SUPPRESSOR CELL MEDIATED REGULATION OF DELAYED-TYPE HYPERSENSITIVITY TO HISTOCOMPATIBILITY ANTIGENS

REGULATIE VAN VERTRAAGD TYPE OVERGEVOELIGHEID TEGEN TRANSPLANTATIE ANTIGENEN DOOR SUPPRESSOR CELLEN

PROEFSCHRIFT

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Aan Hanne Geeske

Aan onze ouders

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VOORWOORD

Het immuunsysteem van gewervelde dieren heeft als taak vreemde elementen (antigenen) die het lichaam binnendringen te signaleren en onschadelijk te maken. Voorbeelden van deze elementen zijn bacteriën, virussen, parasieten en schimmels. Ook getransplanteerde organen en weefsels worden in het algemeen als lichaamsvreemd herkend en dientengevolge afgestoten. Om haar taak uit te oefenen beschikt het immuunsysteem over niet-specifieke cellen die antigenen in zich kunnen opnemen en verteren (fagocyten) en over specifieke cellen (B en T lymfocyten) die alleen die antigenen kunnen herkennen waarvoor zij een specifieke receptor bezitten. Beide typen cellen ontstaan uit hemopoëtische (= bloedvormende) stamcellen in het beenmerg.

B lymfocyten maken het grootste deel van hun rijping door in het beenmerg. Daarna verhuizen zij naar gespecialiseerde lymfatische organen, zoals de milt en de lymfeklieren. Na contact met hun specifieke antigeen gaan zij in deling en ontwikkelen zij zich tot plasmacellen. Deze plasmacellen produceren antistoffen die kunnen binden aan het antigeen, waardoor het antigeen onschadelijk gemaakt en opgeruimd kan worden.

Voorlopers van T lymfocyten maken een deel van hun rijping door in een gespecialiseerd lymfatisch orgaan buiten het beenmerg: de thymus. Hier leren zij het onderscheid tussen lichaamseigen en lichaamsvreemde elementen en ontwikkelen zij karakteristieke celoppervlakte kenmerken. Op grond van hun functie worden drie soorten T lymfocyten onderscheiden: killer T (Tk), helper T (Th) en suppressor T (Ts) lymfocyten. Tk lymfocyten herkennen antigenen op het oppervlak van lichaamseigen cellen (bijvoorbeeld door virus geïnfecteerde cellen). Vervolgens maken zij deze cellen kapot, hetzij via direct cel-cel contact, hetzij door lytische factoren uit te scheiden. Th lymfocyten helpen B lymfocyten en Tk lymfocyten om hun functie optimaal te vervullen. Hiertoe scheiden zij, na contact met antigeen, verschillende stoffen uit met immuunregulerende werking (lymfokines). Th lymfocyten zijn ook de cellen die vertraagd-type overgevoeligheidsreacties veroorzaken. Ts lymfocyten oefenen een remmende werking uit op de activiteit van zowel Th, Tk als B lymfocyten. Zij dienen als veiligheidsmechanisme om te voorkomen dat een immuunrespons te heftig wordt en/of te lang voortduurt. Men veronderstelt dat Ts lymfocyten ook een rol spelen bij het voorkómen van immuunreacties tegen het eigen lichaam.

Een belangrijk onderdeel van het immuunsysteem is het immunologisch geheugen (memory). Als gevolg van het eerste contact met antigeen delen B en T lymfocyten zich. Van deze delende cellen zullen sommige direct een afweerfunctie tegen het antigeen vervullen, terwijl andere zich ontwikkelen tot zogenaamde memorycellen. Bij hernieuwd contact met het antigeen zullen deze memory-cellen een veel snellere en sterkere afweerreactie veroorzaken dan bij een eerste reaktie op dat antigeen. Op dit gegeven zijn vaccinatie programma's gebaseerd.

B lymfocyten en Ts lymfocyten zijn in staat om antigenen te herkennen die zich vrij in het lichaam bevinden. Daarentegen herkennen Th en Tk lymfocyten slechts antigenen die in combinatie met bepaalde lichaamseigen moleculen op het oppervlak van cellen aan de Th en Tk cellen worden 'gepresenteerd'. Deze lichaamseigen moleculen werden het eerst ontdekt bij transplantatie van tumoren van muizen van een stam naar muizen van een andere stam, waarbij men opmerkte dat in die situatie 'vreemde' tumoren werden

afgestoten. Om deze reden werden deze moleculen transplantatie-antigenen of histocompatibiliteitsantigenen genoemd. De genen, die de code voor deze moleculen bevatten, liggen gegroepeerd in het zogenaamde "Major Histocompatibility Complex" (MHC). Zulke complexen zijn onder andere gevonden in de muis (H-2 complex), de rat (Rt-1 complex), de hond (DLA-complex), de rhesusaap (RhLA-) complex en de mens (HLA-complex). De MHC moleculen zijn zeer polymorf. Dit betekent dat ieder individu een unieke set MHC moleculen op zijn lichaamscellen tot expressie brengt.

Histocompatibiliteitsantigenen spelen niet alleen een rol bij de transplantaatafstoting, maar zij reguleren ook de activiteit van Tk en Th cellen. Op grond van hun bouw worden MHC moleculen in twee klassen verdeeld: klasse I en klasse II. Tk lymfocyten herkennen een antigeen alleen wanneer dat tezamen met een lichaamseigen klasse I molecuul aan die Tk cellen wordt gepresenteerd door een 'antigeen-presenterende' cel. Th lymfocyten daarentegen herkennen antigeen alleen in combinatie met een lichaamseigen klasse II molecuul. Bij orgaan- en weefseltransplantatie herkennen Tk cellen van de ontvanger klasse I transplantatie-antigenen van de donor, terwijl Th lymfocyten van de ontvanger klasse II transplantatie-antigenen van de donor herkennen. Onder goed gedefinieerde experimentele omstandigheden zijn er echter ook uitzonderingen op deze algemene regels gevonden.

Getransplanteerde organen en weefsels kunnen zowel door antistoffen als door T lymfocyten worden afgestoten. Het eerste vindt soms plaats wanneer de ontvanger reeds eerder contact heeft gehad met lichaamsvreemde transplantatie-antigenen (bijvoorbeeld tijdens zwangerschap, bloedtransfusie of eerdere transplantatie). Veel vaker worden getransplanteerde organen en weefsels door T lymfocyten afgestoten. Een dergelijke cellulaire afstoting kan gezien worden als het eindresultaat van een immuunrespons waarbij alle vormen van de T cel afweer betrokken zijn. Eén van deze vormen is de vertraagd-type overgevoeligheidsreactie die door transplantatie-antigenen kan worden opgewekt. Dit proefschrift beschrijft ons onderzoek naar de onderdrukking van vertraagd-type overgevoeligheid tegen transplantatie-antigenen door verschillende typen suppressor cellen.

CHAPTER I

GENERAL INTRODUCTION

GENERAL INTRODUCTION

I.1. DELAYED-TYPE HYPERSENSITIVITY

Delayed-type hypersensitivity (DTH) can be defined as an immunologically specific inflammatory reaction which is maximal at 24 to 96 hrs after elicitation and shows a characteristic histological appearance of infiltration with mononuclear cells (reviewed by Turk, 1980). DTH reactivity has been most widely studied in species such as guinea pig, rat and mouse, employing antigens diverging from bacteria, viruses, fungi, protozoa and synthetic polypeptides to xenogeneic erythrocytes and allogeneic histocompatibility (H) antigens (Crowle, 1975; Van der Kwast et al., 1978). In man, DTH related phenomena are believed to play a role during the course of diseases such as tuberculosis, sarcoidosis, leprosy, listeriosis, leishmaniasis and Chagas' disease. Characteristics of DTH such as kinetics of appearance, strength of the response and composition of the infiltrate vary with the species tested and the antigen used (Crowle, 1975).

DTH can be transferred with lymphoid cells from a sensitized donor to a naive syngeneic recipient (Chase, 1945). This important feature of DTH distinguishes DTH from acute hypersensitivity reactions such as the Arthus reaction and anaphylactic hyperreactivity, which can be transferred by immune serum.

T lymphocytes were found to be the lymphoid cells mediating DTH. This was originally described in a murine model by Cooper and Ada (1972) and by Youdim et al. (1973). They reported that removal of T lymphocytes from a lymphoid cell population of a sensitized donor prevented the adoptive transfer of DTH. These early studies already showed that transfer of a small number of T cells (5×10^2) was sufficient to transfer DTH. Indeed, Marchal et al. (1982) showed that local injection of a single, sensitized T cell, together with the specific antigen, was sufficient to initiate a histologically characteristic DTH response in mice.

Classical DTH responses are thought to develop as follows. Antigen-specific precursors of T cells are stimulated by antigen presented in the context of major histocompatibility complex (MHC) encoded molecules on the cell surface of antigen-presenting cells. In response they proliferate and develop into a recirculating population of sensitized T cells. This is called the induction (afferent) phase of DTH. In response to the second encounter with the (locally injected) antigen, presented in the context of MHC molecules, these sensitized T cells release lymphokines, which attract the non-specific, bone marrow derived macrophages that give the DTH infiltrate its characteristic histological appearance (see section I.2.). This is called the expression (efferent) phase of DTH.

Evidence is accumulating (Schreier et al., 1982; Mossmann and Coffman, 1987) that the DTH effector T cells represent a subpopulation of helper T (Th) cells. The T lymphocytes that mediate DTH in mice are usually found to express the Thy-1^+,L3T4^+,Lyt-1^+,Lyt-2^- phenotype and recognize antigen in the context of MHC class II molecules. However, these findings can vary depending on experimental conditions. For instance, virus-specific cloned Tc cells (Lyt-1^-,2^+, class I restricted) have been found to mediate a DTH-like response after local injection together with antigen (Lin and Askonas, 1981). Moreover, the genetic requirements for a DTH response to virus appear to be highly dependent on the virus' replicating nature (Liew, 1982a). They can be either class I restricted (Zinkernagel, 1976) or class II restricted (Nash et al., 1982) or both (Leung et al., 1980).

A more complex scheme for the efferent phase of DTH was proposed by Askenase and Van Loveren (1983). From their studies on contact hypersensi-

tivity to picryl chloride (PCl) they concluded that a cascade of two populations of Lyt-1$^+$ T cells is required for the elicitation of DTH. The first subset of T cells in the cascade can be detected one day after sensitization. These cells release antigen-specific T cell factors that activate mast cells to release serotonin after encounter with antigen. The serotonin opens gaps between endothelial cells, allowing sensitized T cells from the other Lyt-1$^+$ T cell subset to enter the extravascular space. These T cells are than stimulated by the local antigen and release lymphokines which attract non-specific macrophages (Van Loveren and Askenase, 1984). From a biological point of view, the advantage of the latter scheme is that it is more efficient that antigen-specific T cell factors rather than antigen-specific T cells recirculate through the extravascular space to mediate a form of immune surveillance.

I.2. ASSAYS FOR DELAYED-TYPE HYPERSENSITIVITY

I.2.1. The in vivo foot/ear swelling assay

The most widely used assay to measure in vivo DTH reactivity in mice is the foot swelling assay, originally described by Gray and Jennings (1955). They injected a tuberculin solution into a metatarsal pad of the tensed plantar surface of a hind foot of tuberculous mice. Subsequently, a swelling and induration appeared at the site of injection. Typically, such a DTH reaction peaks at 18 to 24 hours, is still present significantly at 48 hours and than disappears within about two days (Halliday and Webb, 1969; Blanden et al., 1969; Anacker et al., 1969). Objective measurements can be performed with a set of calipers (Youdim et al., 1973) or a specialized foot thickness meter (Bonta and De Vos, 1965), or by determining the amount of fluid that the swollen foot will displace in a plethysmograph (Uyeki et al., 1969; Pearson et al., 1971). Since its original description the foot swelling assay has been used to elicit and measure DTH to antigens such as haptens, bacteria and viruses (Crowle, 1975), xenogeneic erythrocytes (Liew, 1977) and allogeneic H antigens (Kon and Klein, 1976; Van der Kwast and Benner, 1978; Liew, 1982b). A major advantage of the foot swelling assay is that it can be rapidly mastered and performed, which permits its use in large scale experiments. A major disadvantage of the foot swelling assay is that its measurement is prone to variability due to intra- and inter-observer variation. For instance, handling of calipers cannot be performed repeatedly with exactly the same force. To circumvent such disadvantage we routinely use a thoroughly balanced foot thickness meter which minimizes the force which is exerted on the foot during foot thickness measurements.

A DTH-like reaction can be induced in a foot also by injecting sensitized lymphocytes together with the specific antigen. This variation of the foot swelling assay is called the immune-lymphocyte-transfer (ILT) assay. ILT assays can be used to monitor alloimmunity. In that case immune lymphocytes from an allosensitized donor are injected into a hind foot of recipients whose tissues express the specific alloantigens.

Histologically a foot swelling due to DTH is characterized by a cellular infiltrate in which mononuclear cells rather than polymorphonuclear cells predominate, and wide spread edema, characterized by the spreading apart of connective tissue fibers. This infiltrate distinguishes a DTH response from an ordinary inflammatory response.

The mouse ear has proven very appropriate to measure contact hypersensitivity (CHS) reactions to topically applied chemicals (Asherson and Zembala, 1970). Ear swelling measurements are usually performed with dial gauge calipers (Dietrich and Hess, 1970). DTH responses can also be elicited in the ear by subcutaneous (s.c.) injection of antigen. Some investigators have claimed that ear testing for DTH is more sensitive than foot testing for DTH (Robinson and Naysmith, 1976). Also other sites to elicit murine DTH responses in vivo have been described, such as the flank (Crowle, 1959), the tail (Hartley et al., 1983) and the abdominal cavity (Tasaka et al., 1985).

I.2.2. The in vivo radioactivity accumulation assay

In 1972, Sabolovic et al. described an assay to measure DTH reactivity in vivo, based on the accumulation of radiolabelled inflammatory cells at the site of elicitation of DTH (for example ear or foot). In principle, a labelling dose of an isotope that can be incorporated into DNA during mitosis is injected into experimental animals some time after sensitization. One day after challenge of the animal with the specific antigen the challenge site is removed and its radioactivity is determined. Compounds such as tritiated thymidine (^{3}H-TdR; Sabolovic et al., 1972) and iodinated uridine (^{125}I-UdR; Pritchard and Micklem, 1974) can be used. Variations of this principle, employing ^{51}Cromium (^{51}Cr) labelled sensitized cells (Sabbadini et al., 1974) or ^{125}I-labelled mouse albumin to measure the extent of the edema at the reaction site (Paranjpe and Boone, 1972) have been described as well.

The advantages of this assay, in comparison with the foot/ear swelling assay are claimed to be: greater objectivity, greater sensitivity, greater reproducibility and greater accuracy for less intense reactions (Lefford, 1974; Vadas et al., 1975). Obvious disadvantages are its greater labouriousness and expensiveness as well as the use of radioactivity.

I.2.3. The in vitro macrophage migration inhibition assay

In response to contact with antigen, sensitized lymphocytes produce a panel of lymphokines with biological activity ranging from inhibitory (e.g. migration inhibiting factor: MIF) to stimulatory (e.g. macrophage activating factor: MAF; interferon: IFN; interleukin 2: IL-2). The biological activity of MIF has been used to establish an in vitro assay for DTH: the macrophage migration inhibition assay. The first report about the use of murine lymphoid cells in this assay was published by George and Vaughan (1962). In principle, living lymphoid cells from a sensitized animal are packed into capillary tubes, which are placed in culture medium with or without the specific antigen. After 24 hours incubation the areas of cell migration from the orifices of the tubes are determined, from which a migration index is calculated (Phillips et al., 1972a). A low migration index correlates with a high degree of DTH in the sensitized donor, and vice versa. This assay has been used to detect DTH to bacteria (Smith and Bigley, 1972) and viruses in mice (Mortensen et al., 1973) and to monitor antigraft immunity (Friedman and Ceglowski, 1973).

This method bears several disadvantages. Firstly, the determination of the migration area ('halo') is subject to inter-observer variation. Secondly, since MIF production is only one of several events occurring during DTH reactions in vivo, a positive MIF test does not always correlate with in

vivo manifestations of DTH (Phillips et al., 1972b). Thirdly, MIF-activity can be masked by the activity of other lymphokines (e.g. macrophage stimulating factor: MStF (MacSween et al., 1982).

I.2.4. The in vitro lymphocyte transformation assay

Another effect of the first encounter with antigen is that lymphocytes may transform into blast cells (characterized by expanded nuclei and cytoplasm) and will proliferate. The resultant increase in DNA synthesis can be assayed in vitro by measuring the uptake of ^{3}H-TdR into proliferating cells (Warnatz et al., 1972). The lymphocyte transformation assay can be used to monitor cell-mediated immunity to allogeneic H antigens and is than called the mixed-lymphocyte-culture (MLC) assay. In principle either highly irradiated (one-way MLC) or unirradiated (two-way MLC) stimulator cells are cocultured with living allogeneic lymphoid responder cells. The responder cells proliferate in response to the alloantigens that are presented on the surface of the stimulator cells. Strongest murine MLC responses are elicited by class II alloantigens (Klein et al., 1972; Meo et al., 1973). The MLC assay is generally considered to be an in vitro model for the early events of DTH induction and T cell activation. Remarkably, injection of culture supernatant of in vitro activated T cells into the dermis of a mouse results in a DTH response which is histologically indistinguishable from cellularly induced DTH (Phillips et al., 1972b).

I.3. SUPPRESSOR CELL MEDIATED REGULATION OF DELAYED-TYPE HYPERSENSITIVITY

DTH reactivity is susceptible for various modes of regulation (Turk, 1980). Regulation by suppressor cells represents one of those modes. Each of the three major cell types of the immune system (i.e. T cells, B cells and macrophages) can inhibit DTH under particular experimental conditions. Macrophages are usually found to exert a non-specific suppressive effect (Nelson, 1976), whereas B and T cells are the mediators of antigen-specific suppression (Koenig and Hoffman, 1979; Green et al., 1983). However, under well defined experimental conditions non-specific suppression exerted by T cells has been reported as well (Bianchi et al., 1983).

I.3.1. T cell mediated suppression of DTH.

The first description of antigen-specific suppression was reported by Gershon and Kondo (1970). They injected mice with a high dose of sheep red blood cells (SRBC) and observed a depressed antibody response to a subsequent challenge with SRBC. Gershon et al. (1972) later identified T lymphocytes to be the mediators of this suppression. Since then Ts cells regulating immune responses such as antibody production, and DTH to haptens (Bach et al., 1979), xenogeneic erythrocytes (Liew, 1977), and allogeneic H antigens (Van der Kwast et al., 1981; Liew, 1982b; Bianchi et al., 1984) have been described. Ts cells are defined by their functional activity in in vitro assays, for example suppression of MLC reactivity and cell-mediated-lymphocytoxicity (CML) when Ts are added in culture, or in in vivo assays, for example the induction of unresponsiveness by the adoptive transfer of Ts cells to naive, syngeneic recipients which are subsequently immunized and assayed for DTH (Hutchinson, 1986).

From such studies it appeared that at least two and possibly three

mutually interacting subsets of Ts cells are involved in the regulation of DTH. After appropriate immunization with haptens, antigen-specific Lyt-1^+2^- Ts inducer (Ts ind, Ts1) cells become activated, which interact with idiotype-specific Lyt-1^+2^+ Ts transducer (Ts trans, Ts2) cells. These Ts2 cells activate antigen-specific Lyt-1^-2^+ Ts effector (Ts eff, Ts3) cells, which in turn elaborate non-specific suppressor factors. These factors ultimately cause the suppressive effect (Dorf and Benacerraf, 1984).

Ts1 cells usually suppress the induction phase of DTH, i.e. the proliferation of draining lymph node cells in response to immunization, whereas Ts2 and Ts3 cells usually suppress the expression phase of DTH (i.e. the activity of already activated DTH effector T cells). With regard to the latter mode of action it has been shown by Ptak et al. (1987) that Lyt-2^+ Ts cells can inhibit the activity of the first T cell in the DTH cascade that has been proposed by Van Loveren and Askenase (see section I.1.).

Studies with Ts hybridomas revealed that suppressor factors are involved in the idiotypic-anti-idiotypic interactions in the Ts cell cascade (Webb et al., 1983). Moreover, the Ts cascade would be subject to regulation by MHC genes and Igh-linked genes. The MHC gene that controls Ts cell activity in mice was previously thought to be located in the serologically defined I-J subregion of the H-2 complex. This was concluded from studies which showed that treatment of Ts cells with anti-I-J antibodies and complement completely blocked the adoptive transfer of suppression (Murphy et al., 1976). However, Steinmetz et al. (1982), employing the technique of gene transfer, showed that there is no DNA within the H-2I subregion that encodes for I-J molecules. The gene(s) encoding for I-J molecules may occur somewhere else in the genome and may be influenced by MHC class II genes, for instance I-E (Klein et al., 1985).

With regard to the regulation of DTH to alloantigens by Ts cells the situation is slightly less complex. So far in vitro as well as in vivo studies of DTH (Beckwith and Rich, 1982; Chaouat et al., 1982; Liew, 1982b) revealed evidence for only two types of mutually interacting Ts cells, which exert their activity through the release of specific and non-specific suppressor factors. The Ts eff cells that suppress DTH reactivity in vivo have been characterized by our group. These Ts cells can be induced by i.v. immunization of mice with a relatively high dose (5×10^7) of irradiated allogeneic spleen cells. They express the Lyt-1^+2^+ phenotype, can inhibit both phases of DTH, and are antigen-specific with regard to the ability to become activated. However, they can also suppress the DTH response to third party alloantigens. This occurs when the latter are administered during the induction or elicitation of DTH, together with the alloantigens that had induced the suppressor cells (Van der Kwast et al., 1981; Bianchi et al., 1983; 1984; 1986).

The above described data all have been obtained in experimental models where Ts cells were induced by some form of immunization. However, Ts cells also appear after non-immunizing treatments, such as total lymphoid irradiation (TLI; Strober, 1984), and ultraviolet (UV) irradiation (Kripke, 1984). In view of its possible application in humans the latter treatment has gained much interest. The UV wave length region can be divided into three subregions: UV-A (320-400 nm), UV-B (280-320 nm) and UV-C (200-280 nm). The first observations concerning the induction of unresponsiveness by UV irradiation came from Fisher and Kripke (1977). They reported that skin tumors arizing after chronic exposure to UV-B irradiation were not rejected by the UV-B exposed, tumor-bearing mice, but were rapidly rejected after transfer to syngeneic, non UV-B exposed recipients. The tumor tolerance

could be transferred to syngeneic recipients with splenic T lymphocytes from UV-B exposed mice (Fisher and Kripke, 1978). Later it was found that CHS responses of mice to chemicals were suppressed after prior exposure to UV-B (Noonan et al., 1981).

Since then each UV subregion has been explored for its immunosuppressive activity (Austad and Mork, 1983; Kripke, 1984). Although under appropriate conditions UV-A and UV-C irradiation were found to exert some degree of immunosuppression, the UV-B region proved to be the most effective. The induction of suppression of tumor rejection required a dose of UV-B ten times higher than the induction of suppression of CHS (Kripke, 1984). The UV-B induced Ts cells are usually detected when UV-B exposed mice are contact-sensitized some days after irradiation (Noonan et al., 1981), and are than found to be antigen-specific for the contact sensitizing agent (Ullrich, 1985).

UV-B irradiation penetrates the skin only very superficially (Kripke, 1984). For the activation of Ts cells in the spleen by this skin-associated treatment two major possibilities have been proposed. Firstly, an effect on Langerhans cells has been suggested (Noonan and Kripke, 1981). However, the effects of UV-B on Langerhans cells and the induction of suppression of CHS appeared to have different wave length dependencies (Noonan, 1984). Secondly, a soluble mediator might be released from the skin. This mediator might activate Ts cells in the spleen. Indeed, evidence for the latter mechanism is accumulating (Swartz, 1984; Harriott-Smith and Halliday, 1986; Schwarz, 1986; 1987; Ross et al., 1986; 1987).

I.3.2. B cell mediated suppression of DTH

Often the decline of a DTH response is associated with a rise in antibody level (Turk, 1975). This could indicate that the mitigation of DTH is due to a B cell dependent mechanism. Indeed, Neta and Salvin (1974) presented evidence for such a mechanism in guinea pigs. Sy et al. (1979) found anti-idiotypic antibodies in the serum of mice several days after mitigation of CHS to dinitrofluorobenzene (DNFB). These antibodies could prevent the adoptive transfer of CHS by binding to the CHS reactive T lymphocytes. A similar finding has been reported by Colizzi et al. (1983) for the inhibition of DTH to purified protein derivative (PPD) by serum from Bacillus-Calmette-Guerin (BCG) infected mice.

