deoxynucleotidase could be distinguished from and correlated better with sensitivity to deoxyadenosine toxicity than ecto-5'NT.

Massaia et al. (18) showed very similar kinase activities in helper and suppressor T lymphocytes, which agrees with the notion mentioned above that deoxycytidine kinase is abundantly present in both human helper and suppressor T lymphocytes. It is not clear whether something similar holds for murine DTH reactive T cells and helper T cells, which are probably identical (8). Cloned murine helper and suppressor T cells seem to be the appropriate material for studies aimed to determine the relevant enzyme activities and accumulation of purine metabolites after administration of Guo and dGuo.

The observation that Guo has no effect merits attention. The lack of similar effects of Guo as compared to dGuo in our in vivo studies might be due to a different turnover of these deoxynucleosides. As a consequence the intracellular concentration of Guo, but not dGuo, in T cells might be too low to cause any significant inhibition of suppressor T cell activation. Since the

pharmacokinetics of Guo and dGuo in plasma are not well known, more studies on these aspects are necessary.

Lelchuk et al. (20) have shown that after infestation of mice with malaria parasites non-specific suppressor T cells are generated which are capable of suppressing the DTH reaction to oxazolone. This suppressor T cell activity was found to be abrogated by daily treatment of the mice with 1 mg dGuo. In a subsequent study these investigators showed that murine antigen-specific suppressor T cell activity capable of suppressing the DTH response to sheep red blood cells was not abrogated by dGuo (21). The latter results seem to contradict our results and those of Dosch and Gelfand (22). The crucial difference between the systems employed, however, seems to be the requirement for proliferation of the T lymphocytes for the expression of their function in our system and in that of Dosch and Gelfand. The finding by Lelchuk et al. (21) that non-specific suppressor T cells induced by the mitogen concanavalin A, which non-specifically suppress the IgM plaque-forming cell response in vitro, are dGuo sensitive, seems to support this notion.

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REFERENCES

1. Giblett, E.R., J.E. Anderson, F. Cohen, B. Pollara, and H.J. Meuwissen. 1972. Adenosine-deaminase deficiency in two patients with severely impaired cellular immunity. Lancet ii : 1067.
2. Giblett, E.R., A.J. Ammann, R. Sandman, D.W. Wara, and L.K. Diamond. 1975. Nucleoside-phosphorylase deficiency in a child with severely defective T cell immunity and normal B cell immunity. Lancet i : 1010.
3. Carson, D.A., E. Lakow, D.B. Wasson, and N. Kamatani. 1981. Lymphocyte dysfunction caused by deficiencies in purine metabolism. Immunol. Today 2 : 234.
4. Gelfand, E.W., J.J. Lee, and H.M. Dosch. 1979. Selective toxicity of purine deoxynucleosides for human lymphocyte growth and function. Proc. Natl. Acad. Sci. 76 : 1998.
5. Dosch, H.M., A. Mansour, A. Cohen, A. Shore, and E.W. Gelfand. 1980. Inhibition of suppressor T cell development following deoxyguanosine administration. Nature 285 : 494.
6. Kwast, Th.H. van der, and R. Benner. 1978. T1 and T2 lymphocytes in primary and secondary delayed type hypersensitivity of mice. I. Contribution in the response to sheep red blood cells and to allogeneic spleen cells. Cell. Immunol. 39 : 194.
7. Wolters, E.A.J., and R. Benner. 1978. Immunobiology of the Graft-versus-Host reaction. I. Symptoms of Graft-versus-Host disease in mice are preceded by delayed type hypersensitivity to host histocompatibility antigens. Transplantation 26 : 40.
8. Bianchi, A.T.J., H. Hooijkaas, R. Benner, R. Tees, A.A. Nordin, and M.H. Schreier. 1981. Clones of helper T cells mediate antigen-specific, H-2 restricted DTH. Nature 290 : 62.
9. Kwast, Th.H. van der, J.G. Olthof, and R. Benner. 1979. Primary and secondary delayed type hypersensitivity to minor histocompatibility antigens in the mouse. Cell. Immunol. 47 : 182.
10. Kwast, Th.H. van der, A.T.J. Bianchi, H. Bril, and R. Benner. 1981. Suppression of antigraft immunity by preimmunization. I. Kinetic aspects and specificity. Transplantation 31 : 79.
11. Bril, H., and Benner, R. 1982. Specific suppression of anti-host immune reactivity in Graft-versus-Host reaction. Adv. Exp. Med. Biol. 149 : 577.
12. Gershon, R.K., D.D. Eardley, S. Durum, D.R. Green, F.W. Shen, K. Yamauchi, H. Cantor and D.B. Murphy. 1981. Contrasuppression a novel immunoregulatory activity. J. Exp. Med. 153 : 1533.
13. Bloom, B.R., L.D. Hamilton, and M.W. Chase. 1964. Effects of mitomycin C on the cellular transfer of delayed type hypersensitivity in the guinea pig. Nature 201 : 689.
14. Wolters, E.A.J., and R. Benner. 1979. Immunobiology of the Graft-versus-Host reaction. II. The role of proliferation in the development of specific anti-host immune responsiveness. Transplantation 27 : 39.
15. Boss, G.R., L.F. Thompson, H.L. Spiegelberg, W.J. Pichler, and J.F. Seegmiller. 1980. Age dependency of lymphocyte ecto-5'Nucleotidase activity. J. Immunol. 125 : 679.

16. Van Laarhoven, J.P.R.M., G.T. Spierenburg, H. Collet, G. Delespesse, and De Bruyn, C.H.M.M. 1983. Enzymes of purine interconversion pathways in T, B, T_γ and T-$T_\gamma$cells from human peripheral blood. Adv. Exp. Med. Biol., in press.
17. Zachowski, A., G. Simonin, J. Aubry, P. Pummier, S.N. Singh, M. Potter, and A. Baraf. 1981. Plasma membrane enzymes in BALB/c lymphomas with either T or B cell properties. I. 5'-Nucleotidase. J. Recept. Res. 2 : 97.
18. Massaia, M., D.D.F. Ma, T.A. Sylvestrowicz, N. Tidman, G. Price, G. Janossy, and A.V. Hoffbrand. 1982. Enzymes of purine metabolism in human peripheral lymphocyte subpopulations. Clin. exp. Immunol. 150 : 148.
19. Carson, D.A., J. Kaye, and D.B. Wasson. 1981. The potential importance of soluble deoxynucleotidase activity in mediating deoxyadenosine toxicity in human lymphoblasts. J. Immunol. 126 : 348.
20. Lelchuk, R., V.M.A. Sprott, and J.H.L. Playfair. 1981. Differential involvement of non-specific suppressor T cells in two lethal murine malaria infections. Clin. exp. Immunol. 45 : 433.
21. Lelchuk, R., A. Cooke, and J.H.L. Playfair. 1982. Differential sensitivity to 2'-deoxyguanosine of antigen-specific and non-specific suppressor T cells in delayed hypersensitivity. Cell. Immunol. 72 : 202.
22. Dosch, H.M., and E.W. Gelfand. 1979. Specific in vitro IgM responses of human B cells: a complex regulatory network modulated by antigen. Immunol. Rev. 45 : 243.