B cells with suppressive activity are also activated by polyclonal B cell stimulators such as bacterial lipopolysaccharide (LPS). LPS activated B cells have been found to inhibit the induction of DTH to SRBC (Lagrange et al., 1975; Gill and Liew, 1979), as well as antibody production to SRBC (Koenig and Hoffmann, 1979).

Besides a direct suppressive effect on immune responses, B cells that play a role in the induction of Ts cells have been found as well. Hausmann et al. (1986) reported the *in vitro* induction of Ts eff cells specific for the 4-hydroxy-3-nitrophenyl acetyl (NP) hapten by *in vitro* activated anti-idiotypic B cells. Campa et al. (1986) reported that idiotypic B cells, induced by BCG infection of mice, activated anti-idiotypic B cells which, in turn, activated idiotypic Ts cells that suppressed DTH to BCG.

I.3.3. Macrophage mediated suppression of DTH

Macrophages mediating suppressive effects on cell-mediated immunity in mammals have been found after injection with Corynebacterium parvum

(Kirchner et al., 1975), after oral carrageenan administration (Cochran and Baxter, 1984), after experimental liver injury (Tajima et al., 1985) and after administration of LPS (Nelson, 1976). Suppression of DTH is usually found to be antigen non-specific and can affect both phases of DTH (Ellner and Spagnuolo, 1979; Nakano and Nakano, 1985).

Moreover, macrophages have been found to present suppressor factor(s) to the T cells in the Ts cascade (Dorf and Benacerraf, 1984).

I.4. DELAYED-TYPE HYPERSENSITIVITY AND ALLOGRAFT REJECTION

In 1958, Brent et al. suggested that DTH and skin allograft rejection in mice could be due to a common mechanism. They noted similarity between the histological picture of DTH and that of skin allograft rejection. They also found that DTH responses could be elicited by intracutaneous injection of allogeneic cells into a previously sensitized recipient. Moreover, skin allografts on tolerized mice were rejected rapidly after infusion with lymphoid cells from syngeneic allosensitized donors (Brent et al., 1962).

After the discovery of Tc cells (Govaerts, 1960), this cell type was believed to be the effector cell of allograft rejection (Cerottini and Brunner, 1974; Berke; 1980). In 1981 the role of DTH in allograft rejection was reexamined by Loveland et al. The latter authors studied the capacity to reject allogeneic skin grafts of different T cell subsets after infusion into adult thymectomized, lethally irradiated, bone marrow reconstituted (ATXBM), hence T cell deprived, mice and found that this could only be accomplished by infusion of Lyt-1^+2^- T cells obtained from a sensitized donor. Their results were confirmed in a study with T cel deprived (ATXBM) rats by Dallman et al. (1982). Moreover, Loveland and McKenzie (1982a) showed a correlation between the ability of T cell deprived mice infused with sensitized Lyt-1^+2^- cells to mount a DTH response to alloantigens and to reject skin and tumor allografts. Other investigators (Greene et al., 1980) found a complete correlation between the development of DTH to the male antigen H-Y and the rejection of male skin allografts. Liew and Simpson (1982) found that the genetic control of male skin allograft rejection appeared indistinguishable from that of DTH to H-Y.

In their study with T cell deprived rats, Dallman et al. (1982) had noted a large proportion of cells with the $OX8^+$ (cytotoxic) phenotype within the rejecting grafts. These cells are thought to originate from radioresistant precursors of Tc cells (Duprez et al., 1982) and apparently escaped the T cell depletion of the animals. This already indicated that the situation is more complex than initially suggested by Loveland et al. (1981). Moreover, Engers et al. (1982) showed that cloned Lyt-2^+ T cells could reject allogeneic tumors in vivo and Tyler et al. (1984) showed that such T cells could reject allogeneic tissue in an immune-lymphocyte-transfer assay.

Evidence for the combined activity of Th cells and Tc cells in allograft rejection has been reported as well. Th cells from non-sensitized donors were not nearly as effective as unseparated non-sensitized T cells in the transfer of the ability to reject skin allografts in T cell deprived mice (Loveland and McKenzie, 1982b). Moreover, both T cell subsets as well as IL-2 were required for the rejection of non-MHC incompatible rat heart allografts (Mason et al., 1984).

Recently, Rosenberg et al. (1987) showed that skin allograft rejection in mice required both lymphokine-producing Th cells and lymphokine-reactive

Tc cells. Remarkably, the phenotype of both T cell subsets was determined by the alloantigens eliciting the rejection. In case of a class I incompatibility, skin graft rejection required Th and Tc cells which expressed the Lyt-2^+ phenotype. In contrast, in case of a class II incompatibility, skin graft rejection required Th and Tc cells which both expressed the L3T4^+ phenotype. Thus, as has been suggested by Swain (1981), there appears to be a better correlation between the phenotype of T cells and the class of MHC antigen they recognize than between T cell phenotype and function.

In conclusion, it appears that neither Th cells nor Tc cells alone mediate allograft rejection. Rather, allograft rejection is accomplished by an interplay between Th and Tc cells. Both these cell types could be susceptible for regulation by Ts cells (Hutchinson, 1986).

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CHAPTER II

INTRODUCTION TO THE EXPERIMENTAL WORK

INTRODUCTION TO THE EXPERIMENTAL WORK

DTH to allogeneic histocompatibility antigens is a T cell dependent reaction, which can be induced in immunologically competent individuals by subcutaneous (s.c.) or intravenous (i.v.) administration of alloantigens (Van der Kwast and Benner, 1978; Bianchi et al., 1984). After immunization, activated antigen-specific T cells and, possibly, T cell derived factors circulate through the body and mediate an effective immune-surveillance. After reencounter with the specific antigen, for example after injection of the relevant allogeneic spleen cells into a hind foot, these T cells or their factors cause the release of vasoactive substances, which lead to the accumulation of granulocytes, mononuclear cells and edemic fluid. This inflammatory response appears as a swelling of the site of reencounter of the antigen, which is maximal between 24 and 96 hours after antigen administration. The delayed onset distinguishes DTH from the more immediate types of hypersensitivity.

DTH can also be induced in immunologically incompetent individuals, by the infusion of allogeneic, immunocompetent T cells. This leads to a state of DTH directed to the host histocompatibility antigens (Wolters and Benner, 1978). Anti-host DTH can be assayed by secondary transfer of lymphoid cells from the irradiated recipients to naive secondary recipients, syngeneic to the original spleen cell donors. The secondary recipients are subsequently challenged in a hind foot with spleen cells expressing the alloantigens of the primary irradiated recipients.

The T cells that mediate DTH probably represent a subset of Th cells (Bianchi et al., 1981; Mossman and Coffman, 1987), although certain cloned Tc cells mediating DTH have been described (Lin and Askonas, 1981; Weiss and Dennert, 1981).

Although the phenomena related to DTH vary with the antigen used and the species tested, the mouse has been widely accepted as a suitable species to investigate DTH (Crowle, 1975). The existence and use of a broad variety of inbred and recombinant strains has greatly facilitated studies aimed at increasing knowledge of regulation of DTH to various types of antigen, such as bacteria, virusses, heterologous erythrocytes and allogeneic histocompatibility antigens (Crowle 1975; Van der Kwast, 1978).

This thesis deals with the regulation of DTH to allogeneic histocompatibility antigens by suppressor cells. Three modes of induction of suppressor cells were employed, namely immunization with allogeneic spleen cells, combined immunization with LPS and allogeneic spleen cells, and exposure to UV-B irradiation.

Chapter III deals with the state of suppression and suppressor cells induced by i.v. or local immunization with allogeneic spleen cells. Sections 1 and 2 of this chapter describe that i.v. immunization with alloantigens induces a population of Ts ind cells. The phenotypical and functional characteristics of these cells have been investigated. It is shown that the generation of these Ts ind cells leads to the activation of Ts effector (Ts eff) cells that suppress DTH. The characteristics of the latter cell type have been investigated extensively by our group (Van der Kwast et al., 1981; Bianchi et al., 1984; 1986). Section 3 of this chapter describes the phenotypical and functional characteristics of Ts eff cells that are induced by local immunization (s.c. in the hind feet) with alloantigens (Hind-Foot-Immunization (HFI)). This method was originally described by

Rich and Rich (1973), who investigated the *in vitro* activity of HFI-induced Ts cells. Our study represents the first extensive investigation of the *in vivo* activity of HFI-induced Ts cells.

Chapter IV describes the suppression induced by simultaneous systemic (i.v.) immunization with bacterial lipopolysaccharide and local immunization (s.c. in the inguinal areas) with alloantigens. The state of suppression induced by this procedure is mediated by B cells. These B cells can suppress the development of DTH under host-versus-graft conditions (sections 1 and 2) as well as under graft-versus-host conditions (section 3). Evidence is presented that this B cell dependent suppression is mediated by antibodies that are directed towards the antigen-receptor of DTH reactive T cells.

Chapter V describes the induction of Ts cells by exposure of mice to UV-B irradiation. Obviously, these Ts cells are non-specific, since they were not induced by means of immunization. UV-B irradiation is known to penetrate the skin only superficially (Kripke, 1984). Still, Ts cells can be detected in the spleen. A clue for the connection between UV-B irradiation and the induction of Ts cells is presented in section 2 of this chapter, where we describe that UV-B induced, skin derived serum factor(s) can activate Ts cells that suppress DTH to alloantigens.

These studies show that T cells mediating DTH to allogeneic histocompatibility antigens are very susceptible for the activity of suppressor cells induced by the various modalities. In view of the role that DTH reactive T cells may play in allograft rejection (see section I.4) these studies may aid to the development of new methods to prevent allograft rejection, based on the selective inhibition of those T cells that are specific for the allogeneic histocompatibility antigens.

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CHAPTER III

INDUCTION OF SUPPRESSION BY IMMUNIZATION WITH ALLOANTIGENS

CHAPTER III.1.

EVIDENCE FOR AN INDUCER-SUPPRESSOR T CELL IN THE REGULATION OF DTH TO ALLOANTIGENS

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INTRODUCTION

Delayed-type hypersensitivity (DTH) to allogeneic histocompatibility (H) antigens in mice is a T cell dependent phenomenon which can be induced by immunization with irradiated or non-irradiated allogeneic spleen cells. DTH responses can be elicited some days later by subcutaneous (s.c.) injection of a dose of the relevant allogeneic spleen cells into a hind foot. Hind foot thickness measured 24 hrs later is than a measure for the strength of the DTH response (1). The DTH reactive T cells probably represent a subset of helper T cells (2, 3). Bianchi et al. (4) reported that DTH to H antigens can be regulated by antigen-specific suppressor T (Ts) cells induced by intravenous (i.v.) tolerization with a high dose of irradiated allogeneic spleen cells. These Ts cells where shown to be Lyt-$1^+,2^+$ (4) and appeared to be capable of suppression of the induction phase (4) as well as the expression phase of DTH (5). Moreover, depending on the experimental conditions the DTH response against third party alloantigens could be suppressed (6). Since it has been shown in other experimental systems that Ts effector (Ts eff) cells can be activated through a cascade of cell interactions (reviewed in ref 7 and 8), we investigated the putative presence of an inducer Ts cell in the regulation of DTH to H antigens.

MATERIALS AND METHODS

Mice

(C57BL/Rij x CBA/Rij)F1 (H-$2^{b/q}$) and C3H/Law (H-2^{k}) female mice were purchased from the Radiobiological Institute TNO, Rijswijk, The Netherlands. DBA/2 (H-2^{d}), BALB/c (H-2^{d}, Igh^{a}), B10.D2 (H-2^{d}) and BALB. K(H-2^{k}) mice were purchased from HARLAN OLAC Ltd., Bicester, U.K. C.B.20 (H-2^{d}, Igh^{b}) female mice were bred and raised at our department. The animals age ranged from 10 to 14 weeks at the start of each experiment.

Preparation of cell suspensions and immunization

Spleens from donor mice were minced with scissors and squeezed through a nylon gauze filter to get a single cell suspension. The cells were suspended in a balanced salt solution (BSS). Nucleated cells were counted with a Coulter Counter model BZI (Coulter Electronics, Harpenden, U.K.). DTH reactivity was induced by s.c. immunization with 1 x 10^{7} allogeneic spleen cells injected into the inguinal areas in a total volume of 100 ul BSS. Suppression of DTH reactivity was induced by i.v. tolerization with 5 x 10^{7} irradiated (20 Gy) allogeneic spleen cells in a volume of 0.5 ml BSS prior to induction of DTH.

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

Cell suspensions were treated for 30 min at 4°C with anti-Thy-1.2 (clone F7D5, HARLAN OLAC Ltd.), anti-Lyt-1.1 (clone 7-20.6/3 Cedarlane Laboratories Ltd., Hornby, Ontario, Canada), anti-Lyt 2 (produced by in vitro culture of an anti-Lyt-2 producing hybridoma, which was kindly provided by Dr. F.W. Fitch, Department of Pathology, University of Chicago, U.S.A.). After incubation the cells were centrifuged, resuspended in BSS and incubated with guinea pig complement (Behringwerke AG, Marburg-Lahn, F.R.G.) for 30 min at 37°C. The cells were washed three times and resuspended in BSS. This procedure eliminated at least 90% of the viable lymphocytes detected by the monoclonal antibody used.

Assay for DTH

DTH responses were elicited by s.c. injection of a challenge dose of 2 x 10^{7} allogeneic spleen cells into the dorsum of the right hind foot of previously immunized mice. Foot thickness was measured 24 hrs later. A group of nonimmunized control mice which only received the challenge injection was included in each experiment. The percentage specific increase of foot thickness was calculated as the mean percentage increase of foot thickness from each experimental group minus the mean percentage increase of foot thickness from the challenge control group. The increase of foot thickness of these control mice varied between 15 and 25%.

RESULTS AND DISCUSSION

Four days after i.v. tolerization of (C57BL x CBA)F1 mice with DBA/2 spleen cells the recipients' spleens were found to contain suppressor cells since adoptive transfer of recipient spleen cells to syngeneic recipient mice (one spleen equivalent per recipient) caused suppression of DTH to DBA/2 H antigens in these recipients (Fig. 1, line 2).

When spleen cells from i.v. tolerized (C57BL x CBA)F1 mice were adoptively transferred to syngeneic recipients one instead of four days after

Responder strain	Donor strain	Transfer		S.C. Immunization		% Specific Increase Foot Thickness	Suppression
		Day 1	Day 4	Day 1	Day 4		
(C57BL × CBA)F_1	(C57BL×CBA)F_1	–	+	–	DBA/2		
(C57BL × CBA)F_1	DBA/2	–	+	–	DBA/2		80 %
(C57BL × CBA)F_1	(C57BL×CBA)F_1	+	–	DBA/2	–		
(C57BL × CBA)F_1	DBA/2	+	–	DBA/2	–		0 %
(C57BL × CBA)F_1	(C57BL×CBA)F_1	+	–	–	DBA/2		
(C57BL × CBA)F_1	DBA/2	+	–	–	DBA/2		69 %
(C57BL × CBA)F_1	(C57BL×CBA)F_1	+(IRR)	–	–	DBA/2		
(C57BL × CBA)F_1	DBA/2	+(IRR)	–	–	DBA/2		2 %

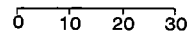

Fig. 1. Transfer of i.v. induced tolerance for alloantigens by spleen cells. (C57BL x CBA)F1 responder mice received an i.v. injection of 5×10^7 irradiated DBA/2 spleen cells. Control mice received an i.v. injection of syngeneic spleen cells. One or four days later spleen cells from these mice were adoptively i.v. transferred to syngeneic recipient mice (one whole spleen equivalent per recipient). DTH reactivity was induced in these recipients by s.c. immunization with DBA/2 spleen cells within two hours after transfer or three days later, as indicated. Five days after s.c. immunization all mice were assayed for DTH. Each column represents the mean response ± 1 SEM (n=5). The degree of suppression was calculated as percentage decrease of DTH response as compared with the relevant control group.

tolerization, DTH reactivity could be induced by immediate s.c. immunization of the recipients with DBA/2 spleen cells (Fig. 1, line 4). However, s.c. immunization three days after transfer could only induce a marginal DTH response (Fig. 1, line 6). Irradiation of the spleen cells to be transferred again facilitated the induction of DTH reactivity in the recipients three days later (Fig. 1, line 8). These data indicate that one day after i.v. tolerization the spleen contains a population of cells that is not suppressive by itself, but that induces a state of suppression in recipient mice.

Transfer of T cell-depleted spleen cells from C3H/Law donor mice i.v. tolerized with (C57BL x CBA)F1 spleen cells one day previously did not induce a state of suppression in the recipients (Fig. 2, line 3). Depletion of Lyt-1^+ cells from the spleen cells to be transferred also prevented the transfer of suppression (Fig. 2, line 4). However, depletion of Lyt-2^+ cells did not prevent the early transfer of suppression (Fig. 2, line 5). A mixture of anti-Lyt-1.1 treated spleen cells (still containing Lyt-2^+ cells) and anti-Lyt-2 treated spleen cells (still containing Lyt-1^+ cells) again did induce a state of suppression of DTH in the recipients (Fig. 2, line 6). This indicates that the cell responsible for the early transfer of

suppression has the Thy-1^+, Lyt-1^+,2^- phenotype. This cell-type will be referred to as inducer Ts (Ts ind) cell.

Evidence for Ts cells of DTH to H antigens has also been reported by Liew (9). He reported the presence of splenic Lyt-1^+,2^- primary Ts cells three days after i.v. tolerization with 1.5 x 10^8 irradiated allogeneic lymphoid cells. According to his interpretation, these cells could suppress DTH. Liew also reported that three days after an intraperitoneal (i.p.) booster immunization secondary splenic Ts cells were found that expressed the Lyt-1^+,2^+ phenotype. The difference in kinetics of appearance of the former cell type in his model and the Ts ind cells described in this paper might be due to the three-fold higher antigen dose applied by Liew. However, the possibility that after adoptive transfer the primary Ts cells induced secondary Ts cells that suppressed the induction and/or expression phase of DTH was not excluded. Thus, this primary Ts cell might well be an example of a Ts ind cell.

Hutchinson (10) reported that in the rat a Ts cell expressing the $CD4^+$,8^- ($W3/25^+$, $OX8^-$) phenotype could be detected three days after receiving a kidney allograft. Transfer of these cells to a syngeneic rat resulted in specific long term survival of a kidney allograft transplanted several days later. In rats receiving donor-specific blood transfusions two populations of $CD8^+$ ($OX8^+$) suppressor cells were found. He suggested that in the rat a Ts cell cascade is involved in suppression of allograft rejection.

Responder	Donor strain	Treatment of transferred spleen cells on day 1	S.C. Immunization on day 4	% Specific Increase Foot Thickness	Suppression
C3H/Law	C3H/Law	C'	(C57BL × CBA)F_1		
C3H/Law	(C57BL × CBA)F_1	C'	(C57BL × CBA)F_1		73 %
C3H/Law	(C57BL × CBA)F_1	anti-Thy-1.2 + C'	(C57BL × CBA)F_1		0 %
C3H/Law	(C57BL × CBA)F_1	anti-Lyt-1.1 + C'	(C57BL × CBA)F_1		14 %
C3H/Law	(C57BL × CBA)F_1	anti-Lyt-2 + C'	(C57BL × CBA)F_1		71 %
C3H/Law	(C57BL × CBA)F_1	anti-Lyt-1.1 + C' + anti-Lyt-2 + C'	(C57BL × CBA)F_1		68 %

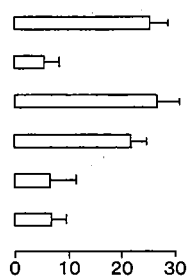

Fig. 2. Phenotype characterization of the Ts ind cell. C3H/Law mice received an i.v. injection of 5 x 10^7 irradiated (C57BL x CBA)F1 or C3H/Law spleen cells. One day later their spleen cells were treated in vitro with monoclonal anti-Thy-1.2, anti-Lyt-1.1 or anti-Lyt-2 antibodies and complement, or complement only. The residual cells were adoptively i.v. transferred to syngeneic recipients. One group of mice received a mixture of anti-Lyt-1.1. treated spleen cells and anti-Lyt-2 treated spleen cells. Three days later DTH-reactivity was induced with (C57BL x CBA)F1 spleen cells. Another five days later all mice were assayed for DTH. Each column represents the mean response $\pm$ 1 SEM (n=5). The degree of suppression was calculated as percentage decrease of DTH response as compared with the relevant control group.

Studies in the mouse on T cell-mediated suppression of DTH to haptenized spleen cells (7), or suppression of antibody production to lactate dehydrogenase (LDH-B,8) showed that several interacting populations of T cells may constitute a suppressor cell cascade. Depending on the experimental conditions a Ts ind cell, usually expressing the Lyt-1^+,2^- phenotype, activates a Lyt-1^-,2^+ Ts eff cell either directly (8) or via an interaction with a Lyt-1^+,2^+ transducer Ts (Ts trans) cell (7).

The Ts ind cell of DTH to alloantigens might well correlate with the Ts ind cell described in these systems. In our system the induction phase as well as the expression phase of DTH to alloantigens can be suppressed by Ts eff cells expressing, just like Ts trans cells, the Lyt-1^+,2^+ phenotype (4,5). As yet we have no evidence for a third Ts cell population in the suppression of DTH to alloantigens.

The above described interactions between Ts ind cells and other cells of the Ts cascade in the mouse are subject to MHC- and Igh-restriction (7, 8). Furthermore, Hutchinson (10) reported that a sonicate of $CD4^+$ Ts cells induced suppression of allograft rejection in a manner restricted by minor but not MHC-antigens. We investigated whether the interaction between the Ts ind cells and the Ts eff cells that are involved in the regulation of DTH to alloantigens are also subject to MHC- and Igh-restriction. Therefore, naive BALB/c recipient mice received spleen cells either from MHC-incompatible BALB.K mice tolerized with B10.D2 spleen cells or from Igh-incompatible C.B.20 mice tolerized with (C57BL x CBA)F1 spleen cells one day previously. As shown in Fig. 3, lines 2 and 4, neither the presence of a MHC barrier nor the presence of an Igh-barrier prevented the transfer of suppression by Ts ind cells. These findings suggest that the Ts ind cells can induce a state of suppression across MHC- and Igh-barriers. However, since unfractionated spleen cell suspensions were transferred, the possibi-

Responder strain	Donor strain	Recipient (day 1)	S.C. Immunization (day 4)	% Specific Increase Foot Thickness	Suppression
BALB.K (H-2^k)	BALB.K	BALB/c (H-2^d)	B10.D2		
BALB.K	B10.D2	BALB/c	B10.D2		64 %
CB.20 (IgH^b)	CB.20	BALB/c (Igh^a)	(C57BL × CBA)F_1		
CB.20	(C57BL × CBA)F_1	BALB/c	(C57BL × CBA)F_1		70 %

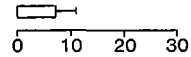

Fig. 3. Transfer of the state of suppression over MHC and Igh barriers. BALB.K and C.B.20 mice received an i.v. injection of 5×10^7 B10.D2 or (C57BL x CBA)F1 spleen cells, respectively. Control mice received syngeneic spleen cells. One day later their spleen cells were obtained and adoptively i.v. transferred to naive BALB/c recipients. Three days later DTH reactivity was induced by s.c. immunization with B10.D2 or (C57BL x CBA)F1 spleen cells, respectively. Another five days later all mice were assayed for DTH. Each column represents the mean response $\pm$ 1 SEM (n=5). The degree of suppression was calculated as percentage decrease of DTH response as compared with the relevant control group.

lity remains that the Ts ind cells interacted with other T cells present in the transferred inoculum and activated these cells to become the Ts eff cells which have been described to suppress DTH across MHC- and Igh-barriers (4). Experiments directed to this critical question are in progress. Anyhow, the present experiments do indicate that the suppressive effect can be exerted across MHC- and Igh-barriers.

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CHAPTER III.2.

MHC-UNRESTRICTED INTERACTION BETWEEN L3T4$^+$,LYT-2$^-$ Ts INDUCER CELLS AND L3T4$^-$,LYT-2$^+$ Ts EFFECTOR CELLS IS REQUIRED FOR T CELL DEPENDENT SUPPRESSION OF DTH TO HISTOCOMPATIBILITY ANTIGENS

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SUMMARY

This paper describes the characteristics of T suppressor inducer (Ts ind) cells and their interaction with T suppressor effector (Ts eff) cells that regulate delayed-type hypersensitivity (DTH) to alloantigens. Adoptive transfer of spleen cells from mice intravenously (i.v.) injected with allogeneic spleen cells one day earlier induced an antigen-specific state of suppression in the recipients. This became obvious when DTH was induced by subcutaneous (s.c.) immunization of the recipients three days after transfer. The induction of suppression after adoptive transfer required Thy-1^+,L3T4^+,Lyt-2^- cells, which will be referred to as Ts ind cells. These cells, which by themselves could not exert a suppressive effect, did induce a state of suppression in the recipient mice by activation of recipient-derived Ts eff cells. When athymic nude mice were used as recipients, Lyt-2^+ cells had to be transferred together with the Ts ind cells to induce a state of suppression in these mice. The Ts ind cells could also induce a state of suppression in MHC and Igh incompatible recipients. The results are discussed in relation to previously described immunoregulatory T cell pathways.

INTRODUCTION

Delayed-type hypersensitivity (DTH) to allogeneic histocompatibility (H) antigens in mice can be induced by intravenous (i.v.) or subcutaneous (s.c.) injection of irradiated or non-irradiated allogeneic spleen cells. DTH responses can be elicited some days later by s.c. injection of the relevant allogeneic spleen cells into, for instance, a hind foot. Hind foot thickness measured 24 hr or 48 hr later can be used as a reliable measure for the strength of the DTH response (1,2). The DTH reactive cells probably belong to a subset of helper T (Th) cells (3,4), although certain cytotoxic T (Tc) cell clones have also been reported to mediate DTH (5,6).

DTH to H antigens can be regulated by alloantigen-specific T suppressor (Ts) cells, that can be induced by i.v. injection with a relatively high dose of irradiated allogeneic spleen cells (7). These Ts cells can suppress the induction phase as well as the expression phase of DTH, and express the Lyt-1^+2^+ phenotype (2,8). Depending on the experimental conditions also the response against third party alloantigens can be suppressed by such Ts cells. This occurs when the third party alloantigens are administered during either the induction or the elicitation of DTH together with the alloantigens that had induced the Ts cells (9,10). Moreover, the state of suppression could be transferred across MHC and Igh barriers (8).

Other investigators reported about Ts cell subsets that regulate DTH to alloantigens. For instance, Liew reported that Lyt-1^+2^- primary Ts cells appeared after i.v. injection of mice with a high dose of irradiated allogeneic lymphoid cells, whereas secondary Lyt-1^+2^+ Ts cells appeared three days after a subsequent intraperitoneal (i.p.) injection with irradiated allogeneic lymphoid cells (11).

Recently, we reported evidence for a radiation-sensitive T suppressor inducer (Ts ind) cell in the regulation of DTH to alloantigens (12). This Ts ind cell expressed the Lyt1^+2^- phenotype and induced a state of suppression of DTH across MHC and Igh barriers. This paper extends these studies and shows that the Ts ind cells are antigen-specific, express the L3T4 cell surface marker, but by themselves cannot exert a suppressive effect. To induce a state of suppression, they interact with L3T4$^-$,Lyt-2^+ T suppressor effector (Ts eff) cells in a MHC and Igh unrestricted manner.

MATERIALS AND METHODS

Mice

(C57BL/Rij x CBA/Rij)F1 (H-$2^{b/q}$) and C3H/Law (H-2^k) female mice, 4 to 6 weeks of age, were purchased from the Radiobiological Institute TNO, Rijswijk, The Netherlands. BALB.K (H-2^k), BALB.B (H-2^b) and B10.D2 (H-2^d) female mice, 4 to 6 weeks of age, were purchased from HARLAN OLAC Ltd., Bicester, Oxon, U.K. BALB/c (H-2^d, Igh^a), C57BL/6 (H-2^b) and B10.nu/nu (H-2^b) female mice, 4 to 6 weeks of age, were purchased from Bomholtgard, Ry, Denmark. C.B.-20 (H-2^d, Igh^b), C57BL/Ka-BL-1 (H-2^b) and C57BL/Ka (H-2^b) female mice, were bred and raised at our department. All mice had access to pelleted food (Hope Farms, Woerden, The Netherlands) and acidified water ad libitum. The animals age ranged from 10 to 14 weeks at the start of each experiment.

Preparation of cell suspensions

Spleens from donor mice were minced with scissors and squeezed through a nylon gauze filter to get a single cell suspension. The cells were

suspended in a balanced salt solution (BSS). Nucleated cells were counted with a Coulter Counter model BZI (Coulter Electronics, Harpenden, Herts, U.K.). Cell viability always exceeded 95%.

Induction of suppression

Suppression of DTH was induced by i.v. injection of 5 x 10^7 irradiated (20 Gy) allogeneic spleen cells in a volume of 0.5 ml BSS. Radiation of spleen cells was performed in a Philips-Müller MG 300 X-ray apparatus, as described (13).

Transfer of suppression

Suppression of DTH was i.v. transferred either one or four days after tolerization of the donors by i.v. injection of allogeneic spleen cells. Each recipient received a number of spleen cells equivalent to one whole spleen, suspended in a volume of 1.0 ml BSS.

Selective elimination of Thy-1.2$^+$, L3T4$^+$, and Lyt-2.2$^+$ cells

Cell suspensions were treated with either monoclonal IgM anti-Thy-1.2 (clone F7D5, HARLAN OLAC, Ltd.), monoclonal IgG2b anti-L3T4 (produced by *in vitro* culture of clone GK1.5, obtained from the American Type Culture Collection, Rockville, MD, USA) or monoclonal IgG2a anti-Lyt-2.2 (clone AD4(15), Cedarlane Laboratories Ltd., Hornby, Ontario, Canada) for 30 min at 4°C. After incubation the cells were centrifuged, resuspended in BSS and incubated with Rabbit Low Tox 'M' complement (Cedarlane Laboratories Ltd.) for 25 min at 37°C. The cells were washed three times and resuspended in BSS. This procedure eliminated at least 90% of the viable lymphocytes detected by the monoclonal antibody used, as determined by FACS analysis (FACS-II, Becton Dickinson, Sunnyvale, CA, USA).

Estimation of DNA synthesis in draining lymph node cells

Incorporation of tritiated thymidine (3H-TdR) was used to measure DNA synthesis by cells in the lymph nodes that drain the site of s.c. immunization as a reflection of the proliferative activity of these cells in response to immunization. Briefly, on day 5 after s.c. immunization three mice from each experimental group were sacrificed, their inguinal lymph nodes were removed and single cell suspensions were prepared. Measurement of DNA synthesis was performed using a modification (14) of the method described by Moorhead (15).

Immune-lymphocyte-transfer assay

The immune-lymphocyte-transfer (ILT) reactivity of lymph node cells from immunized donors, directed against alloantigens of a particular mouse strain, was determined by s.c. injection of 5 x 10^6 of these lymph node cells into the dorsum of the right hind foot of mice of the relevant recipient strain. To control for background reactivity all mice were injected into the left hind foot with 5 x 10^6 normal lymph node cells from non-immunized donor mice. The latter injection results in so-called normal-lymphocyte-transfer (NLT) reactivity. The specific ILT-reactivity was calculated as ILT minus NLT and expressed in 10^{-2} mm.

Induction of DTH

DTH reactivity was induced by s.c. injection of a dose of 1 x 10^7 viable allogeneic spleen cells in a volume of 100 ul BSS. A volume of 50 ul of this suspension was injected into each inguinal area.

Assay for DTH

DTH responses were elicited by s.c. injection of a challenge dose of 2 x 10^7 allogeneic spleen cells in a volume of 50 ul BSS into the dorsum of the right hind foot of previously immunized mice. Foot thickness was measured 24 hr later using a foot thickness meter with a 0.05 mm accuracy. To correct for background swelling a group of nonimmunized control mice which only received the challenge injection was included in each experiment. The percentage specific increase of foot thickness was calculated as the mean percentage increase of foot thickness from each experimental group minus the mean percentage increase of foot thickness from the challenge control group. The background response of the control mice varied between 15 and 25%.

RESULTS

Ts inducer cells of DTH to alloantigens are antigen-specific

In a previous paper (12) we reported that 24 hr after i.v. tolerization with allogeneic spleen cells radiation-sensitive Ts ind cells can be obtained from the spleen of the tolerized mice. After adoptive transfer, these cells induce a state of suppression of DTH in the recipient mice. To investigate whether the Ts ind cells are antigen specific, (C57BL x CBA)F1 mice were i.v. tolerized with either BALB/c or C3H/Law spleen cells. One day later their spleen cells were adoptively transferred to syngeneic recipients. Three days later these mice were s.c. immunized with BALB/c or C3H/Law spleen cells to induce DTH. Another five days later all mice were

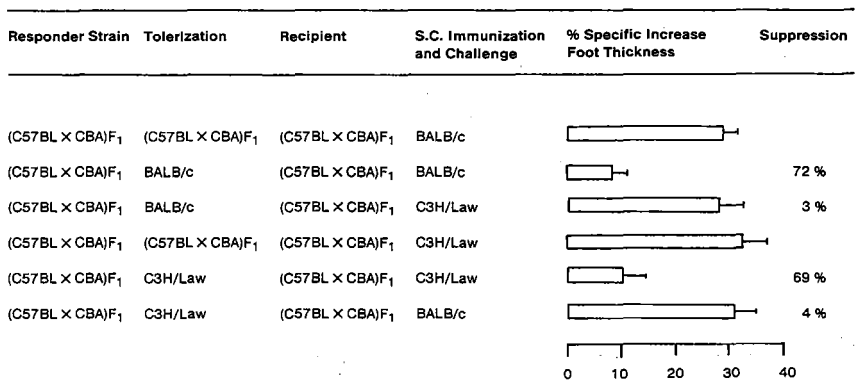


Fig. 1. Antigen-specificity of Ts ind cells. Groups of (C57BL x CBA)F1 mice were i.v. injected with either (C57BL x CBA)F1, BALB/c or C3H/Law spleen cells. One day later all mice were sacrificed, their spleen cells were obtained and i.v. transferred to naive (C57BL x CBA)F1 recipients. Three days later the recipient mice were s.c. immunized with BALB/c or C3H/Law spleen cells. Five days later the mice were challenged with BALB/c or C3H/Law spleen cells to assay for DTH. Each bar represents the mean percentage specific increase of foot thickness $\pm$ 1 SEM (n=5). The degree of suppression was calculated as percentage decrease of DTH as compared to the relevant DTH control group.

challenged to assay for DTH. As shown in Fig. 1, lines 2 and 3, the recipients of Ts ind cells induced by i.v. tolerization with BALB/c spleen cells could not mount a significant DTH response to BALB/c alloantigens, but showed normal DTH reactivity to C3H/Law alloantigens. Similar results were obtained with the reversed (lines 5 and 6) and other (data not shown) donor-recipient combinations.

L3T4 phenotype of Ts ind and Ts eff cells

The Ts ind cells of DTH to alloantigens express the Lyt-1^+2^- phenotype (12), whereas the Ts eff cells express the Lyt-1^+2^+ phenotype (2,8). To investigate whether these Ts inducer cells and Ts effector cells express the L3T4 cell surface marker, groups of (C57BL x CBA)F1 mice were i.v. tolerized with BALB/c spleen cells. Either one day or four days later their spleen cells were treated with anti-L3T4 monoclonal antibodies and complement or complement only. The residual cells were adoptively transferred to syngeneic recipients, which were s.c. immunized with BALB/c spleen cells at

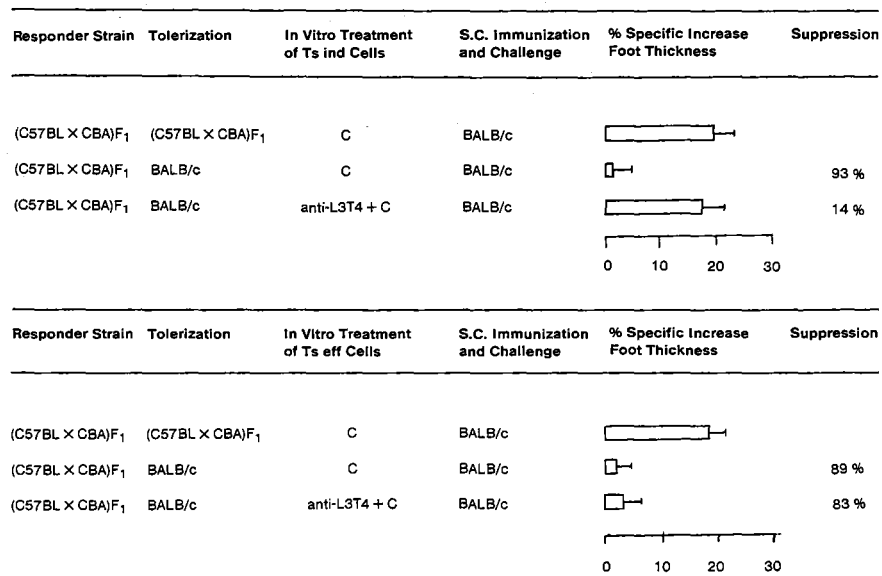
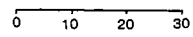


Fig. 2. L3T4 phenotype of Ts ind and Ts eff cells. Groups of (C57BL x CBA)F1 mice were i.v. injected with either (C57BL x CBA)F1 or BALB/c spleen cells. Mice were sacrificed one (upper part) or four (lower part) days later to harvest Ts ind cells and Ts eff cells, respectively. The spleen cells were obtained, treated with anti-L3T4 monoclonal antibodies and complement (C), or complement only, and the residual cells were i.v. transferred to naive (C57BL x CBA)F1 recipients. Either three days (upper part) or immediately (lower part) after transfer the recipients were s.c. immunized with BALB/c spleen cells. Five days later the mice were challenged with BALB/c spleen cells to assay for DTH. Each bar represents the mean percentage specific increase of foot thickness $\pm$ 1 SEM (n=5). The degree of suppression was calculated as percentage decrease of DTH as compared to the relevant DTH control group.

Responder Strain	Tolerization	Recipient	S.C. Immunization and Challenge	% Specific Increase Foot Thickness	CPM
(C57BL × CBA)F_1	(C57BL × CBA)F_1	(C57BL × CBA)F_1	DBA/2		5789 ± 312
(C57BL × CBA)F_1	DBA/2	(C57BL × CBA)F_1	DBA/2		5647 ± 419

Fig 3A



Recipient	Tolerization of (C57BL×CBA)F_1 Spleen Donors	Ts Cell Population Tested for Suppression	Immune lymphocytes	Specific ILT	Suppression
DBA/2	(C57BL × CBA)F_1	-	(C57BL × CBA)F_1-anti-DBA/2		
DBA/2	DBA/2	Ts ind	(C57BL × CBA)F_1-anti-DBA/2		6 %
DBA/2	(C57BL × CBA)F_1	-	(C57BL × CBA)F_1-anti-DBA/2		
DBA/2	DBA/2	Ts eff	(C57BL × CBA)F_1-anti-DBA/2		76 %

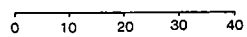


Fig. 3. Ts ind cells cannot exert suppressive activity during the induction and expression phase of DTH. Upper part: To test the effect of Ts ind cells on the induction phase of DTH, groups of (C57BL x CBA)F1 mice were i.v. injected with DBA/2 spleen cells. One day later their spleen cells, containing Ts ind cells, were i.v. transferred to syngeneic naive recipients, which were s.c. immunized with DBA/2 spleen cells immediately after transfer. Five days later some of these mice were challenged with DBA/2 spleen cells to assay for DTH, whereas the other mice were sacrificed to measure the proliferative activity of their draining lymph node cells. Each bar represents the mean percentage specific increase of foot thickness ± 1 SEM (n=5). The proliferative activity is expressed in cpm ± 1 SEM (n=5). The degree of suppression was calculated as percentage decrease of DTH as compared to the relevant DTH control group.
Lower part : To test the effect of Ts ind cells on the expression phase of DTH, spleen cell suspensions containing Ts ind cells were tested for their capacity to suppress ILT reactivity. Immune lymphocytes were obtained from the draining lymph nodes of (C57BL x CBA)F1 mice five days after s.c. immunization with DBA/2 spleen cells. To evaluate suppression by Ts ind cells, 5 x 10^6 (C57BL x CBA)F1-anti-DBA/2 immune lymphocytes were s.c. injected into the right hind foot of DBA/2 recipients, together with 1 x 10^7 (C57BL x CBA)F1 spleen cells containing DBA/2 induced Ts ind cells, or together with 1 x 10^7 spleen cells from naive (C57BL x CBA)F1 mice. To control for background reactivity all mice were similarly injected into the left hind foot with a mixture of normal (C57BL x CBA)F1 lymphocytes and (C57BL x CBA)F1 spleen cells containing Ts ind cells. Each bar represents the mean specific ILT response ± 1 SEM (n=5). The degree of suppression was calculated as percentage decrease of specific ILT response as compared to the ILT control group.

three days or immediately after transfer respectively. Five days later all mice were challenged to assay for DTH. As shown in Fig. 2, upper part, line 3, depletion of $L3T4^+$ cells from the spleen cell suspension obtained one day after tolerization (and thus containing Ts ind cells) completely prevented the induction of suppression in the recipients. In contrast, similar depletion from the spleen cell suspension obtained 4 days after tolerization (and thus containing Ts eff cells) did not affect the transfer of suppression (Fig. 2, lower part, line 3). This indicates that Ts ind cells are $L3T4^+$, in contrast to Ts eff cells which are $L3T4^-$.

Ts ind cells cannot exert suppressive activity

To investigate whether the Ts ind cells themselves can exert suppressive activity on DTH to alloantigens, (C57BL x CBA)F1 mice were i.v. tolerized with DBA/2 spleen cells. One day later their spleen cells were either adoptively transferred to syngeneic recipients, which were immediately s.c. immunized to test for suppression of the induction phase of DTH (2), or tested in the ILT assay for suppression of the expression phase of DTH (8). As shown in Fig. 3, upper part, line 4, Ts ind cells suppressed neither the induction of DTH to DBA/2 alloantigens nor the proliferative activity of the draining lymph node cells in response to s.c. immunization with DBA/2 spleen cells. Likewise, the ILT reactivity of (C57BL x CBA)F1-anti-DBA/2 immune lymphocytes could not be suppressed by simultaneous injection of these cells with spleen cells containing Ts ind cells (Fig. 3, lower part, line 2). Thus, there is no evidence that Ts ind cells themselves can suppress DTH.

Ts ind cells activate recipient-derived Ts eff cells

In order to induce suppression of DTH, Ts ind cells might either differentiate into Ts eff cells or activate precursors of Ts eff cells. To discriminate between these possibilities, C57BL/Ka-BL-1 mice (whose T cells are Thy-1.1^+) were i.v. tolerized with DBA/2 spleen cells. One day later their spleen cells were obtained and adoptively transferred to C57BL/Ka recipients (whose T cells are Thy-1.2^+). Three days later the recipients' spleen cells were obtained, treated with anti-Thy-1.2 monoclonal antibodies and complement or complement only, and adoptively transferred to syngeneic recipients. Immediately after transfer these secondary recipients were s.c. immunized with DBA/2 spleen cells. Five days later all mice were challenged to assay for DTH. As shown in Fig. 4, line 4, depletion of Thy-1.2^+ cells from the inoculum to be transferred to the secondary recipients completely prevented the transfer of suppression to these mice. This indicates that Ts ind cells activate recipient-type precursors of Ts eff cells, leading to activated Ts eff cells that can suppress DTH.

Induction of suppression in nude mice by Ts ind cells requires donor Lyt-2^+ cells

The previous experiments do not exclude the possibility that after transfer Ts ind cells interact with donor-derived precursors of Ts eff cells, which can be expected to be present in the spleen cell inoculum to be transferred. To investigate this possibility, C57BL/Ka mice were i.v. tolerized with DBA/2 spleen cells. One day later their spleen cells were obtained, from which one part was adoptively transferred to either athymic B10 nude mice, which do not possess T cells, or to C57BL/Ka mice. The other part was either treated with anti-Lyt-2.2 monoclonal antibodies and complement or complement only, and similarly transferred to B10 nude mice. Three

Responder Strain	Tolerization	Primary Recipient	In Vitro Treatment of Spleen Cells	Secondary Recipient	S.C. Immunization and Challenge	% Specific Increase Foot Thickness	Suppression
C57BL/Ka-BL1	C57BL/Ka-BL1	C57BL/Ka	C	C57BL/Ka	DBA/2		
C57BL/Ka-BL1	DBA/2	C57BL/Ka	C	C57BL/Ka	DBA/2		66 %
C57BL/Ka-BL1	C57BL/Ka-BL1	C57BL/Ka	anti-Thy-1.2 + C	C57BL/Ka	DBA/2		
C57BL/Ka-BL1	DBA/2	C57BL/Ka	anti-Thy-1.2 + C	C57BL/Ka	DBA/2		9 %

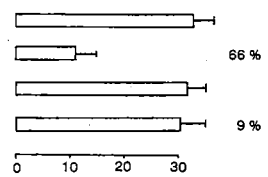


Fig. 4. Ts ind cells activate recipient-derived Ts eff cells. Groups of C57BL/Ka-BL 1 mice were i.v. injected with C57BL/Ka-BL-1 or DBA/2 spleen cells. One day later their spleen cells were obtained and i.v. transferred to C57BL/Ka-BL-1 (Thy-1.1$^+$) or C57BL/Ka (Thy-1.2$^+$) recipients. Three days later these recipients were sacrificed, their spleen cells were obtained, treated with anti-Thy-1.2 monoclonal antibodies and complement or complement only, and i.v. transferred to naive syngeneic recipients. Immediately after transfer the recipients were s.c. immunized with DBA/2 spleen cells. Five days later all mice were challenged with DBA/2 spleen cells to assay for DTH. Each bar represents the mean percentage specific increase of foot thickness $\pm$ 1 SEM (n=5). The degree of suppression was calculated as percentage of DTH as compared to the relevant DTH control group.

days later the spleen cells from all primary recipients were adoptively transferred to secondary C57BL/Ka recipients. Immediately after transfer these recipients were s.c. immunized with DBA/2 spleen cells. Five days later DTH responses were elicited. As shown in Fig. 5, upper part, line 4, Ts ind cells can induce a state of suppression in athymic nude mice. However, this required the presence of Lyt-2$^+$ cells in the inoculum to be transferred to the nude recipients, since depletion of the Lyt-2$^+$ cells from the inoculum completely prevented the induction of suppression in these nude mice (Fig. 5, lower part, line 8).

Interaction between Ts ind cells and Ts eff cells is not MHC or Igh restricted

We previously reported that Ts ind cells can induce a state of suppression in MHC and Igh incompatible recipients and should therefore be regarded as being not MHC or Igh restricted (12). However, in view of the previous experiments it might well be that after adoptive transfer to MHC or Igh incompatible recipients, Ts ind cells interact with donor-derived Lyt-2$^+$ Ts eff cells, rather than with recipient-type Ts eff cells. This may account for their putative unrestrictedness. Thus, we investigated whether Ts ind cells require donor-derived Lyt-2$^+$ cells to induce suppression in MHC or Igh incompatible recipients. Therefore BALB.K (H-2^k) mice were i.v. tolerized with C57BL/6 spleen cells. One day later their spleen cells were treated with anti-Lyt-2.2 monoclonal antibodies and complement or complement only, and adoptively transferred to either BALB.K, or MHC-incompatible BALB/c (H-2^d) recipients. Three days later all mice were s.c. immunized with C57BL/6 spleen cells. Another five days later DTH responses were

elicited. As shown in Fig. 6, line 8, depletion of Lyt-2^+ cells from the inoculum to be transferred to MHC-incompatible BALB/c recipients did not prevent the induction of suppression in these mice. Similar results were found when Ts ind cells from BALB/c (Igh^a) donors were transferred to Igh-incompatible C.B.-20 (Igh^b) recipients (Fig. 7, line 8). Thus, the Ts ind cells are truly MHC and Igh unrestricted.

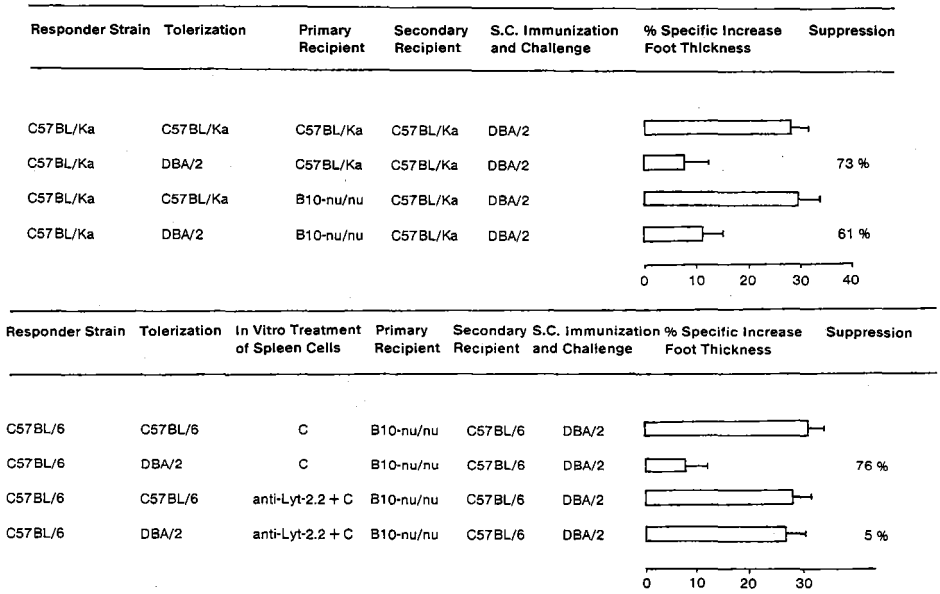


Fig. 5. Ts ind cells induce suppression in nude mice, which requires donor derived Lyt-2^+ cells. Groups of C57BL/6 mice were i.v. injected with either C57BL/6 or DBA/2 spleen cells. One day later their spleen cells were obtained and either i.v. transferred to naive C57BL/6 mice or B10 athymic nude mice (upper part), or treated with anti-Lyt-2.2 monoclonal antibodies and complement or complement only, and than similarly transferred to B10 nude mice (lower part). Three days later all mice were sacrificed and their spleen cells were i.v. transferred to secondary C57BL/6 recipients, which were s.c. immunized with DBA/2 spleen cells immediately after transfer. Five days later DTH responses were elicited. Each bar represents the mean percentage specific increase of foot thickness $\pm$ 1 SEM (n=5). The degree of suppression was calculated as percentage decrease of DTH as compared to the relevant DTH control group.

DISCUSSION

The studies described in this paper show that i.v. injection of mice with allogeneic spleen cells induces a population of Ts ind cells which interact with precursors of Ts eff cells, ultimately leading to a state of tolerance for these alloantigens. This state of tolerance prevents the development of DTH reactivity when the mice are s.c. immunized with the

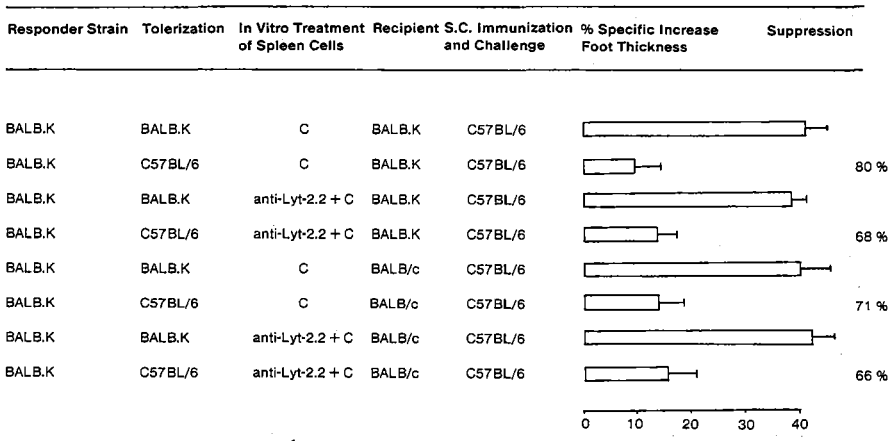


Fig. 6. Interaction between Ts ind cells and Ts eff cells is not MHC-restricted. Groups of BALB.K mice were i.v. injected with either BALB.K or C57BL/6 spleen cells. One day later their spleen cells were obtained, treated with anti-Lyt-2.2 monoclonal antibodies and complement or complement only and i.v. transferred to either BALB.K or BALB/c recipients. Three days later all mice were s.c. immunized with C57BL/6 spleen cells to induce DTH. Five days later all mice were challenged with C57BL/6 spleen cells to assay for DTH. Each bar represents the mean percentage specific increase of foot thickness $\pm$ 1 SEM (n=5). The degree of suppression was calculated as percentage decrease of DTH as compared to the relevant DTH control group.

relevant allogeneic spleen cells. After adoptive transfer of Ts ind cells, only DTH reactivity against the alloantigens that had been used to induce them became suppressed (Fig. 1). Interestingly, the Ts ind cells do not exert suppressive activity themselves (Fig. 2). In a previous study (12) we have shown that the Ts ind cells are Lyt-1^+2^-, while the Ts eff cells are Lyt-1^+2^+. In the present study we show (Fig. 3) that Ts ind cells, but not Ts eff cells, also express the helper/inducer T cell specific cell surface marker L3T4. To induce a state of suppression in recipient mice the Ts ind cells preferentially interact with recipient-type precursors of Ts eff cells (Fig. 4). However, when such precursors are not available, as is the case in athymic nude mice, the Ts ind cells interact with donor-type, Lyt-2^+ precursors of Ts eff cells, that are present in the transferred inoculum (Fig. 5). Activation of Ts eff cells by Ts ind cells migh occur through idiotypic-antiidiotypic interactions, which is subject of investigation.

Ts cells regulating DTH to alloantigens in mice have also been described by Liew (11). He also found evidence for two antigen-specific Ts cell types. One Ts cell type could be detected in the spleen three days after i.v. tolerization with 1.5 x 10^8 irradiated allogeneic lymphocytes, and displayed the Lyt-1^+2^- phenotype. Adoptive transfer of these cells to syngeneic recipients resulted in a state of suppression of DTH, which appeared when the recipients were s.c. immunized immediately after transfer with 1 x 10^8 of the relevant irradiated allogeneic lymphocytes. The second Ts cell type appeared in the spleen three days after an intraperitoneal

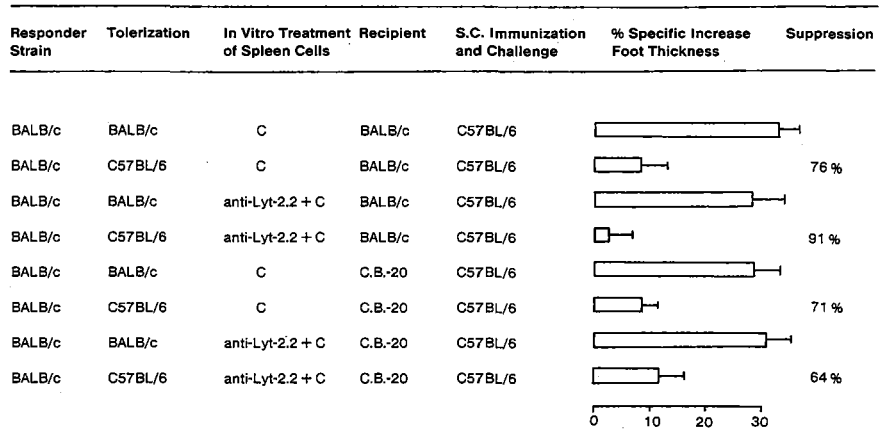


Fig. 7. Interaction between Ts ind cells and Ts eff cells is not Igh-restricted. Groups of BALB/c mice were i.v. injected with either BALB/c or C57BL/6 spleen cells. One day later their spleen cells were obtained, treated with anti-Lyt-2.2 monoclonal antibodies and complement or complement only, and i.v. transferred to either BALB/c or C.B.-20 recipients. Three days later all mice were s.c. immunized with C57BL/6 spleen cells to induce DTH. Five days later all mice were challenged with C57BL/6 spleen cells to assay for DTH. Each bar represents the mean percentage specific increase of foot thickness $\pm$ 1 SEM (n=5). The degree of suppression was calculated as percentage decrease of DTH as compared to the relevant DTH control group.

(i.p.) booster immunization with 2 x 10^8 of the relevant irradiated allogeneic spleen cells. These cells displayed the Lyt-1^+2^+ phenotype. Although Liew did not investigate whether the i.p. immunization was a prerequisite for the generation of the Lyt-1^+2^+ cells, his data are quite compatible with the combined data presented in this paper and those reported by Bianchi et al. (8). The Lyt-1^+2^- Ts cells described by Liew (11) are probably comparable to the L3T4$^+$,Lyt-1^+2^- Ts ind cells described here. Liew concluded that these Ts cells suppressed the DTH reactivity. However, we could not detect any suppressive activity by Ts ind cells (Fig. 2). It cannot be excluded, that the Lyt-1^+2^- Ts cells described by Liew (11) activated, after transfer, Lyt-1^+2^+ Ts effector cells which subsequently suppressed DTH. Liew did not investigate whether these Ts cells were MHC-restricted with regard to their mutual interaction, or their interaction with DTH effector T cells. However, Hewitt and Liew did report that the culture supernatant from a Ts hybridoma exerted suppression of DTH to sheep red blood cells (SRBC) across a MHC barrier (16).

Recently, Aoki et al. (17) also reported that two types of Ts cells are involved in the regulation of DTH to alloantigens. Five days after i.v. tolerization with 5 x 10^7 unirradiated allogeneic spleen cells they found antigen-specific, MHC-unrestricted Lyt-2^- Ts cells that, after adoptive transfer, suppressed the induction phase of DTH. Although the time point after tolerization at which these Ts cells were detected seems to be late

as compared to our studies, their Lyt-2^- Ts cells appear to be comparable with the Ts ind cells that we found. Unfortunately, Aoki's conclusion about suppression of the induction phase of DTH was only based on the time point of adoptive transfer of the Ts cells, in relation to the time point of induction of DTH. In contrast, Van der Kwast et al. (2) and Bianchi et al. (7) carefully examined the proliferative activity of draining lymph node cells in response to alloimmunization as a measure for suppression of the induction phase of DTH. They concluded that the suppression of the induction phase of DTH was due to the activity of Lyt-1^+2^+ Ts cells and that Lyt-2^- cells were unable to suppress this reponse.

The other Ts cell type, described by Aoki et al. (17) was found at the same time point as the former, displayed the Lyt-2^+ phenotype and suppressed the expression phase of DTH in a MHC-restricted manner. Bianchi et al. (2,8) also reported that Ts cells that suppress the expression phase of DTH appear in the spleen at the same time point after tolerization as Ts cells that suppress the induction phase of DTH. However, several dissimilarities between these studies exist. Firstly, in contrast to Aoki et al. (17), Bianchi et al. (2) could not find suppressor cell activity when spleen cells from tolerized mice were i.v. transferred to previously primed mice which were subsequently challenged for DTH. Instead, for evaluation of suppression of the expression phase of DTH the immune-lymphocyte-transfer assay had to be employed (8). Secondly, in contrast to Aoki et al., (17) Bianchi et al. found the induction phase Ts cells phenotypically indistinguishable from the expression phase Ts cells. Both displayed the Lyt-1^+2^+ phenotype (2,8). Thirdly, in contrast to Aoki et al. (17), Bianchi et al. found that expression phase Ts cells were MHC-unrestricted (8). The only apparent difference in protocol that both groups employed for the induction of suppression is the use of irradiated spleen cells by Bianchi et al., whereas Aoki et al. used unirradiated spleen cells. It is difficult to explain the discrepancies between these reports solely by this difference.

A major regulatory role for MHC-encoded molecules has been described in other Ts cell pathways. Especially molecules encoded by the elusive I-J subregion of the MHC complex have been reported to regulate the interactions between Ts cells, and between Ts cells and antigen-presenting cells. Data suggesting this were obtained in studies on the contact hypersensitivity response to 4-hydroxy-3-nitrophenyl acetyl (NP,18), the DTH response to azobenzene arsonate (ABA,18), and the in vitro antibody response to SRBC (19). In addition, in these models molecules encoded by genes located in the Igh region have been described to regulate Ts cell interactions. However, the data presented in Figs 6 and 7, and those reported by Bianchi et al. (8) about the Ts cell mediated regulation of DTH to alloantigens indicate that in this regulation neither MHC nor Igh encoded molecules play a major role.

Evidence for two Ts cell types has also been reported for the in vitro regulation of alloimmunity. For instance, Chaouat et al. (20) reported two interacting Ts cell types that regulate mixed-lymphocyte-reactivity (MLR). They showed that both Lyt-2^- and Lyt-2^+ cells were required for suppression. Furthermore, the supernatant of cultured, allosensitized Lyt-2^- cells inhibited MLR, provided Lyt-2^+ cells were present in the responder cell population. In line with the above discussed data they suggested that a suppressor-inducer/suppressor-effector cell system is involved in the regulation of alloimmunity. Their data have been supported by other investigators (21).

In addition, Hutchinson (22) reported three, possibly interacting, Ts

cell populations regulating kidney allograft rejection in the rat. Three days after transplantation of a kidney allograft, W3/25$^+$, OX8$^-$ Ts cells (which compare to L3T4$^+$,Lyt-2$^-$ Ts ind cells in the mouse) could be obtained from the spleen of the kidney recipient. These cells could not prevent rejection of the primary kidney allograft but, after transfer to syngeneic recipients, induced a permanent, alloantigen-specific state of suppression of allograft rejection in the recipient. Furthermore, after donor-specific blood transfusion of rats subsequently grafted allogeneic kidneys were found to survive indefinitely. This long-term survival was ascribed to the activity of two types of W3/25$^-$,OX8$^+$ Ts cells (which compare to L3T4$^-$,Lyt-2$^+$ Ts cells in the mouse), that might interact through an idiotypic-anti-idiotypic interaction.

In conclusion, our data, together with the literature cited, strongly suggest that at least two mutually interacting Ts cell types are involved in the regulation of alloimmunity. We are currently investigating the nature of these interactions and the possible role of suppressor factor(s) herein.

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CHAPTER III.3.

GENERATION OF SUPPRESSOR T CELLS AFTER LOCAL IMMUNIZATION WITH HISTOCOMPATIBILITY ANTIGENS

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Submitted for publication.

SUMMARY

Subcutaneous (s.c.) hind-foot-immunization (HFI) of mice with allogeneic spleen cells can induce a state of delayed-type hypersensitivity (DTH) as well as a state of suppression of DTH. This paper deals with the suppression induced by HFI. The state of suppression could be adoptively transferred by spleen cells and lymph node cells between day 3 and day 7 after HFI only. However, in the hind-foot-immunized mice themselves the state of suppression lasted at least 25 days. The suppressor cells expressed the Thy-1^+, Lyt-1^-2^+ phenotype and suppressed DTH antigen-specifically. The suppressor cells, however, also suppressed DTH responses to unrelated third-party alloantigens, provided the latter were administered during the induction of DTH together with the same alloantigens that were used for HFI. The HFI-induced Ts cells suppressed the induction phase of DTH (i.e. the proliferative activity of the draining lymph node cells after secondary s.c. immunization), but not the expression phase of DTH (i.e. the activity of already activated DTH effector T cells). H-2D compatibility between the donors of the HFI-induced Ts cells and the recipients was required for the adoptive transfer of suppression. The differences in effect of local immunization versus systemic immunization on the induction and functional activity of Ts cells are discussed.

INTRODUCTION

Delayed-type hypersensitivity (DTH) against allogeneic histocompatibility (H) antigens can be induced in mice by subcutaneous (s.c.) or intravenous (i.v.) immunization with allogeneic spleen cells. DTH-responses can be elicited in such mice by injecting a dose of the relevant allogeneic spleen cells into, for instance, a hind foot or an ear. The DTH-response appears several hours later as a measurable swelling of the injected site, which reaches its maximum at 24 to 48 hours after injection (1,2).

The induction of DTH-reactivity to H antigens appears to be highly dependent on the injected dose of allogeneic cells, on the route of immunization and other experimental conditions. S.c. immunization with allogeneic spleen cells in the inguinal and/or axillary areas (1) as well as i.v. immunization (2) can induce a good DTH-reactivity. However, when the s.c. immunization is preceded by an i.v. immunization with a relatively high dose of the relevant allogeneic spleen cells, a state of unresponsiveness is induced rather than a state of DTH. The state of unresponsiveness is mediated by antigen-specific suppressor T (Ts) cells, whose activity is not restricted by major histocompatibility complex (MHC) encoded molecules and/or molecules encoded by Igh-linked genes (3,4). Induction of unresponsiveness does not occur after s.c. immunization in the inguinal or axillary areas. Instead, dependent on the alloantigenic differences between donor and recipient, after two s.c. immunizations in the inguinal or axillary areas secondary type DTH reponses can occur (5).

In contrast, Rich and Rich have reported that s.c. immunization in the hind feet (hind-foot-immunization; HFI) induces Ts cells that nonspecifically suppress *in vitro* mixed-lymphocyte-culture (MLC)-reactivity in a MHC *restricted* manner (6,7). These authors stated that these Ts cells have similar characteristics as Ts cells that are induced by i.v. immunization (7). However, as stated before, Van der Kwast et al. (3) and Bianchi et al. (4) reported that i.v. immunization with allogeneic spleen cells induces *in vivo* active alloantigen-specific Ts cells that suppress DTH-reactivity in a *non-restricted* manner. In view of the apparent dissimilarities between the *in vitro* studies of Rich and Rich (6,7) and the *in vivo* studies of Van der Kwast et al. (3) and Bianchi et al. (4) we studied the effects of HFI on DTH to H antigens.

MATERIALS AND METHODS

Mice

(C57BL/Rij x CBA/Rij)F1 (H-$2^{b/q}$), CBA/Rij (H-2^{q}) and C3H/Law (H-2^{k}) female mice, 4 weeks of age, were purchased from the Radiobiological Institute TNO, Rijswijk, The Netherlands. BALB/c (H-2^{d}), DBA/2 (H-2^{d}), C57BL/6 (H-2^{b}), B10.A (H-2^{a}), B10.AQR (H-2^{y1}), B10.BR (H-2^{k}) and B10.T(6R) (H-2^{y2}) female mice, 4-8 weeks of age, were purchased from HARLAN OLAC Ltd., Bicester, Oxon, U.K. B10.BYR (H-2^{by1}) and Swiss (H-2^{s}) female mice were bred and raised at our department. All mice were kept in well-ventilated, light cycled rooms and had access to pelleted food (Hope Farms, Woerden, The Netherlands) and acidified water *ad libitum*. At the start of each experiment the animals' age ranged from 10-16 weeks.

Preparation of cell suspensions

Mice were sacrificed by exposure to carbon dioxide. The lymphoid organs

were removed, placed in a balanced salt solution (BSS), minced with scissors and squeezed through a nylon gauze filter to get a single cell suspension. Nucleated cells were counted with a Coulter Counter model BZI (Coulter Electronics, Harpenden, U.K.).

Immunization with allogeneic spleen cells

Mice were immunized in either of three ways: (a) S.c. injection of a dose of allogeneic spleen cells into the inguinal areas. A volume of 50 ul was injected into each area; (b) S.c. injection of a dose of allogeneic spleen cells into the hind feet. A volume of 50 ul was injected into each hind foot; (c) I.v. injection of a dose of allogeneic spleen cells suspended in a volume of 0.5 ml BSS. In some experiments the spleen cells to be injected were irradiated with 20 Gy X-irradiation in a Philips-Müller MG 300 X-ray apparatus, as described (8).

Transfer of suppression

The state of suppression was transferred to recipient mice by i.v. injection of spleen or lymph node cells from mice which had been immunized in their hind feet several days previously. The interval between HFI and transfer is given in the legends to the figures. A few hours after transfer the recipients were s.c. immunized.

Selective elimination of Thy-1.2, Lyt-1.2 and Lyt-2.2 positive cells

Cell suspensions were treated with monoclonal IgM anti-Thy-1.2 (clone F7D5; HARLAN OLAC Ltd.), monoclonal IgG2a anti-Lyt-1.2 (clone CG16; Cedarlane Laboratories Ltd., Hornby, Ontario, Canada) or monoclonal IgG2a anti-Lyt-2.2 (clone AD4(15); Cedarlane Laboratories Ltd.) for 30 min at 4°C. After incubation the cells were washed twice, resuspended in BSS and incubated with guinea pig complement (Behringwerke, Marburg-Lahn, F.R.G.) for 20 min at 37°C. The cells were then washed three times, resuspended in BSS and adoptively transferred. This procedure eliminated at least 90% of the viable lymphocytes detected by the monoclonal antibody used.

Estimation of DNA synthesis in draining lymph node cells

Incorporation of tritiated thymidine (3H-TdR) was used to measure DNA synthesis by cells in the draining lymph nodes as a reflection of their proliferative activity in response to immunization with allogeneic spleen cells. Briefly, on day 5 after s.c. immunization three mice from each experimental group were sacrificed, their inguinal lymph nodes were removed and single cell suspensions were prepared. Measurement of DNA-synthesis was subsequently performed using a modification (9) of the method described by Moorhead (10).

Immune-Lymphocyte-Transfer assay

The immune-lymphocyte-transfer (ILT) reactivity of lymph node cells from immunized donors, directed against alloantigens of a particular mouse strain, was determined by s.c. injection of 5×10^6 of these lymph node cells into the dorsum of the right hind foot of mice of the relevant recipient strain. A control group of syngeneic recipient mice received a similar dose of normal lymphocytes from non-immunized donor mice. The latter injection results in so-called normal-lymphocyte-transfer (NLT) reactivity. The specific ILT-reactivity was calculated as ILT minus NLT and expressed in 10^{-2} mm.

Assay for DTH

DTH reactions were determined by measuring the difference in thickness of the hind feet of responder mice 24 hours after s.c. injection of a challenge dose of 2 x 10^7 of the appropriate allogeneic spleen cells into the dorsum of the right hind foot. To correct for non-specific background swelling in each experiment a group of non-immunized syngeneic mice was included which only received the challenge dose. The specific DTH response was calculated as the relative increase in foot thickness of the experimental group minus the relative increase in foot thickness of the challenge control group. The increase of foot thickness of the control mice ranged between 15 and 25%.

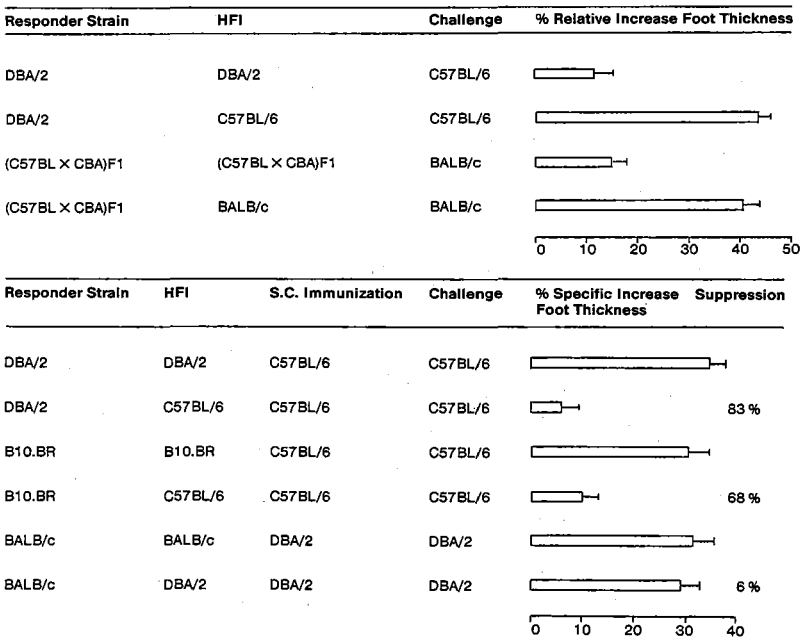

Fig. 1. Effect of HFI on DTH to allogeneic spleen cells. Two experimental systems were employed. Upper part: DBA/2 and (C57BL x CBA)F1 mice were s.c. injected into their hind feet with a dose of 2 x 10^7 C57BL/6 and BALB/c spleen cells, respectively. Control mice received the same dose of syngeneic spleen cells. Five days later all mice were challenged to assay for DTH. Each bar represents the mean relative increase of hind foot thickness $\pm$ 1 SEM (n=5). Lower part: DBA/2, B10.BR and BALB/c mice were s.c. injected into their hind feet with a dose of 2 x 10^7 C57BL/6 (groups 2 and 4) and DBA/2 (group 6) spleen cells. Control mice received syngeneic spleen cells. Seven days later all mice were s.c. immunized in the inguinal areas to induce DTH. Another five days later all mice were challenged to assay for DTH. Each bar represents the mean specific increase of hind foot thickness $\pm$ 1 SEM (n=5). The degree of suppression was calculated as percentage of specific DTH response as compared with the relevant control group.

RESULTS

HFI can induce DTH as well as suppression of DTH to alloantigens

To investigate the effects of HFI on DTH two experimental systems were employed. One consisted of HFI of mice with 2 x 10^7 allogeneic spleen cells followed five days later by a s.c. hind foot challenge with the relevant spleen cells to assay for the development of DTH. The other consisted of HFI of mice with a similar dose of allogeneic spleen cells, seven days later followed by s.c. immunization with the relevant allogeneic spleen cells in the inguinal areas. Another five days later the mice were challenged to assay for DTH. As shown in Fig. 1, upper part, lines 2 and 4, HFI of DBA/2 and (C57BL x CBA)F1 mice with H-2 and non-H-2 incompatible C57BL/6 and BALB/c spleen cells, respectively, induced a clear DTH response. However, when DBA/2 mice were s.c. immunized with C57BL/6 spleen cells in the inguinal areas seven days after HFI with similar spleen cells and were challenged to assay for DTH another five days later, DTH responses could hardly be detected (Fig. 1, lower part, line 2). This suppression of DTH was also found after HFI of B10.BR mice with H-2 incompatible but non-H-2 compatible C57BL/6 spleen cells (Fig. 1, lower part, line 4). However, HFI of BALB/c mice with H-2 compatible but non-H-2 incompatible DBA/2 spleen cells did not result in suppression of DTH (Fig. 1, lower part, line 6). Also in the reversed and other donor-recipient combinations suppression of DTH could not be found (data not shown). Together, these data indicate that, dependent on the experimental conditions, HFI can induce a state of DTH as well as a state of suppression of DTH, except for immunization with non-H-2 alloantigens only.

Dose-response relationship of HFI-induced suppression of DTH

The optimal dose of allogeneic spleen cells to induce suppression by HFI was investigated by HFI of (C57BL x CBA)F1 mice with either irradiated or nonirradiated DBA/2 spleen cells using doses ranging from 1 x 10^6 to 3 x 10^7 cells per hind foot. As shown in Fig. 2, maximal suppression was found at doses of 3 x 10^6 and 1 x 10^7 spleen cells, regardless of irradiation of these spleen cells. In all subsequent experiments a dose of 1 x 10^7 unirradiated spleen cells was injected into each hind foot.

Adoptive transfer of HFI-induced suppression of DTH

The underlying cause of the suppression of DTH induced by HFI was investigated in adoptive transfer experiments. Therefore, spleens and axillary, inguinal and mesenteric lymph nodes were obtained from DBA/2 mice four days after HFI with (C57BL x CBA)F1 spleen cells and single cell suspensions were prepared. These spleen cells or pooled lymph node cells were i.v. injected into naive DBA/2 recipients, which were s.c. immunized with (C57BL x CBA)F1 spleen cells within two hours after transfer. As shown in Fig. 3, lines 2 and 4, the state of suppression could be adoptively transferred by spleen cells as well as by pooled lymph node cells.

Time-course of HFI-induced suppression

The time-course of HFI-induced suppression of DTH-reactivity was studied in (C57BL x CBA)F1 mice. At various intervals after HFI with DBA/2 spleen cells a group of mice was s.c. immunized with DBA/2 spleen cells, whereas from another group of mice the spleen cells were obtained and transferred to naive syngeneic recipients (one spleen equivalent per recipient). A few hours later these recipients were also s.c. immunized with

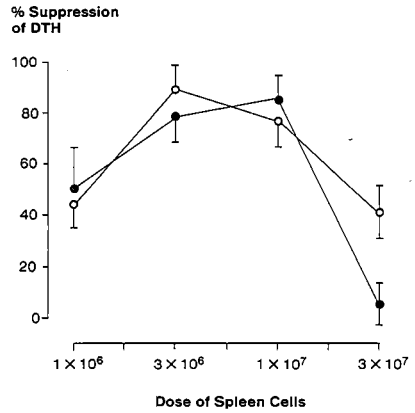

Fig. 2. Dose-dependency of the HFI-induced suppression of DTH. Groups of (C57BL x CBA)F1 mice were s.c. injected with 1 x 10^6, 3 x 10^6, 1 x 10^7 or 3 x 10^7 irradiated (o) or unirradiated (●) DBA/2 spleen cells per hind foot. Control mice received syngeneic spleen cells. Seven days later all mice were s.c. immunized to induce DTH. Another five days later all mice were challenged to assay for DTH. Each experimental point represents the mean percentage suppression of DTH ± 1 SEM (n=5). The degree of suppression was calculated as percentage decrease of specific DTH response as compared with the relevant control group.

Responder Strain	HFI	Transfer	S.C. Immunization and Challenge	% Specific Increase Foot Thickness	Suppression
DBA/2	DBA/2	LNC	(C57BL × CBA)F1		
DBA/2	(C57BL × CBA)F1	LNC	(C57BL × CBA)F1		71 %
DBA/2	DBA/2	SpC	(C57BL × CBA)F1		
DBA/2	(C57BL × CBA)F1	SpC	(C57BL × CBA)F1		79 %

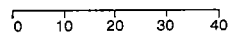

Fig. 3. Adoptive transfer of the HFI-induced suppression of DTH. Groups of DBA/2 responder mice were s.c. injected into their hind feet with either DBA/2 or (C57BL x CBA)F1 spleen cells. Four days later all mice were sacrificed, their spleens (SpC) and inguinal, axillary and mesenteric lymph nodes (LNC) were removed and i.v. injected as single cell suspensions into syngeneic recipient mice. Each recipient received one whole spleen equivalent or the equivalent of the pooled lymph node cells of one donor. Within two hours the recipients were s.c. immunized to induce DTH. Another five days later all mice were challenged to assay for DTH. Each bar represents the mean response ± 1 SEM (N=5). The degree of suppression was calculated as percentage decrease of specific DTH response as compared with the relevant control group.

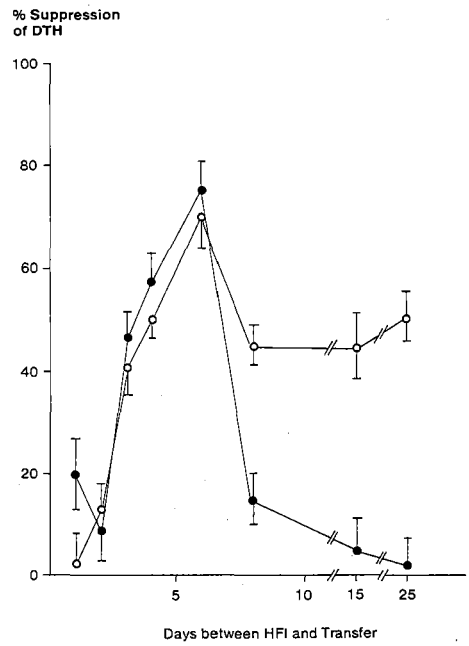

Fig. 4. Time-course of the HFI-induced suppression of DTH. Groups of (C57BL x CBA)F1 mice were s.c. injected into their hind feet with either (C57BL x CBA)F1 or BALB/c spleen cells. At various intervals one group of mice was s.c. immunized to induce DTH (o), whereas another group of mice was sacrificed and their spleen cells were adoptively transferred to syngeneic recipients which were subsequently s.c. immunized (●). Five days after s.c. immunization the mice were challenged to assay for DTH. Each experimental point represents the mean degree of suppression ± 1 SEM (n=5). The degree of suppression was calculated as percentage decrease of specific DTH response as compared with the relevant control group.

DBA/2 spleen cells. The results, shown in Fig. 4, reveal that the suppressive effect could be adoptively transferred by spleen cells from day 3 until day 7 after HFI, but not at later time points. However, in the hind foot immunized mice themselves, suppression could still be detected at day 25 after HFI. In all subsequent experiments suppressed spleen cells were adoptively transferred at day 4 after HFI.

Phenotype of the HFI-induced suppressor cells

The phenotype of the HFI-induced suppressor cells was studied by selective cytotoxic depletion of spleen cell subsets during adoptive transfer experiments. BALB/c mice were used as donors of spleen cells four days after HFI with (C57BL x CBA)F1 spleen cells. Thy-1.2, Lyt-1.2 or Lyt-2.2 positive cells were eliminated *in vitro* by treatment with specific monoclonal antibodies and complement, and the residual cells were i.v. transferred to syngeneic recipients. Immediately thereafter the recipients were s.c.

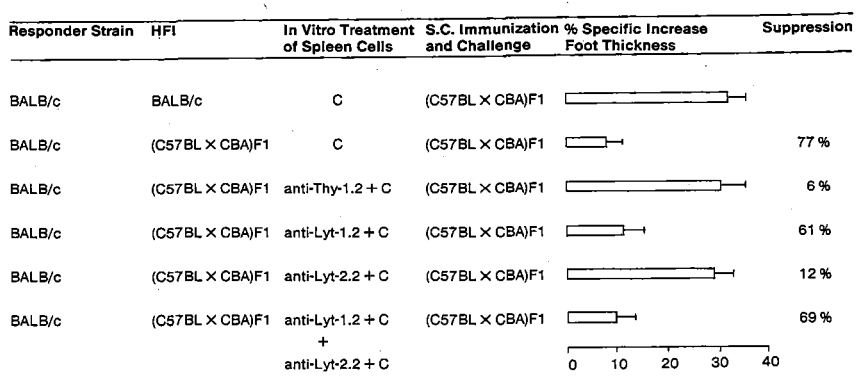

Fig. 5. Phenotype of the HFI-induced suppressor cells. Groups of BALB/c mice were s.c. injected into their hind feet with either BALB/c or (C57BL x CBA)F1 spleen cells. Four days later their spleens were obtained and treated with monoclonal anti-Thy-1.2, anti-Lyt-1.2 or anti-Lyt-2.2 antibodies and complement (C), or complement only. The residual cells were adoptively transferred to syngeneic recipients, which were s.c. immunized to induce DTH within two hours after transfer. Five days later all mice were challenged to assay for DTH. Each bar represents the mean response $\pm$ 1 SEM (n=5). The degree of suppression was calculated as percentage decrease of specific DTH response as compared with the DTH control group.

immunized with (C57BL x CBA)F1 spleen cells to induce DTH. As shown in Fig. 5, lines 3 and 5, elimination of Thy-1.2 positive cells as well as elimination of Lyt-2.2 positive cells completely abrogated the adoptive transfer of suppression. Elimination of Lyt-1.2 positive cells did not affect the adoptive transfer of suppression (Fig. 5, line 4). Recipients of a mixture of Lyt-1.2 depleted cells (still containing Lyt-2.2 positive cells) and Lyt-2.2 depleted cells (still containing Lyt-1.2 positive cells) also showed a suppressed DTH response (Fig. 5, line 6). This experiment was repeated twice, yielding similar results. Together these data indicate that the HFI-induced suppressor cells are T cells expressing the Thy-1^+, Lyt-1^- 2^+ phenotype.

Target cells of the HFI-induced Ts cells

In principle, the HFI-induced Ts cells might exert their effect on the induction phase of DTH (i.e. the proliferation of draining lymph node cells after immunization) as well as on the expression phase of DTH (i.e. the activity of already activated DTH effector T cells). To investigate the former mode of action, (C57BL x CBA)F1 recipients received spleen cells from syngeneic suppressed donors four days after HFI of these donors with DBA/2 spleen cells. Immediately thereafter the recipients were s.c. immunized in the inguinal areas with DBA/2 spleen cells. Five days later, in separate groups of mice either DTH responses were elicited or the proliferative activity of the inguinal lymph node cells was assessed. As shown in Fig. 6A, line 2, the recipients of HFI-induced Ts cells showed a suppressed DTH response concomitantly with a suppressed proliferative response in the draining lymph nodes.

To investigate the effect of HFI-induced Ts cells on the expression phase of DTH we used the immune-lymphocyte-transfer (ILT) assay. (C57BL x CBA)F1 mice were s.c. immunized with DBA/2 spleen cells in their inguinal and axillary areas. Five days later their draining lymph nodes were used as a source of immune lymphocytes. Spleens from other (C57BL x CBA)F1 mice were used as source of Ts cells four days after HFI with DBA/2 spleen cells. To evaluate their suppressive capacity, a mixture of 5 x 10^6 immune lymphocytes together with 1 x 10^7 suppressed spleen cells was injected into the right hind foot of naive DBA/2 recipients. As shown in Fig. 6B, line 2, HFI-induced suppressor cells did not suppress ILT-reactivity. Also when HFI-induced suppressor cells from (C57BL x CBA)F1 mice were i.v. transferred to syngeneic recipients, which had been s.c. immunized five days earlier, suppression of DTH was not observed after subsequent challenge for DTH (data not shown).

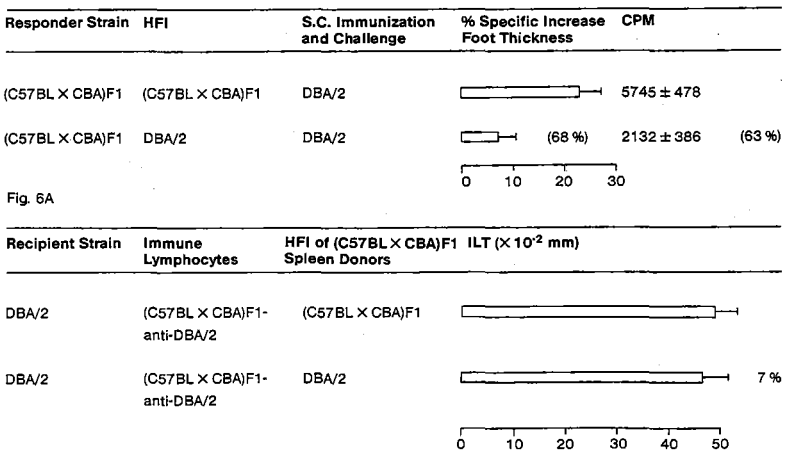

Fig. 6. Target cells of the HFI-induced Ts cells. Upper part: Effect on the induction phase of DTH. Groups of 8 (C57BL x CBA)F1 mice were s.c. injected in their hind feet with either (C57BL x CBA)F1 or DBA/2 spleen cells. Seven days later all mice were s.c. immunized to induce DTH. Another five days later the proliferative activity in the lymph nodes draining the site of immunization from 3 mice was assessed and DTH responses were elicited in the other 5 mice. Each bar represents the mean response ± 1 SEM (n=5). Radioactivity is expressed as cpm ± 1 SEM. The degree of suppression was calculated as percentage decrease of the response as compared to the control group. Lower part: Effect on the expression phase of DTH. Immune lymph node cells were obtained from (C57BL x CBA)F1 mice that had been s.c. immunized with 1 x 10^7 DBA/2 spleen cells in the inguinal areas 5 days prior to the ILT-assay. Suppressed spleen cells were obtained from (C57BL x CBA)F1 donors that had been s.c. injected into their hind feet with 1 x 10^7 DBA/2 spleen cells 4 days prior to the ILT-assay. The immune lymph node cells (5 x 10^6) and the suppressed spleen cells (1 x 10^7) were injected as a mixture into the dorsum of the right hind foot of naive DBA/2 mice. Responses were calculated as (ILT-NLT) and expressed in 10^{-2} mm. Each bar represents the mean response ± 1 SEM (n=5).

Specificity of the HFI-induced Ts cells

To investigate the specificity of the HFI-induced Ts cells, responder mice were s.c. immunized with spleen cells from donor mice that were H-2 and non-H-2 incompatible with the donor mice used as spleen cell donor for HFI. Thus, seven days after HFI of C57BL/6 mice with BALB/c or C3H/Law spleen cells, these mice were s.c. immunized with either BALB/c or C3H/Law spleen cells. As shown in Fig. 7, lines 2 and 3, DTH to BALB/c spleen cells was suppressed after HFI with BALB/c spleen cells but not after HFI with C3H/Law spleen cells, and vice versa (Fig. 7, lines 5 and 6).

Subsequently we investigated the effect of HFI on DTH to third party alloantigens using the procedure described by Bianchi et al. (11,12). Thus, seven days after HFI of BALB/c mice with CBA spleen cells these mice were s.c. immunized with (C57BL x CBA)F1 spleen cells. Five days later the development of DTH to the third party C57BL/6 alloantigens was evaluated. As shown in Fig. 7, line 8, DTH to C57BL/6 alloantigens was suppressed by the Ts cells induced by HFI with CBA spleen cells. Similar suppression of

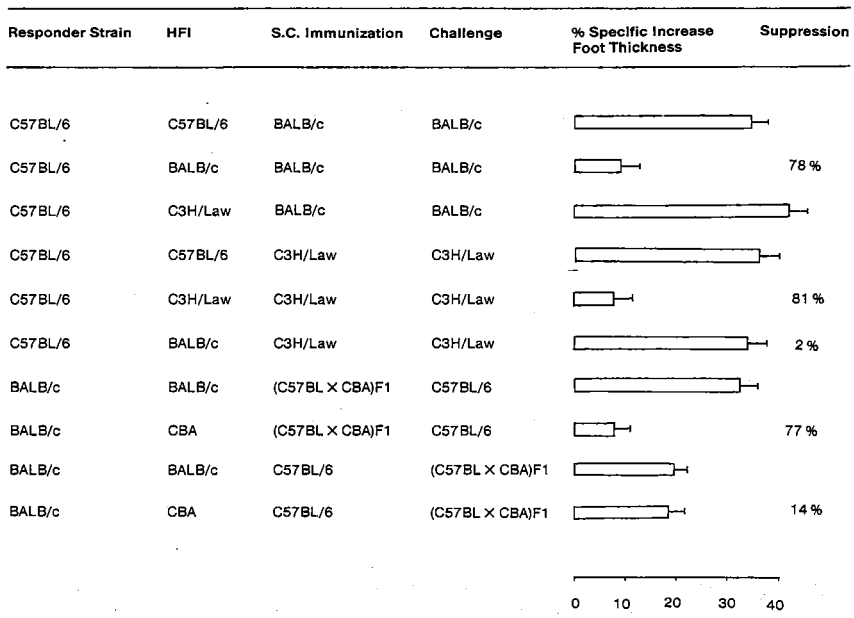

Fig. 7. Specificity of the HFI-induced Ts cells. Responder mice were s.c. injected into their hind feet with the indicated allogeneic spleen cells. Seven days later all mice were s.c. immunized with the indicated allogeneic spleen cells and challenged to assay for DTH another five days later. Each bar represents the mean response $\pm$ 1 SEM (n=5). The degree of suppression was calculated as percentage decrease of DTH as compared to the relevant DTH control group.

DTH to third party alloantigens was observed in other donor-recipient combinations (data not shown). However, when the suppression-inducing alloantigens were administered together with the third party alloantigens during the expression (challenge) phase of DTH, suppression was not found (Fig. 7, line 10).

MHC-restriction of HFI-induced Ts cells

To investigate whether HFI-induced Ts cells suppress DTH in a MHC-restricted manner, B10.AQR mice were immunized in their hind feet with Swiss spleen cells. Four days later their spleen cells were obtained and i.v. transferred to syngeneic B10.AQR, H-2K incompatible B10.A, H-2I incompatible B10.T6R and H-2D incompatible B10.BYR recipients, respectively. Within two hrs after transfer all mice were s.c. immunized with Swiss spleen cells to induce DTH. Five days later DTH responses were elicited. As shown in Fig. 8, lines 4 and 6, HFI-induced Ts cells caused a suppressed DTH response in H-2K as well as in H-2I incompatible recipients. However, suppression was not observed in H-2D incompatible recipients of HFI-induced Ts cells (Fig. 8, line 8). Thus, the HFI-induced Ts cells appear to suppress DTH in a H-2D restricted manner.

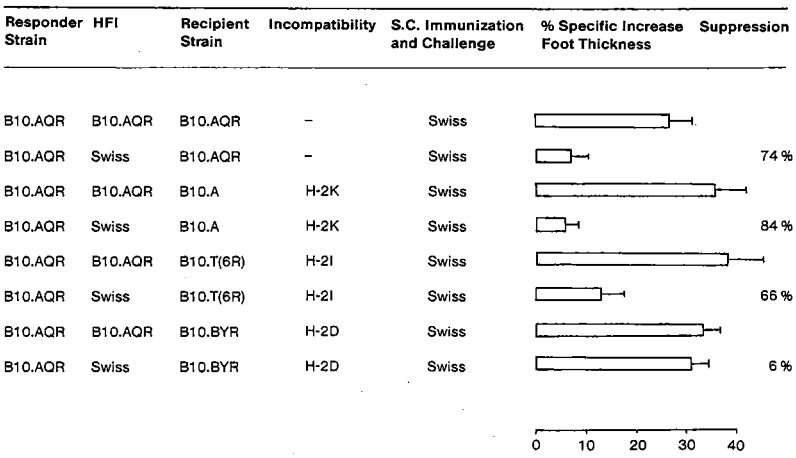

Fig. 8. MHC-restriction of HFI-induced Ts cells. Groups of B10.AQR mice were s.c. injected into their hind feet with Swiss spleen cells. Four days later all mice were killed, their spleen cells were pooled and i.v. injected into B10.AQR, B10.A, B10.T(6R) and B10.BYR recipients, respectively. Within two hrs after transfer all recipients were s.c. immunized with Swiss spleen cells. Five days later DTH responses were elicited. Each bar represents the mean response $\pm$ 1 SEM (n=5). The degree of suppression was calculated as percentage decrease of DTH as compared to the relevant control group.

DISCUSSION

Suppression of alloimmunity can be induced by the systemic administration of supraimmunogenic doses of alloantigen, which preferentially leads to induction of immune unresponsiveness. Strikingly, the present study shows that s.c. injection of a moderate antigen dose of 1 x 10^7 allogeneic spleen cells into the hind feet of mice induces not only a long-lasting state of suppression of DTH to these alloantigens, but also induces DTH reactivity. Whether DTH reactivity (Fig. 1, upper part) or suppression of DTH reactivity (Fig. 1, lower part) is found, merely depends on the protocol employed after the HFI. Thus, when the hind-foot-immunized mice were s.c. challenged in a hind foot five days after HFI, DTH responses were found. However, when such mice were s.c. immunized in their inguinal areas seven days after HFI, and DTH responses were elicited another five days later, DTH reactivity appeared to be suppressed. Suppression of DTH was also found after s.c. immunization in the fore-feet seven days prior to s.c. immunization in the inguinal areas (data not shown). Apparently, there is a delicate balance between activation and suppression of DTH to alloantigens.

Suppression by HFI could be induced in donor-responder combinations that were either H-2 and non-H-2 incompatible, or H-2 incompatible only, but not in combinations that were non-H-2 incompatible only (Fig. 1, lower part). In contrast, i.v. injection of allogeneic spleen cells induced suppression of DTH in all donor-recipient combinations, independent of their alloantigenic differences (3,12).

The induction of suppression appeared to be dose dependent. A dose of 3 x 10^6 to 1 x 10^7 allogeneic spleen cells injected per hind foot, induced a state of suppression of DTH, regardless of whether or not the injected spleen cells had been irradiated (Fig. 2). This stands in clear contrast to the induction of suppression by i.v. injection of allogeneic spleen cells. In the case of i.v. immunization unirradiated allogeneic spleen cells optimally induce suppression at a dose ten times lower than irradiated allogeneic spleen cells do (2,3). This difference is probably due to the site of sequestration of the allogeneic spleen cells used for immunization. I.v. injected cells that are damaged by irradiation are probably more prone to scavenge by the capillary beds in lung, liver and kidney than unirradiated cells, thus leaving a smaller dose that can effectively induce suppression in the lymphoid organs. This problem is less encountered in HFI, where the induction of suppression likely takes place predominantly in the regional (popliteal) lymph nodes, from where the suppressor cells disseminate through the body.

Rich and Rich (6) reported that from day 3 to day 7 after HFI Ts cells could be obtained from the spleen. These Ts cells suppressed MLC-reactivity. As shown in Figs 3 and 4, HFI-induced suppression of DTH could also be adoptively transferred from day 3 to day 7 after HFI. This appeared possible with spleen cells and pooled lymph node cells (Fig. 3), but not with serum (data not shown). This resemblance in time course occurred in spite of the fact that in MLC the suppressor cells are in close proximity to the cells whose activity they have to regulate, whereas after adoptive transfer the suppressor cells first have to migrate to the lymphoid organ(s) where they have to exert their function. A similar time course for the transfer of suppression by spleen cells was reported for Ts cells induced by i.v. immunization (2). Bril et al. attributed the long-lasting maintenance of suppression in the actively suppressed mice to recirculating Ts memory

cells (13). Whether similar Ts memory cells are involved in the maintenance of HFI-induced suppression of DTH is subject of investigation. Phenotypical analysis of the in vivo acting HFI-induced suppressor cells revealed that they expressed the Thy-1^+, Lyt-1^-2^+ phenotype (Fig. 5) and thus can be referred to as Ts cells. Rich and Rich reported that after specific in vitro reactivation, HFI-induced Lyt-1^-2^+ Ts cells produced a genetically restricted suppressor factor (7,14). This factor would activate Lyt-1^+2^+ Ts cells that are present in the MLC responder population to deliver the ultimate suppressive signal (15).

Several groups (16,17) reported evidence for regulation of in vivo cell-mediated immunity by an idiotypic-anti-idiotypic cascade of Ts cells. Through interaction of Lyt-1^+2^- Ts inducer cells with Lyt-1^+2^+ Ts transducer cells, Lyt-1^-2^+ Ts effector cells would become activated. The phenotype of the HFI-induced Ts cells would make these cells comparable to these Ts effector cells. However, functionally there appears to be a major difference. The HFI-induced Ts cells can only suppress the induction phase, but not the expression phase of DTH (Fig. 6), whereas the Ts effector cells operative in other systems usually suppress the expression phase of various immune responses (16,17). The Ts cells induced by i.v. injection of allogeneic spleen cells expressed the Lyt-1^+2^+ phenotype, and suppressed the induction phase as well as the expression phase of DTH (2,4). It therefore appears that the detection of a certain immunoregulatory pathway is highly dependent on the applied protocol for induction of unresponsiveness and on the assay used to monitor suppression.

Our studies on the specificity of the HFI-induced Ts cells showed that they are antigen-specific (Fig. 7). DTH responses were suppressed only when the donors of the spleen cells that had been used for HFI were syngeneic to the donors of the spleen cells used for the induction of DTH. Similar results were found by Van der Kwast et al. (3) for the Ts cells induced by i.v. immunization with allogeneic spleen cells, and by Liew (18) for Ts cells induced by i.v. immunization with xenogeneic red blood cells. In contrast, Rich and Rich reported that HFI-induced Ts cells suppressed MLC-reactivity antigen-non-specifically (6). Bianchi et al. (11,12) described experimental conditions by which also the response against so called 'third party' alloantigens could be suppressed. When they administered the alloantigens that had been used for i.v. immunization to the third party during either the induction phase of DTH or the expression phase of DTH alloantigens, together with the DTH response to the third party alloantigens appeared to be suppressed. When after HFI of responder mice (Fig. 7) third party alloantigens were administered together with the suppression-inducing alloantigens during the induction phase of DTH, we also found suppression of DTH to third party alloantigens. Thus, the HFI-induced Ts cells are antigen-specific as far as their activation is concerned, but once activated they can also suppress the response against third party alloantigens. Our finding that DTH to third party alloantigens could not be suppressed when both sets of alloantigens were simultaneously injected at challenge only, emphasizes that the HFI-induced Ts cells can only operate during the induction phase of DTH.

The phenomenon of bystander-suppression might explain the discrepancy between the specificity-data of Rich and Rich (6) and those of Van der Kwast et al. (3). It cannot be excluded that in the experiments of Rich and Rich C57BL/6 alloantigens from the suppression-inducing inoculum were still present in the spleen of the suppressed BALB/c mice when they were sacrificed and used as donors of suppressor cells. If so, these alloantigens

were simultaneously added to the MLC used to test the proliferative response to third party A/J alloantigens. Retrospectively, a likely correct interpretation of their data would be that the contaminating C57BL/6 alloantigens reactivated C57BL/6-specific Ts cells. Once reactivated these Ts cells may have suppressed the response against the A/J alloantigens as well. Indeed, Rich and Rich reported that for in vitro production of non-specific suppressor factor the HFI-induced Lyt-1^{-}2^{+} Ts cells could only be reactivated with the specific alloantigens (7).

With regard to the MHC restriction of the in vivo activity of HFI-induced suppressor cells we found that, after adoptive transfer, these Ts cells could not exert their DTH-suppressing effect in H-2D incompatible recipients. Rich and Rich (7) reported that the in vitro activity of Ts cells induced by HFI of BALB/c mice with completely allogeneic C57BL/6 spleen cells was controlled by H-2D and/or H-2I-C encoded molecules. Miller et al. (20) reported that the induction and expression of Ts cells regulating contact hypersensitivity to 1-fluoro-2,4 dinitrobenzene was restricted by H-2D encoded molecules. On the other hand, Rich (19) reported that the in vitro activity of HFI-induced, MHC class I-specific Ts cells was restricted by I-C and I-J determinants. A role for I-J determinants in the regulation of Ts cell cascades has been reported for in vivo cell-mediated immune responses such as contact hypersensitivity to 4-hydroxy-3-nitrophenyl-acetyl and DTH to azobenzene arsonate (17). However, it has been reported that no MHC-molecule is encoded by the I-C subregion (21), whereas DNA-studies have shown that I-J molecules are not encoded by a definite H-2I subregion (22). In contrast to the HFI-induced Ts cells, the Ts cells induced by i.v. immunization with allogeneic spleen cells exerted their effect across MHC- as well as Igh-barriers (4).

In conclusion, HFI with allogeneic spleen cells induces Ts cells that under the appropriate experimental conditions can suppress DTH to alloantigens. Several differences in in vivo activity were found between Ts cells induced by HFI and Ts cells induced by i.v. immunization. This indicates that the route of immunization might dictate the characteristics of the immunoregulatory pathway which becomes activated.

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CHAPTER IV

INDUCTION OF SUPPRESSION BY SIMULTANEOUS IMMUNIZATION WITH LIPOPOLYSACCHARIDE AND ALLOANTIGENS

CHAPTER IV.1.

LIPOPOLYSACCHARIDE-INDUCED SUPPRESSION OF DTH REACTIVITY TO HISTOCOMPATIBILITY ANTIGENS. I. KINETIC ASPECTS AND SPECIFICITY

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SUMMARY

The effects of bacterial lipopolysaccharide (LPS) on the development of DTH-reactivity to alloantigens in mice were investigated. DTH to a particular set of alloantigens could be suppressed by treatment of responder mice with a single intravenous (i.v.) injection of 100 ug LPS and a simultaneous subcutaneous (s.c.) injection of the appropriate allogeneic spleen cells. The suppression lasted at least 60 days and affected the afferent limb of the DTH response as well as the efferent limb. The suppression could be adoptively transferred to naive syngeneic recipient mice by spleen cells, but not by immune serum, and proved to be antigen-specific. In spite of this specificity, the DTH-response against unrelated "third-party" alloantigens could be suppressed as well, provided the latter were presented during the induction-phase of DTH together with the alloantigens that had been used for the induction of suppression.

INTRODUCTION

Delayed-type-hypersensitivity (DTH) can be elicited in rodents against a variety of antigens such as bacteria, sheep red blood cells, contact-sensitizing agents and histocompatibility antigens (reviewed by Crowle (1)) and is a T cell-dependent phenomenon. Most studies on the nature of DTH reactive T cells suggest that predominantly helper T (Th) cells cause DTH reactions (2-4), although some studies have shown that under certain conditions also cytotoxic T (Tc) cells can cause DTH (5, 6).

In previous papers we have dissected the DTH response to histocompatibility antigens (7-11). Subcutaneous (s.c.) or intravenous (i.v.) immunization of mice with allogeneic spleen cells and a challenge with similar allogeneic spleen cells in a hind foot 5 or 6 days later causes a specific thickening of the injected hind foot due to DTH.

Van der Kwast et al. (8) showed that this DTH reactivity can be suppressed by an i.v. tolerizing injection of the responder mice with the relevant non-irradiated or irradiated allogeneic spleen cells. This suppression proved to be antigen dose dependent, and could be demonstrated up to 70 days after the tolerizing injection. Bianchi et al. (9) showed that this suppression was mediated by Lyt-1+,2+ suppressor T (Ts) lymphocytes whose mode of action is to reduce the proliferative activity of the DTH reactive T cells. Specificity studies revealed that these Ts cells are antigen-specific as far as their activation is concerned. However, once activated they can also suppress the DTH reaction to third-party antigens, provided the latter are presented in combination with, and at the same site as the specific antigens that have activated the Ts cells (10,11).

Gill and Liew (12), Uchiyama et al. (13) and Colizzi (14) presented evidence showing that immune responses can be regulated not only by Th and Ts cells, but also by non-T cells, especially when the latter are activated by lipopolysaccharide (LPS). These LPS activated cells suppressed a variety of responses. Remarkably, Franzl and McMaster (15), Hoffman et al. (16) and Lagrange et al. (17,18) reported that administration of LPS together with antigen can enhance as well as suppress the immune response. The actual outcome, however, is highly dependent on the experimental conditions, as reviewed by Nelson (19).

Rosenstreich (20) reported that the enhancing effect is most likely caused by stimulation of macrophage activity. On the other hand, Colizzi (14) and others (21,22) showed that the suppressive effect can be attributed to the induction of suppressor B cells.

In this study we determined the suppressive effect of LPS on DTH against histocompatibility antigens and established the kinetic aspects and specificity of the suppression.

MATERIALS AND METHODS

Mice

(C57BL/Rij x CBA/Rij)F1 (H-$2^{b/q}$), C3H/Law (H-2^{k}), CBA/Rij (H-2^{q}) and AKR (H-2^{k}) female mice were purchased from the Radiobiological Institute TNO, Rijswijk, The Netherlands. DBA/2 (H-2^{d}) and BALB/c (H-2^{d}) female mice were purchased from Bomholtgard, Ry, Denmark. B10.AQR (H-2^{y1}), C57BL/6 (H-

2^b), B10.ScSn ($H\text{-}2^b$) and BALB.B ($H\text{-}2^b$) female mice were purchased from HARLAN OLAC Ltd, Bicester, England. B6.C-$H\text{-}2^{bm1}$ ($H\text{-}2^{bm1}$), B6.C-$H\text{-}2^{bm12}$ ($H\text{-}2^{bm12}$) and (B6.C-$H\text{-}2^{bm1}$ x B6.C-$H\text{-}2^{bm12}$)F1 ($H\text{-}2^{bm1/bm12}$) female mice were bred and raised at our own department. All mice were 10-12 weeks of age at the start of the experiments.

Preparation of cell suspensions and serum

Spleen and/or lymph nodes were removed, placed in a balanced salt solution (BSS), minced with scissors and squeezed through a nylon gauze filter to provide single cell suspensions. Nucleated cells were counted with a Coulter counter model BZI (Coulter Electronics, Harpenden, England). Blood was obtained from donor mice from the axillary and brachial arteries under ether anesthesia, allowed to clot at room temperature for 30 min and centrifuged for 20 min at 3000 rpm after which the serum was collected.

Immunization with alloantigens

Induction of DTH reactivity was performed by s.c. immunization with 1 x 10^7 of the appropriate allogeneic spleen cells, suspended in a volume of 100 ul. A volume of 50 ul of this suspension was injected into each inguinal area.

Suppression of DTH to alloantigens was induced either by the LPS-protocol (see below) or by i.v. immunization of responder mice with 5 x 10^7 irradiated (20Gy) allogeneic spleen cells prior to induction of DTH. Radiation characteristics of the equipment used have been described in a previous paper (23).

Induction of suppression by LPS

LPS-B (Salmonella typhosa 0901) was purchased from Difco Laboratories, Detroit, Mich., U.S.A. Experimental mice received one i.v. injection of LPS dissolved in BSS. Control mice received similar injections of BSS only. The schedules of LPS treatment in relation to the s.c. injection of allogeneic spleen cells and DTH assay are indicated in the legend to the figures. Unless stated otherwise, a dose of 100 ug LPS was i.v. injected in combination with a s.c. injection of 1 x 10^7 allogeneic spleen cells.

Estimation of DNA synthesis in draining lymph node (LN) cells

Incorporation of tritiated thymidine (3H-TdR) was used to measure DNA-synthesis of draining LN cells as a reflection of proliferation of these LN cells, according to the method described by Moorhead (24). Briefly, on day 5 after transfer of spleen cells and s.c. immunization three mice that received spleen cells from suppressed mice and three mice that received spleen cells from BSS treated control mice were sacrificed, the draining lymph nodes were removed and single cell suspensions were prepared. To measure DNA synthesis, quintuple cultures of 1 x 10^6 cells/ml in tissue culture medium (RPMI 1640 plus 10% fetal calf serum) from each group were pulsed with 1 uCi of 3H-TdR for 5 hr in vitro (specific activity 5 c mM).

After 5 hr incubation, the cells were washed with BSS, acid insoluble material was precipitated on glass fiber filters and radioactivity was counted in a liquid scintillation counter (Packard model 3375). Radioactivity was corrected for background and quenching, and expressed as cpm.

Assay for immune-lymphocyte-transfer (ILT) reactivity

To measure ILT reactivity a modification of the method described by

Bianchi et al. (25) was used. Briefly, 1 x 10^7 immune lymph node cells obtained from immunized donors 5 days after immunization with a particular set of alloantigens were s.c. injected into the dorsum of the right hind foot of relevant recipients. The resulting DTH response is called the immune-lymphocyte-transfer (ILT) response. Syngeneic control mice received 1 x 10^7 lymph node cells from non-immunized donor mice. This injection results in a normal lymphocyte-transfer (NLT) response. The specific ILT reactivity was calculated as mean ILT (n=5) minus mean NLT (n=5) and is expressed in 10^{-2} mm.

Assay for DTH

The assay for measuring DTH to alloantigens has been described in detail in previous papers (7,8). Briefly, DTH responses were elicited by s.c. injection of a challenge dose of 2 x 10^7 allogeneic spleen cells into the dorsum of the right hind foot of previously immunized mice. Foot thickness was measured 24 and 48 hrs later. In each experiment a group of non-immunized control mice also received a challenge injection. The percentage specific foot swelling was calculated as the mean percentage of swelling from each experimental group minus the mean percentage of swelling from the challenge control group. The swelling of these control mice varied between 15 and 25%.

RESULTS

Induction of suppression

A state of suppression of DTH was induced in (C57BL x CBA)F1 mice by i.v. injection of 100 ug LPS dissolved in 0.5 ml BSS and simultaneous s.c. immunization with 1 x 10^7 H-2 and non-H-2 incompatible BALB/c spleen cells. Control mice received either BSS i.v. plus s.c. injections of BALB/c spleen cells, LPS i.v. only, 5 x 10^7 irradiated BALB/c spleen cells i.v. or no treatment at all. Six days later all mice were s.c. immunized with 1 x 10^7 BALB/c spleen cells. Another 6 days later DTH responses were elicited. As shown in Figure 1A, line 3 the combined treatment with LPS i.v. and allogeneic spleen cells s.c. caused a clear suppression of DTH whereas the treatment with either LPS alone (Fig. 1A, line 4) or BSS and allogeneic spleen cells (Fig. 1A, line 2) did not reduce the DTH response. Since the DTH response in the group which received BSS and allogeneic spleen cells and the response in the group which did not receive any treatment (Fig. 1A, line 1) appeared to be of comparable strength the former treatment was applied as control treatment in all subsequent experiments. The same protocol was used with B10.AQR responder mice and H-2 incompatible B10.ScSn spleen cells as immunogen and with C3H/Law responder mice and non-H-2 incompatible AKR spleen cells as immunogen. In each combination a clear suppression of DTH was achieved by administration of LPS plus alloantigens (Fig.1, expts. B and C). Also the DTH response against H-2 subregion coded alloantigens could be suppressed with this protocol. B10.AQR mice treated with LPS and spleen cells from either H-2K incompatible B10.A donors or H-2I incompatible B10.T(6R) donors showed a poor DTH response against these alloantigens (Fig. 1, expts. D en E). The extent of suppression induced by the combined treatment with LPS and alloantigens was the same as could be achieved by i.v. tolerization of responder mice with the relevant allogeneic spleen cells (last bar of each experiment in Fig. 1), which treatment has been shown to induce Ts cells (9).

	Responder Strain	Treatment	S.C. Immunization and Challenge	% Specific Increase Foot Thickness	Suppression
A.	(C57BL × CBA)F_1	-	BALB/c		
	(C57BL × CBA)F_1	BSS i.v. BALB/c s.c.	BALB/c		
	(C57BL × CBA)F_1	LPS i.v. BALB/c s.c.	BALB/c		70 %
	(C57BL × CBA)F_1	LPS i.v.	BALB/c		2 %
	(C57BL × CBA)F_1	BALB/c i.v.	BALB/c		77 %
B.	B10.AQR	BSS i.v. B10.ScSn s.c.	B10.ScSn		
	B10.AQR	LPS i.v. B10.ScSn s.c.	B10.ScSn		70 %
	B10.AQR	LPS i.v.	B10.ScSn		18 %
	B10.AQR	B10.ScSn i.v.	B10.ScSn		78 %
C.	C3H/Lw	BSS i.v. AKR s.c.	AKR		
	C3H/Lw	LPS i.v. AKR s.c.	AKR		81 %
	C3H/Lw	LPS i.v.	AKR		10 %
	C3H/Lw	AKR i.v.	AKR		75 %
D.	B10.AQR	BSS i.v. B10.A s.c.	B10.A		
	B10.AQR	LPS i.v. B10.A s.c.	B10.A		80 %
	B10.AQR	LPS i.v.	B10.A		5 %
	B10.AQR	B10.A i.v.	B10.A		71 %
E.	B10.AQR	BSS i.v. B10.T(6R) s.c.	B10.T(6R)		
	B10.AQR	LPS i.v. B10.T(6R) s.c.	B10.T(6R)		56 %
	B10.AQR	LPS i.v.	B10.T(6R)		1 %
	B10.AQR	B10.T(6R) i.v.	B10.T(6R)		75 %

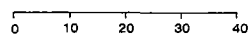


Fig. 1. Suppression of DTH by combined treatment with LPS and allogeneic spleen cells. Responder mice received an i.v. injection of 100 ug LPS in 0.5 ml BSS or 0.5 ml BSS only with a simultaneous s.c. injection of allogeneic spleen cells. Other mice received an i.v. injection of 5×10^7 irradiated allogeneic spleen cells. In Exp.A. one group of mice received no injection prior to the induction of DTH. Six days later all mice were s.c. immunized with 1×10^7 allogeneic spleen cells. Challenge for DTH was performed on day 6 after s.c. immunization. Each column represents the mean response ± 1 SEM (n=6). The donor-responder combinations were: Exp. A: BALB/c - (C57BL x CBA)F1 (H-2 and non-H-2 incompatible); Exp. B: B10.ScSn - B10.AQR (H-2 incompatible); Exp. C: AKR - C3H/Law (non-H-2 incompatible); Exp. D: B10.A-B10.AQR (H-2K incompatible); Exp. E: B10.T(6R)-B10.AQR (H-2I incompatible).

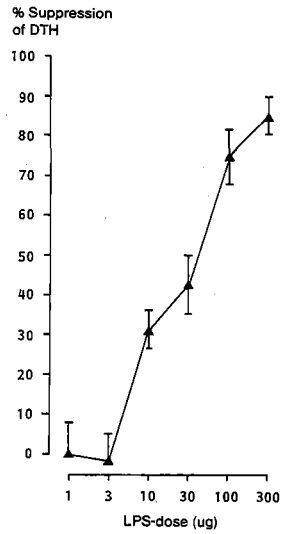


Fig. 2A

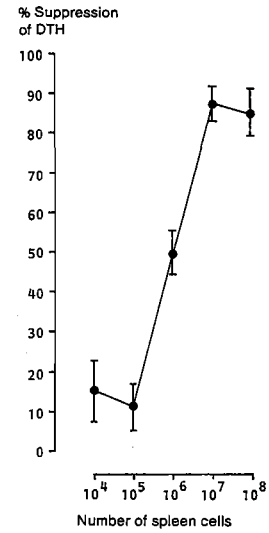


Fig. 2B

Fig. 2. Determination of the optimal dose of LPS (A) and dose of alloantigens (B) for suppression of DTH. (A): (C57BL x CBA)F1 mice were i.v. injected with 1, 3, 10, 30, 100 or 300 ug LPS together with a s.c. injection of 1 x 10^7 BALB/c spleen cells. Six days later all mice were s.c. immunized with 1 x 10^7 BALB/c spleen cells. Another six days later all mice were challenged for DTH. Each experimental point represents the arithmetic mean of the suppressive effect $\pm$ 1 SEM (n=6), calculated as percentage of the positive control. (B): (C57BL x CBA)F1 mice were i.v. injected with 100 ug LPS and simultaneously s.c. injected with 10^4, 10^5, 10^6, 10^7 or 10^8 BALB/c spleen cells. Another six days later all mice were challenged for DTH. Each experimental point represents the arithmetic mean of the suppressive effect $\pm$ 1 SEM (n=6), calculated as percentage of the positive control.

Dose-response relationship

The optimal dose of LPS to induce a state of suppression was investigated by treatment of (C57BL x CBA)F1 mice with either 1, 3, 10, 100 or 300 ug LPS i.v. and 1 x 10^7 BALB/c spleen cells s.c. Six days later all mice were s.c. immunized with BALB/c spleen cells to induce a state of DTH. Fig. 2A shows that increasing doses of LPS in combination with a fixed dose of 1 x 10^7 allogeneic spleen cells caused a decreasing DTH response. A dose of 100 ug to 300 ug LPS was sufficient to cause almost complete suppression of DTH. Doses of LPS higher than 100 ug were frequently lethal for the responder mice. Therefore, in all subsequent experiments a dose of 100 ug LPS was used. Hereafter we investigated the optimal dose of allogeneic spleen cells which, together with a fixed dose of 100 ug LPS would result in suppression of DTH. (C57BL x CBA)F1 responder mice received 100 ug LPS i.v. and either 10^4, 10^5, 10^6, 10^7, or 10^8 DBA/2 spleen cells s.c. As shown in Fig. 2B, optimal suppression of DTH was achieved with a dose of 10^7 allogeneic spleen cells although substantial reduction of DTH was already observed at a dose of 10^6 allogeneic spleen cells. The combined treatment

with 100 ug LPS i.v. and 1 x 10^7 allogeneic spleen cells s.c. will be referred to as the LPS-protocol.

Relationship between LPS injection and s.c. injection of alloantigens

To determine the optimal interval between the LPS injection and the injection of allogeneic spleen cells groups of BALB/c responder mice received LPS either 10, 6, 4 or 2 days before or on the same day as the s.c. administration of (C57BL x CBA)F1 spleen cells. Control mice were treated as aforementioned. Six days later in all groups DTH reactivity was induced with the relevant spleen cells. As shown in Fig. 3A the interval between the LPS injection and the first s.c. injection of allogeneic spleen cells should be no longer than 2 days to achieve maximal suppression. Similar kinetics were found for C3H/Law mice responding to non-H-2 incompatible AKR spleen cells.

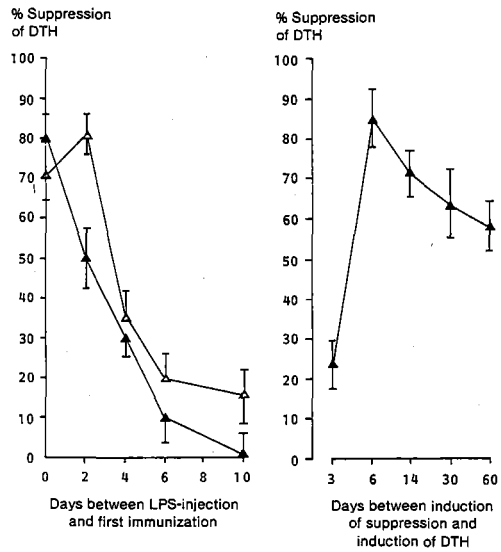


Fig. 3A Fig. 3B

Fig. 3. Determination of the optimal time interval between LPS injection and s.c. injection of allogeneic spleen cells (A) and the time course of the suppression (B). (A): Groups of mice were i.v. injected with 100 ug LPS in BSS or BSS only at 10, 6, 4 or 2 days before or on the same day as the s.c. injection of 1 x 10^7 allogeneic spleen cells. Six days later all groups were s.c. immunized and another six days later all groups were challenged to assay for DTH. Each point represents the arithmetic mean of the suppressive effect $\pm$ 1 SEM (n=6) calculated as a percentage of the positive control. The donor-responder combinations were (open triangles): (C57BL x CBA)F1 - BALB/c (H-2 and non-H-2 incompatible); and (closed triangles) AKR - C3H/Law (non-H-2 incompatible). (B): Groups of (C57BL x CBA)F1 mice were i.v. injected with 100 ug LPS and were simultaneously s.c. injected with 1 x 10^7 BALB/c spleen cells. Each group was s.c. immunized with BALB/c spleen cells either 3, 6, 14 or 60 days later. Six days after the latter s.c. immunization each group was challenged to assay for DTH. Each experimental point represents the arithmetic mean of the suppressive effect $\pm$ 1 SEM (n=6) calculated as percentage of the positive control.

Time course of suppression

The duration of the LPS-induced suppression of DTH was studied in (C57BL x CBA)F1 mice treated with 100 ug LPS i.v. and 1 x 10^7 BALB/c spleen cells s.c. The ability of these mice to mount a DTH response was tested 3, 6, 14 and 60 days later. To circumbent the development of secondary type DTH responses in the control group at the last time point the control groups were treated with BSS and (C57BL x CBA)F1) spleen cells. As shown in Fig. 3B, the LPS treated groups showed a clearly suppressed DTH response from day 6 onwards. Although the suppression gradually decreased, even at 60 days after its induction a significant suppression of DTH was observed.

Transfer of the state of suppression

To investigate the nature of the LPS induced suppression, DBA/2 and (C57BL x CBA)F1 mice were treated with LPS and either (C57BL x CBA)F1 or B10.AQR spleen cells. Six days later, spleens were removed, single cell suspensions were prepared and injected i.v. into naive DBA/2 and (C57BL x CBA)F1 recipients, respectively. Subsequently the recipients were s.c. immunized with (C57BL x CBA)F1 or B10.AQR spleen cells and challenged six days later with the relevant allogeneic spleen cells. As shown in Fig. 4, lines 2 and 4, the mice which received spleen cells from LPS plus alloantigen-treated donors displayed a markedly suppressed DTH reaction against the

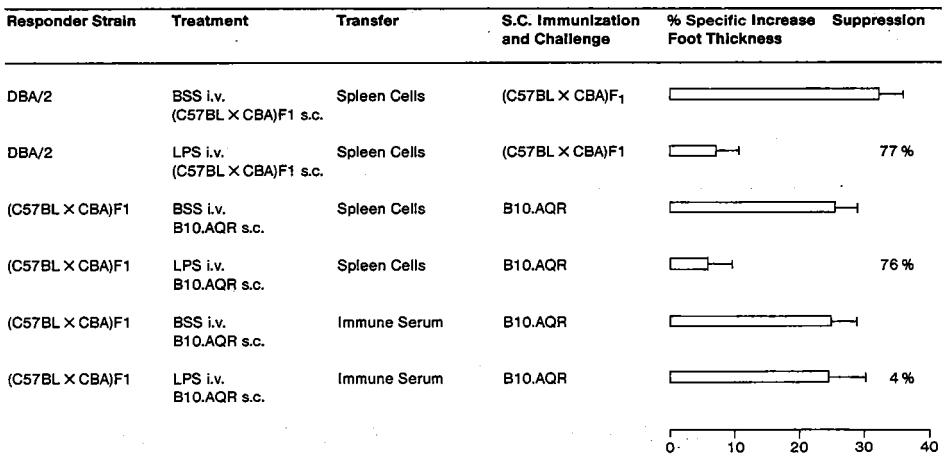


Fig. 4. Determination of the transferability of the LPS induced suppression of DTH. Mice were i.v. injected with 100 ug LPS and s.c. injected with 1 x 10^7 allogeneic spleen cells. Control mice were injected with BSS and allogeneic spleen cells. Four days later all mice were killed, and either a single cell suspension of the spleens of each group of mice or their pooled sera (0.5 ml per recipient) were transferred to naive syngeneic recipient mice. Each recipient received the total spleen cell yield of one experimental animal. A few hours later all mice were s.c. immunized with the appropriate allogeneic spleen cells and six days later all mice were tested for DTH. Each column represents the mean response $\pm$ 1 SEM (n=6). The donor-responder combinations were: (C57BL x CBA)F1 - DBA/2 (H-2 and non-H-2 incompatible); and B10.AQR - (C57BL x CBA)F1 (H-2 and non-H-2 incompatible).

immunizing alloantigens, indicating that the LPS-protocol induces suppressor cells.

On the other hand, transfer of 0.5 ml of serum from the LPS plus alloantigen-treated (C57BL x CBA)F1 donor mice on the day of s.c. immunization could not cause suppression of DTH in the recipient mice (Fig. 4, line 6).

Target cells of the LPS-induced suppressor cells

To test whether LPS-induced suppressor cells can inhibit the afferent limb of DTH (i.e., the activation and clonal growth of DTH reactive T cells), we determined the proliferative activity in the regional lymph nodes of (C57BL x CBA)F1 mice 5 days after adoptive transfer of suppressor cells and subsequent induction of DTH with BALB/c spleen cells.

As shown in Fig. 5A, line 2, the proliferative activity in the regional lymph nodes was significantly reduced concomitantly with a suppressed DTH response in this group.

To test whether LPS-induced suppressor cells can also inhibit the efferent limb of DTH we transferred DTH effector T cells, which had been generated by s.c. immunization of DBA/2 mice with C57BL/6 spleen cells into DBA/2 mice which had been treated with LPS i.v. and C57BL/6 spleen cells s.c. As shown in Fig. 5B, upper part, the suppressed mice that received DTH effector cells showed a greatly suppressed DTH-response. The degree of suppression was of the same extent as in the suppression control group of DBA/2 mice that were both suppressed and s.c. immunized (data not shown). Also when applied in the immune-lymphocyte-transfer assay the LPS-induced suppressor cells suppressed the activity of already activated DTH effector cells (Fig. 5B, lower part).

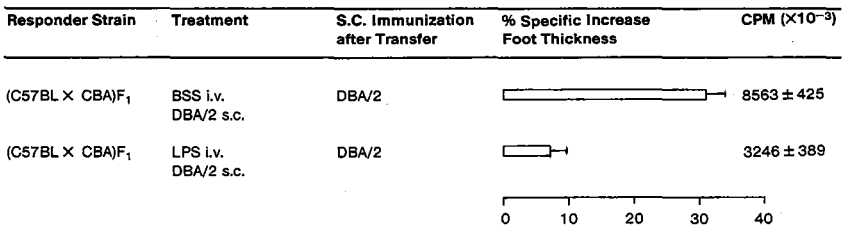
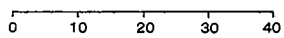


Fig. 5A. Effect of LPS-induced suppressor cells on the induction phase of DTH. Groups of 8 (C57BL x CBA)F1 responder mice were treated with either 100 ug LPS i.v. and 1 x 10^7 DBA/2 spleen cells or 0.5 ml BSS i.v. and 1 x 10^7 DBA/2 spleen cells s.c. Six days later all mice were s.c. immunized with DBA/2 spleen cells. Another five days later the proliferative activity in the lymph nodes draining the site of immunization was assessed and in other mice, DTH responses were elicited with DBA/2 spleen cells. Radioactivity is expressed in cpm $\pm$ 1 SEM (n=3). DTH responses are expressed as percentage specific increase of foot thickness (n=5).

Specificity of the LPS-induced suppression

The specificity of the LPS induced suppression was investigated by using as a source of allogeneic spleen cells for use in the LPS protocol a donor strain that is H-2 and non-H-2 incompatible with the donor strain

Recipient Strain	Treatment	Infused Immune Lymphocytes	Challenge	% Specific Increase Foot Thickness	Suppression
DBA/2	BSS i.v. C57BL/6 s.c.	DBA/2-anti-C57BL/6	C57BL/6		
DBA/2	LPS i.v. C57BL/6 s.c.	DBA/2-anti-C57BL/6	C57BL/6		76%



Recipient Strain	Immune Lymphocytes	Treatment of DBA/2 Spleen Donors	ILT-NLT (X10^{-2}mm)	Suppression
C57BL/6	DBA/2-anti-C57BL/6	BSS i.v. C57BL/6 s.c.		
C57BL/6	DBA/2-anti-C57BL/6	LPS i.v. C57BL/6 s.c.		70%

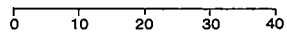


Fig. 5B. Effect of LPS-induced suppressor cells on the expression phase of DTH. Two experimental systems were employed: (A): Infusion of DTH effector cells into suppressed recipients which were subsequently challenged for DTH. DBA/2-anti C57BL/6 immune lymphocytes generated in DBA/2 mice by s.c. immunization with C57BL/6 spleen cells 5 days previously were infused into DBA/2 recipients that had been treated with 100 ug LPS or BSS and 1 x 10^7 C57BL/6 spleen cells 4 days prior to infusion. Within two hours after infusion the recipients were challenged to assay for DTH to C57BL/6 alloantigens. Each bar represents the mean response $\pm$ 1 SEM (n=6). (B): The immune-lymphocyte-transfer (ILT) assay; Immune lymphocytes (5 x 10^6) generated in DBA/2 mice by s.c. immunization with 1 x 10^7 C57BL/6 spleen cells 5 days prior to transfer were injected together with 10^7 spleen cells from naive DBA/2 donors or 10^7 spleen cells from DBA/2 donors that had been treated with 100 ug LPS i.v. and 1 x 10^7 C57BL/6 spleen cells s.c. 4 days prior to transfer into the dorsum of the right hind foot of naive C57BL/6 mice. Responses were calculated as (ILT-NLT) and expressed in 10^{-2} mm. Each bar represents the mean response $\pm$ 1 SEM (n=5).

used for s.c. immunization to induce DTH reactivity. Thus, (C57BL x CBA)F1 mice received LPS and were simultaneously s.c. immunized with B10.AQR spleen cells. Using BALB.B spleen cells for induction and elicitation of DTH, suppression of DTH against these alloantigens could not be demonstrated (Fig. 6A, line 4). This was in clear contrast to the observed suppression in (C57BL x CBA)F1 mice that were given B10.AQR spleen cells during the induction of suppression as well as for the induction and elicitation of DTH (Fig. 6A, line 2).

DTH responses against H-2 subregion coded alloantigens also proved to be specifically suppressed because suppression was observed neither in C57BL/6 responder mice treated with LPS and H-2k incompatible B6.C-H-2^{bm1} spleen cells and s.c. immunized and challenged with H-2I-A incompatible B6.C-H-2^{bm12} spleen cells (Fig. 6B, line 4) nor in C57BL/6 responder mice treated with LPS and B6.C-H-2^{bm12} spleen cells and s.c. immunized and challenged with B6.C-H-2^{bm1} spleen cells (Fig. 6C, line 4).

	Responder Strain	Treatment	S.C. Immunization	Challenge	% Specific Increase Foot Thickness	Suppression
A.	(C57BL × CBA)F_1	BSS i.v. B10.AQR s.c.	B10.AQR	B10.AQR		
	(C57BL ×CBA)F_1	B10.AQR s.c.	B10.AQR	B10.AQR		73 %
	(C57BL × CBA)F_1	BSS i.v. B10.AQR s.c.	BALB.B	BALB.B		
	(C57BL × CBA)F_1	LPS i.v. B10.AQR s.c.	BALB.B	BALB.B		9 %
B.	C57BL/6	BSS i.v. B6.C-H-2^{bm1} s.c.	B6.C-H-2^{bm1}	B6.C-H-2^{bm1}		
	C57BL/6	LPS i.v. B6.C-H-2^{bm1} s.c.	B6.C-H-2^{bm1}	B6.C-H-2^{bm1}		65 %
	C57BL/6	BSS i.v. B6.C-H-2^{bm1} s.c.	B6.C-H-2^{bm12}	B6.C-H-2^{bm12}		
	C57BL/6	LPS i.v. B6.C-H-2^{bm1} s.c.	B6.C-H-2^{bm12}	B6.C-H-2^{bm12}		0 %
C.	C57BL/6	BSS i.v. B6.C-H-2^{bm12} s.c.	B6.C-H-2^{bm12}	B6.C-H-2^{bm12}		
	C57BL/6	LPS i.v. B6.C-H-2^{bm12} s.c.	B6.C-H-2^{bm12}	B6.C-H-2^{bm12}		75 %
	C57BL/6	BSS i.v. B6.C-H-2^{bm12} s.c.	B6.C-H-2^{bm1}	B6.C-H-2^{bm1}		
	C57BL/6	LPS i.v. B6.C-H-2^{bm12} s.c.	B6.C-H-2^{bm1}	B6.C-H-2^{bm1}		3 %
D.	BALB/c	BSS i.v. CBA/Rij s.c.	(C57BL × CBA)F_1	C57BL/6		
	BALB/c	LPS i.v. CBA/Rij s.c.	(C57BL × CBA)F_1	C57BL/6		67 %
	BALB/c	BSS i.v. CBA/Rij s.c.	C57BL/6 and CBA/Rij	C57BL/6		
	BALB/c	LPS i.v. CBA/Rij s.c.	C57BL/6 and CBA/Rij	C57BL/6		70 %
E.	C57BL/6	BSS i.v. B6.C-H-2^{bm1} s.c.	(B6.C-H-2^{bm1} × B6.C-H-2^{bm12})F_1	B6.C-H-2^{bm12}		
	C57BL/6	LPS i.v. B6.C-H-2^{bm1} s.c.	(B6.C-H-2^{bm1} × B6.C-H-2^{bm12})F_1	B6.C-H-2^{bm12}		69 %
F.	C57BL/6	BSS i.v. B6.C-H-2^{bm12} s.c.	(B6.C-H-2^{bm1} × B6.C-H-2^{bm12})F_1	B6.C-H-2^{bm1}		
	C57BL/6	LPS i.v. B6.C-H-2^{bm12} s.c.	(B6.C-H-2^{bm1} × B6.C-H-2^{bm12})F_1	B6.C-H-2^{bm1}		68 %

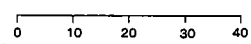


Fig. 6

Fig. 6. Specificity of LPS-induced suppression of DTH. (A): Responder mice were i.v. injected with 100 ug LPS and simultaneously s.c. injected with 1 x 10^7 allogeneic spleen cells. Six days later all mice were s.c. immunized with allogeneic spleen cells and challenged to assay for DTH another six days later. Each column represents the mean DTH $\pm$ 1 SEM (n=6). (B): Responder mice were i.v. injected with 100 ug LPS and simultaneously s.c. injected with 1 x 10^7 allogeneic spleen cells. Control mice received BSS i.v. and allogeneic spleen cells s.c. Six days later all mice were s.c. immunized with allogeneic spleen cells as indicated and challenged for DTH reactivity another six days later. Each column represents the mean DTH response $\pm$ 1 SEM (n=6).

In spite of the apparent specificity of the LPS-induced suppression we investigated whether the LPS-induced suppressor cells could also suppress the DTH reactivity against third-party alloantigens using the procedure described by Bianchi et al. (11). Thus, BALB/c responder mice were treated with LPS and CBA/Rij spleen cells. Six days later these mice were s.c. immunized with either (C57BL/6 x CBA/Rij)F1 spleen cells or with a mixture of irradiated C57BL/6 and CBA/Rij spleen cells and another six days later challenged with C57BL/6 spleen cells which express the third party set alloantigens. As shown in Fig. 6D, lines 2 and 4, the DTH response against C57BL/6 alloantigens was suppressed although these alloantigens had not been administered at the time of the induction of suppression.

Suppression was also found when DTH responses were elicited with B6.C-H-2^{bml2} spleen cells in C57BL/6 mice that had been s.c. immunized with (B6.C-H-2^{bml} x B6.C-H-2^{bml2})F1 spleen cells 6 days after induction of suppression with LPS and B6.C-H-2^{bml} spleen cells. Similar results were obtained in C57BL/6 mice using LPS and B6.C-H-2^{bml2} spleen cells for induction of suppression, (B6.C-H-2^{bml} x B6.C-H-2^{bml2})F1 spleen cells for induction of DTH and B6.C-H-2^{bml} spleen cells for elicitation of DTH (Figs 6E and 6F).

DISCUSSION

To our knowledge this study is the first that reports the modulation of murine DTH to alloantigens (H-2 as well as non-H-2 coded) by LPS. The optimal protocol for the induction of suppression of DTH in mice appeared to be 100 ug of LPS injected i.v. immediately thereafter followed by a s.c. injection of 1 x 10^7 allogeneic spleen cells (Fig. 2). Most authors describing suppressive effects of LPS apply comparable doses of LPS, given around the time of immunization. Gill and Liew (12), Altmann and Blyth (26) and Colizzi (14) injected 100 ug of LPS i.v. just prior to or at the time of antigen administration. Winchurch (27) injected 100 ug of LPS intraperitoneally (i.p.) at the time of graft implantation. We found that i.p. injection of 100 ug of LPS together with s.c. immunization with alloantigens resulted in a suppressed state of DTH to these alloantigens (data not shown). However, in several experiments this suppression was less marked and consistent than the suppression observed after i.v. administration of LPS.

Lagrange et al. (17) injected 100 ug of LPS s.c. in a hind foot together with a dose of sheep red blood cells (SRBC). In our hands s.c. injection of LPS together with allogeneic spleen cells into the inguinal areas

did not suppress the development of DTH (data not shown). Furthermore, s.c. immunization in the hind feet with alloantigens already causes suppression of DTH mediated by antigen-specific Ts cells (Molendijk et al., submitted for publication). This makes it difficult to compare Lagranges' data and ours.

Galleli (28) injected only 1 ug of LPS i.v. 24 hr prior to infection with Listeria monocytogenes, a dose that was found to induce suppression of anti-Listeria-DTH. An explanation for the effectiveness of this low dose of LPS might be that bacterial products from the infecting inoculum potentiated the LPS effect, analogous to the Schwartzmann-reaction.

The interval between LPS injection and first administration of antigen appears to be crucial for the induction of suppression. Only when this interval amounted to two days or less, with the LPS being injected first a suppressed state of DTH was observed (Fig. 3A). Similar data were obtained by others (12, 17, 26, 27). Skopinska (29) found suppression of cell-mediated immunity (CMI) to alloantigens after s.c. injection of 20 ug LPS on seven consecutive days just prior to skin allografting. Lagrange and Mackaness (18), on the other hand, reported enhancement of DTH when the LPS was injected after the administration of SRBC. In our hands administration of LPS after immunization with alloantigens did neither suppress nor enhance the ensuing DTH response (data not shown).

Few authors reported about the time course of LPS-induced suppression of CMI. Hunter et al. (30) found that suppression of DTH to peptidoglycans induced in rats by injection of a purified isolate from streptococcal cell walls lasted for at least 90 days. This is compatible with our finding that even at 60 days after the injection of LPS and alloantigens DTH responses could hardly be elicited (Fig. 3B).

The LPS-induced state of suppression of DTH to alloantigens can be adoptively transferred by spleen cells (Fig. 4). Several authors reported similarly about suppression of CMI by LPS. Macrophages (31, 32), T-cells (26,33) and B-cells (12,21,22,34) have all been implicated as the responsible cell-type. Our finding that suppression could not be transferred with serum from LPS treated mice contradicts the findings of Thomson et al. (35). They found that both spleen cells and serum from LPS treated mice inhibited the development of graft-versus-host reactivity. Studies are in progress to investigate this discrepancy and to characterize the nature of the LPS-induced suppressor cell. Preliminary results indicate that Ts cells are not involved.

Suppression of the proliferation phase of DTH appears to be the primary mechanism by which the LPS-induced suppressor cells inhibit the development of DTH (Fig. 5A). Suppressive effects of bacterial products on the proliferation of T lymphocytes have been reported by Ellner and Spagnuolo (36) and Deschennes et al. (37). Lagrange et al. (17) suggested that suppression of the induction phase of DTH to SRBC by LPS is due to an accelerated antibody production which causes an earlier onset of antigen-specific T cell regulatory mechanisms. Bianchi et al. (9) showed that Ts cells induced by i.v. tolerization with alloantigens likewise suppress DTH to these alloantigens by inhibition of the proliferative activity in the regional lymph nodes.

We found that, apart from suppression of the afferent arm of DTH, LPS-induced suppressor cells can also suppress the activity of already activated DTH effector T cells (Fig. 5B). Enhanced entrapment of i.v. transferred DTH effector T cells in LPS treated mice due to the LPS-induced hyperplasia of the reticulo endothelial system might explain the reduced DTH response. However, the data from the ILT-NLT experiment clearly show

that the LPS induced suppressor cells can inhibit the activity of already activated DTH effector cells.

Suppression of the efferent arm of DTH to alloantigens was also reported by Bianchi et al. (25) for the Ts cells induced by i.v. tolerization. In contrast, Lagrange and Mackaness (18) reported that LPS enhanced the efferent phase of DTH. A possible explanation for this discrepancy could reside in the different route of administration of LPS applied by Lagrange and Mackaness and by us. They injected LPS locally in the same hind foot that had previously been injected with antigen, whereas we injected LPS systemically. Local administration of LPS might stimulate macrophages in the draining lymph nodes, which play a pivotal role during the efferent phase of DTH.

Our finding that the LPS-induced suppression of DTH is antigen-specific (Fig. 6, expts A,B and C) confirms the reports by Altmann and Blyth (26) and Gill and Liew (12). However, suppressive effects after administration of bacteria-like products have been attributed to non-specific suppressor cells. For instance, Leclerc et al. (38) reported the induction of non-specific Ts cells by repeated injection of large doses (>100 ug) of muramyl dipeptide that suppressed the antibody response to SRBC.

As shown in Fig. 6, expts. D,E and F, the LPS-induced suppressor cells can also suppress the DTH response against third party alloantigens. This phenomenon of bystander suppression only becomes obvious when the third party antigens are administered during the induction of DTH together with the alloantigens that together with LPS had induced the suppressor cells. Moreover, physical association between these alloantigens and the third-party alloantigens does not appear to be required for the occurrence of bystander suppression. Altmann and Blyth (26) and Gill and Liew (12) did not report experiments which were appropriate to demonstrate this bystander suppression. On the other hand, Petit et al. (39) showed that i.v. injection of mice with Pseudomonas aeruginosa suppressed their resistance to infection with Listeria monocytogenes as well as anti-Listeria DTH, which, in view of our data, could possibly be explained by the expression of common determinants on both microorganisms.

We conclude that the LPS-induced suppressor cells are antigen-specific as far as their activation is concerned. However, their ultimate suppressive effect is not antigen-specific. A similar conclusion was drawn by Bianchi et al. for the suppressive effect of Ts cells on allogeneic DTH (10,11). The data presented in this paper could therefore possibly be explained by postulating Ts cells as final mediators of the LPS-induced suppression. However, preliminary results indicate that Ts cells are not required for the systemic transfer of the LPS-induced suppression.

Studies to characterize the LPS-induced suppressor cells and to elucidate the suppressive mechanism are in progress.

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CHAPTER IV.2.

LIPOPOLYSACCHARIDE-INDUCED SUPPRESSION OF DTH REACTIVITY TO HISTOCOMPATIBILITY ANTIGENS. II. REQUIREMENTS FOR THE INDUCTION OF THE SUPPRESSION, CHARACTERIZATION OF THE SUPPRESSOR CELLS AND EVIDENCE FOR THE INVOLVEMENT OF AN ANTI-IDIOTYPIC ANTIBODY

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SUMMARY

We have previously reported that simultaneous treatment of mice with bacterial lipopolysaccharide (LPS) and allogeneic spleen cells induces an antigen-specific, suppressor cell mediated state of suppression of delayed-type hypersensitivity (DTH) to histocompatibility (H) antigens. The present study describes the requirements for the induction of the suppression, the characterization of the suppressor cells and their mechanism of action. The LPS-induced suppressor cells could be obtained from the spleen and adoptively transferred between day 3 and day 12 after LPS-treatment, but not at later time points. The lipid A component of LPS appeared to be essential for the induction of suppression. LPS-treatment did not induce suppression in the LPS low responder strains CBA/N and B10.ScCr. The LPS-induced suppressor cells were resistant to treatment with monoclonal anti-Thy-1.2 antibodies and complement, and were not adsorbed during passage through a Sephadex-G10 column. However, they specifically adhered to plastic tissue culture dishes coated with an anti-kappa light chain monoclonal antibody. In vitro culture of spleen cells from suppressed mice and subsequent purification of the secreted immunoglobulins from the culture supernatant yielded an antigen-specific immunosuppressive preparation, which suppressed activated DTH reactive T cells. The suppressive activity could be adsorbed by syngeneic alloantigen-specific immune lymphocytes, but not by allogeneic lymphocytes expressing the specific alloantigens, or by syngeneic lymphocytes with specificity for third party alloantigens. These results suggest that the LPS-induced suppression of DTH to alloantigens is mediated by an anti-idiotypic antibody to the receptor of the DTH reactive T cells.

INTRODUCTION

Immune responses to histocompatibility (H) antigens can be induced by subcutaneous (s.c.) or intravenous (i.v.) immunization with allogeneic cells (1,2). An example of such a response is the DTH response against H antigens which can be elicited in previously sensitized mice by a challenge with allogeneic spleen cells. This DTH reactivity is a function of sensitized T lymphocytes that probably predominantly belong to a subpopulation of helper T lymphocytes (3,4).

Several investigators have reported about the modulation of humoral and cellular immune responses by bacterial products such as lipopolysaccharide (LPS) (5-7). Remarkably, enhancement (6) as well as suppression (7) were reported after treatment with LPS.

In a previous paper (8) we reported that simultaneous i.v. injection of LPS and s.c. immunization with allogeneic spleen cells induces a state of suppression of DTH in mice. Induction of this suppression appeared to be dependent on both the dose of LPS and the dose of allogeneic spleen cells. Under optimal conditions this suppression lasted for at least 60 days after LPS treatment. The suppression proved to be specific for the alloantigens that were injected together with the LPS. However, the response against unrelated third party alloantigens could also be suppressed. This became obvious when the third party alloantigens were administered during either the induction or the elicitation of DTH together with the alloantigens that, together with LPS, had induced the suppression some days before. The state of suppression could be adoptively transferred to recipient mice by i.v. infusion of spleen cells obtained four days after the combined treatment with LPS and allogeneic spleen cells, but not by transfer of immune serum.

The present paper extends these studies to the requirements for the induction of the LPS-induced suppression, the characterization of the suppressor cells and their mechanism of action.

MATERIALS AND METHODS

Mice

(C57BL/Rij x CBA/Rij)F1 (H-$2^{b/q}$), CBA/N (H-2^{k}) and CBA/T6 (H-2^{k}) female mice, 4 to 8 weeks of age, were purchased from the Radiobiological Institute TNO, Rijswijk, The Netherlands. BALB/c (H-2^{d}), DBA/2 (H-2^{d}), B10.ScCr (H-2^{b}) and B10.ScSn (H-2^{b}) female mice, 4 to 8 weeks of age, were purchased from Bomholtgard, Ry, Denmark. All animals were kept in light-cycled rooms and had access to pelleted food (Hope Farms, Woerden, The Netherlands) and acidified water ad libitum. The animals' age at the start of each experiment ranged from 10 - 12 weeks.

LPS-treatment

Mice received a single i.v. injection of 100 ug LPS-B (Salmonella typhosa 0901; Difco Laboratories, Detroit, MI, U.S.A.) dissolved in 0.5 ml balanced salt solution (BSS). Control mice received 0.5 ml BSS without LPS. Lipid A was kindly provided by Dr. C. Galanos, Freiburg, F.R.G. Detoxified LPS was prepared according to the method described by Neter et al. (9). Mice received 100 ug of either preparation i.v., dissolved in 0.5 ml BSS.

Preparation of cell suspensions

Mice were rapidly killed by carbon dioxide exposure and their spleens were obtained, minced with scissors and squeezed through nylon gauze filters to get single cell suspensions. Cell numbers were counted with a Coulter counter model BZI (Coulter Electronics Ltd., Harpenden, Herts, U.K.). This procedure always yielded cell suspensions with over 95% viability.

Cell separation techniques

T cell depletion: Spleen cells were incubated with anti-Thy-1.2 monoclonal antibodies (clone F7D5, HARLAN OLAC Ltd., Bicester, Oxon, U.K.) for 30 min at 4°C. After two washings the cells were incubated with guinea pig complement (Behringwerke, Marburg-Lahn, F.R.G.) for 30 min at 37°C. This procedure eliminated over 90% of the splenic T cells.

Macrophage depletion: Sephadex-G10 (Pharmacia, Uppsala, Sweden) columns were prepared according to the method described by Ly and Mishell (10). A total number of 10^8 spleen leukocytes, obtained after red cell lysis with an ammonium-chloride buffer, were loaded per 8 ml column and incubated for 30 min at 37°C. Thereafter, Sephadex nonadherent cells were eluted with 15 ml of warm (37°C) medium (BSS plus 2% fetal calf serum (FCS)) at a rate of one drop per sec. This method eliminated over 98% of the splenic macrophages and between 15 and 20% of the splenic B cells.

B cell depletion: B cells were eliminated by panning of spleen leukocytes on plastic tissue culture dishes (100 x 15 mm, Nunclon, Roskilde, Denmark) that had been coated with rat-anti-mouse-kappa monoclonal antibodies according to the method described by Mage et al. (11). The anti-kappa monoclonal antibody was produced by the hybridoma 226, which was kindly provided by Prof.Dr. M.D. Scharff, Albert Einstein College of Medicine, Yeshiva University, New York, U.S.A. A volume of 4 ml of a 5 x 10^7 cells/ml suspension was placed on each plate and incubated for 30 min at room temperature (RT). After gentle swirling for 30 sec the plates were incubated for another 30 min at RT. Thereafter, nonadherent cells were removed and the plates were washed twice with BSS to further remove all nonadherent cells. Adherent cells were subsequently removed with a rubber policeman. The nonadherent cells contained less than 10% Ig^+ cells whereas the adherent cells contained more than 95% Ig^+ cells. The cellular composition of each suspension was determined by FACS-analysis (FACS II, Becton Dickinson, Sunnyvale, CA, U.S.A.). Other spleen cells were similarly panned on anti-Lyt-2 coated plates (subclone 53-6.72, kindly provided by Drs. J. Ledbetter and L.A. Herzenberg, Department of Genetics, Stanford University of Medicine, Stanford, CA, USA).

Preparation of control and suppressive immunoglobulin

Spleen cells from donor mice that had been treated with LPS only, or with both LPS and allogeneic spleen cells were cultured in RPMI 1640 medium supplemented with glutamine (4mM), penicillin (100 U/ml), streptomycin (50 ug/ml) and FCS (15%) at 10^7 cells/ml for three days. Culture supernatants were obtained and adsorbed (pH 8.0) to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) coupled with affinity purified rat-anti-mouse kappa light chain monoclonal antibody 226. The binding fraction was eluted with 0.1 M glycine/HCl (pH 2.3). The solution was buffered with 1 M Tris (pH 7) and afterwards dialyzed with phosphate buffered saline (PBS), concentrated to 0.5 - 1.0 mg/ml with a stirred ultra filtration cell (Amicon, Canvers, MA, U.S.A.) and stored in small aliquots (1 ml) at -20°C.

Adoptive transfer

Spleen cells were i.v. transferred to recipient mice in a volume of 1.0 ml BSS. Recipient mice were treated with 15 U heparin (Liquémine; Hoffmann-La Roche & Co., Ltd., Basel, Switzerland) 30 min before transfer to prevent embolism. Usually recipient mice received a number of spleen cells equivalent to the cell yield of one whole spleen.

Induction of DTH

DTH reactivity was induced by s.c. injection of 1×10^7 allogeneic spleen cells suspended in 100 ul BSS, equally divided over both inguinal areas.

Assay for DTH

DTH responses were elicited by a s.c. challenge injection of 2×10^7 allogeneic spleen cells in a volume of 50 ul into the dorsum of the right hind foot. Hind foot swelling was measured 24 and 48 h later using a foot thickness meter with a 0.05 mm accuracy. In each experiment a group of non-immunized syngeneic control mice was included which only received the challenge injection to correct for background swelling. The increase of foot thickness of these control mice varied between 16 and 25 per cent.

Immune-lymphocyte-transfer assay

A number of 1×10^7 immune lymph node cells from immunized donors were s.c. injected into the dorsum of the right hind foot of recipient mice, expressing the relevant H antigens. The donor mice had been s.c. immunized with recipient type allogeneic spleen cells 5 days previously. The resulting DTH response is called the immune-lymphocyte-transfer (ILT) response. To control for background reactivity all recipients were simultaneously s.c. injected with 1×10^7 non-immune lymph node cells into the dorsum of the left hind foot. This injection results in a normal-lymphocyte-transfer (NLT) response. The specific ILT reactivity was calculated as ILT minus NLT and expressed in 10^{-2} mm.

RESULTS

Systemic transfer of LPS-induced suppressor cells

Groups of (C57BL x CBA)F1 mice were i.v. injected with 100 ug LPS and s.c. immunized with 1×10^7 BALB/c spleen cells two hours later. Control groups received BSS and syngeneic spleen cells. At variable intervals after LPS treatment one subgroup of mice was s.c. immunized with BALB/c spleen cells. Another subgroup was sacrificed and their spleen cells were adoptively transferred to syngeneic recipient mice (one spleen equivalent per recipient) that were subsequently s.c. immunized with BALB/c spleen cells. All groups were challenged with BALB/c spleen cells to assay for DTH six days later. As shown in Fig 1, in the LPS-treated animals the state of suppression of DTH rapidly developed and was maximal at day 6 after LPS-treatment. Although the state of suppression gradually waned, DTH reactivity was still significantly suppressed at day 55. On the other hand, adoptive transfer of suppression by spleen cells was possible between day 3 and day 15 after LPS-treatment only, with its optimum on day 4.

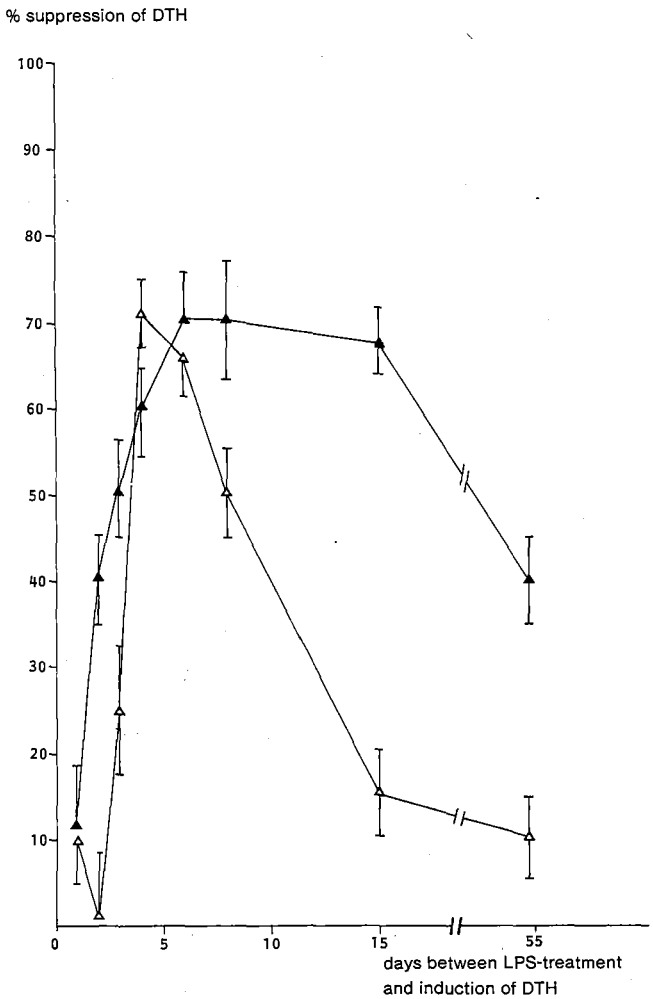

Fig. 1. Time course of the adoptive transfer of LPS-induced suppression. Groups of (C57BL x CBA)F1 mice were i.v. injected with 100 ug LPS or BSS and s.c. immunized with BALB/c spleen cells. Control groups received BSS and syngeneic spleen cells. At various intervals after treatment DTH responses to BALB/c H antigens were elicited (▲) in a subgroup of the LPS-treated mice and in a subgroup of the control mice. At each time point spleen cell suspensions from other LPS treated mice and from other BSS treated mice were prepared and adoptively transferred (△) to syngeneic recipients, which were s.c. immunized with BALB/c spleen cells within two hours after transfer. Six days later DTH responses were elicited in all mice. Each experimental point represents the percentage suppression of DTH ± 1 SEM (n=5). The percentage suppression was calculated as the percentage decrease of DTH response as compared to the relevant DTH control group.

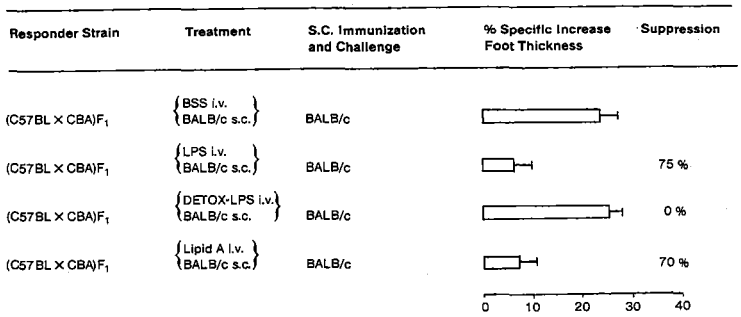

Fig. 2. The effective part of LPS for the induction of suppression. Groups of (C57BL x CBA)F1 mice were i.v. injected with 100 ug LPS, 100 ug detoxified LPS, 100 ug purified lipid A or BSS and s.c. immunized with BALB/c spleen cells. Six days later DTH to BALB/c H antigens was induced. DTH responses were elicited another six days later. Each bar represents the mean DTH response $\pm$ 1 SEM (n=5). The percentage suppression was calculated as the percentage decrease of DTH response as compared to the relevant DTH control group.

The effective part of LPS for the induction of suppression

The role of the lipid A part of the LPS molecule for the induction of suppression of DTH was investigated. Therefore, groups of (C57BL x CBA)F1 mice were i.v. injected with either 100 ug LPS, 100 ug purified lipid A, 100 ug detoxified LPS, which lacks lipid A, or BSS and immediately thereafter s.c. immunized with BALB/c spleen cells. Six days later all groups were s.c. immunized to induce DTH. Another six days later the mice were challenged to assay for DTH. As can be seen in Fig 2, lines 2 and 4, DTH responses were suppressed in the LPS-treated mice as well as in the lipid A-treated mice. Detoxified LPS, however, did not induce suppression of DTH (Fig 2, line 3).

Similar results were obtained when spleen cells from similarly treated (C57BL x CBA)F1 mice were adoptively transferred to syngeneic recipients four days after treatment (data not shown). Apparently it is the mitogenic lipid A part of LPS and not the immunogenic polysaccharide part of LPS that, together with allogeneic immunization, induces the state of suppression.

The influence of LPS responsiveness on the induction of suppression by LPS

Several strains of mice have been described (12,13) which lack responsiveness to LPS due to a genetic defect. We investigated whether the LPS-protocol for induction of suppression can induce a state of unresponsiveness in the LPS low responder strains CBA/N and B10.ScCr. Therefore, groups of CBA/N and B10.ScCr mice and their respective control strains CBA/T6 and B10.ScSn received an i.v. injection of LPS together with a s.c. injection of DBA/2 or BALB/c spleen cells. Another six days later DTH reactivity was induced. As shown in Fig 3, treatment with LPS and allogeneic cells could not induce a state of suppression in the LPS low responder strains CBA/N (Fig 3, line 4) and B10.ScCr (Fig 3, line 8).

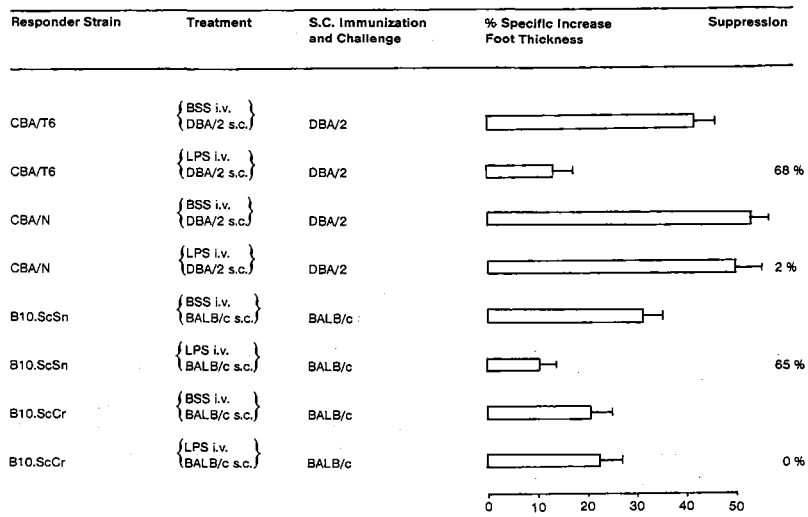

Fig. 3. The influence of LPS responsiveness on the induction of suppression by LPS. Groups of CBA/T6, CBA/N, B10.ScSn and B10.ScCr mice were i.v. injected with LPS or BSS and s.c. immunized with DBA/2 and BALB/c spleen cells, respectively. Six days later DTH was induced and another six days later DTH responses were elicited. Each bar represents the mean DTH response ± 1 SEM (n=5). The percentage suppression was calculated as the percentage decrease of DTH reponse as compared to the relevant DTH control group.

Phenotype of the LPS-induced suppressor cells

The nature of the splenic LPS-induced suppressor cells was investigated by the selective in vitro elimination of spleen cell subsets during adoptive transfer experiments. Firstly, it was investigated whether the suppressor cells belong to the T cell lineage. Therefore, groups of (C57BL x CBA)F1 mice received i.v. injections of LPS or BSS together with s.c. injections of BALB/c spleen cells. Four days later all mice were sacrificed and their spleen cells were obtained, from which the T cells were eliminated by treatment with anti-Thy-1.2 monoclonal antibodies and complement. The residual non-T cells were adoptively transferred to syngeneic recipients, which were s.c. immunized with BALB/c spleen cells. Six days later DTH responses were elicited. As shown in Fig 4, upper part, line 3, depletion of T cells from the inoculum to be transferred did not prevent the transfer of suppression. This indicates that Ts cells are not involved in the adoptive transfer of LPS-induced suppression of DTH.

Secondly, the role of splenic macrophages in the transfer of the LPS-induced suppression was investigated. Therefore, spleen cells from (C57BL x CBA)F1 donors which had been treated with LPS or BSS and DBA/2 spleen cells four days earlier were filtered through Sephadex G-10 columns to eliminate macrophages and monocytes. The nonadherent cells were adoptively transferred to syngeneic recipients which were subsequently s.c. immunized with DBA/2 spleen cells. DTH responses were elicited six days later. As shown in Fig 4, lower part, line 4, elimination of macrophages and monocytes from

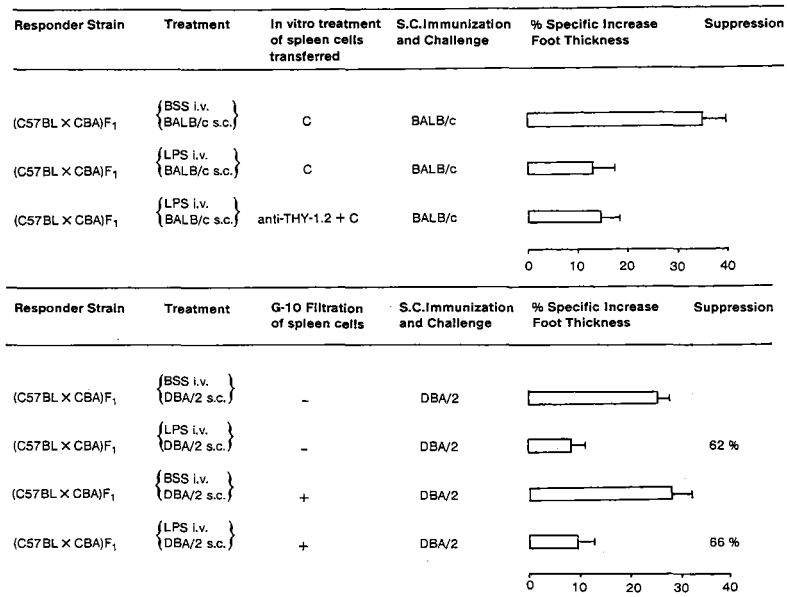

Fig. 4. LPS-induced suppression of DTH is not mediated by T cells (upper part) or macrophages (lower part). Groups of (C57BL x CBA)F1 mice were i.v. injected with LPS or BSS and s.c. immunized with BALB/c or DBA/2 spleen cells. Four days later all mice were killed and their spleen cells were treated with anti-Thy-1.2 monoclonal antibodies and complement or complement only, or their splenic leucocytes were filtered through Sephadex G-10 columns. The residual cells were adoptively transferred to syngeneic recipients. Within two hours these recipients were s.c. immunized to induce DTH and six days later DTH responses were elicited. Each bar represents the mean DTH response $\pm$ 1 SEM (n=5). The percentage suppression was calculated as the percentage decrease of DTH response as compared to the DTH control group.

the spleen cells to be transferred did not abrogate the transfer of suppression.

Thirdly, the role of splenic B cells was investigated. Therefore, spleen cells from (C57BL x CBA)F1 mice that had been treated with LPS or BSS and DBA/2 spleen cells four days earlier were panned on plastic tissue culture dishes coated with either anti-kappa monoclonal antibodies or anti-Lyt-2 monoclonal antibodies. The latter dishes served as controls for the panning procedure. After incubation the non-adherent cells were obtained by thorough washing of the plates and adoptively transferred to syngeneic recipients. Adherent cells were obtained from the anti-kappa coated plates with a rubber policeman and similarly transferred. Six days after transfer and subsequent s.c. immunization with DBA/2 spleen cells DTH responses were elicited. As shown in Fig 5, upper part, line 4, depletion of Lyt-2^+ cells did not abrogate the transfer of suppression. In contrast, depletion of

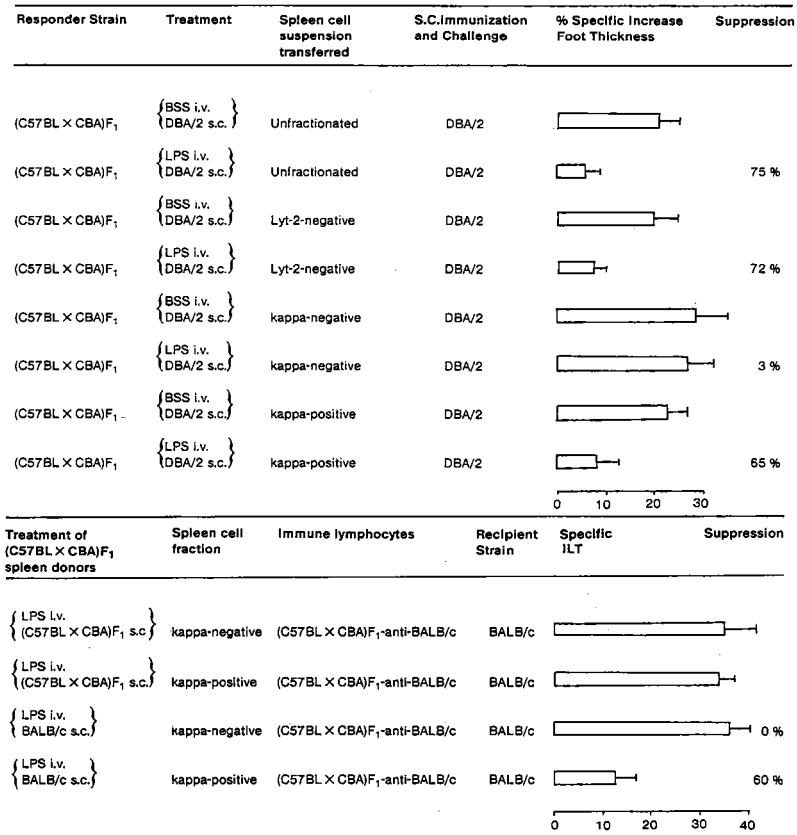

Fig. 5. LPS-induced suppression is mediated by B cells. Upper part: Groups of (C57BL x CBA)F1 mice were i.v. injected with LPS or BSS and s.c. immunized with DBA/2 spleen cells. Four days later their spleen leukocytes were obtained and Lyt-2^+ cells and Ig^+ cells were separated by panning as described in the Materials and Methods section. The various fractions were adoptively transferred to syngeneic recipients as indicated. Within two hours after transfer all recipients were s.c. immunized with DBA/2 spleen cells and six days later DTH responses were elicited. Each bar represents the mean DTH response $\pm$ 1 SEM (n=5). The percentage suppression was calculated as the percentage decrease of DTH response as compared to the relevant DTH control group. Lower part:(C57BL x CBA)F1 mice were used as donors of (C57BL x CBA)F1-anti-BALB/c immune lymph node cells 5 days after s.c. immunization with BALB/c spleen cells. Other (C57BL x CBA)F1 mice were used as donors of suppressor cells four days after treatment with LPS and BALB/c spleen cells. Suppressed and control spleen leucocyte suspensions were separated into kappa-negative and kappa-positive fractions as described in the Materials and Methods section. For evaluation of suppression 5 x

10^6 immune lymph node cells were s.c. injected into the dorsum of the right hind foot of BALB/c recipients together with 1 x 10^7 fractionated suppressed spleen cells. To control for background reactivity all mice received a s.c. injection of 5 x 10^6 normal lymph node cells together with 1 x 10^7 fractionated suppressed spleen cells into the dorsum of the left hind foot. Each bar represents the mean specific ILT response ± 1 SEM (n=5). The degree of suppression was calculated as the percentage decrease of specific ILT response as compared to the relevant ILT control group.

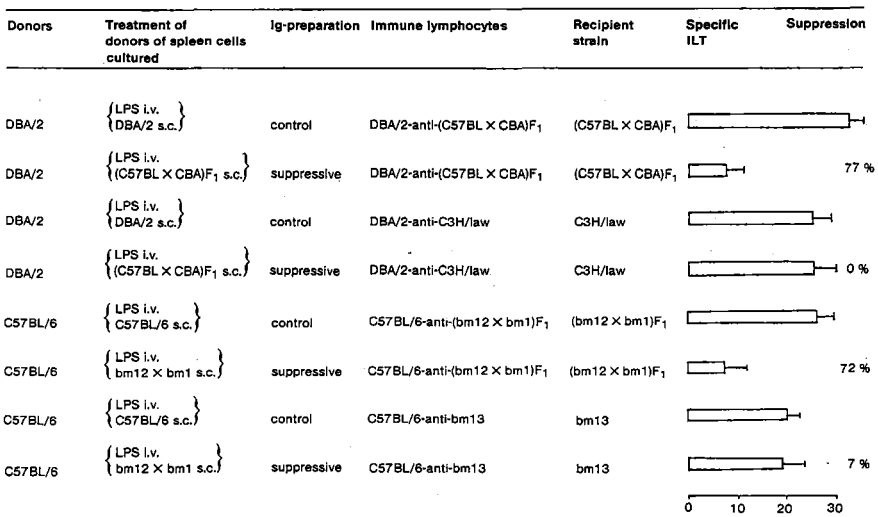

Fig. 6. LPS-induced suppression of ILT reactivity is mediated by molecules binding to an anti-kappa affinity column. Upper part: DBA/2 mice were used as donors of either DBA/2-anti (C57BL x CBA)F1 immune lymph node cells or DBA/2-anti-C3H/Law immune lymph node cells 5 days after s.c. immunization with (C57BL x CBA)F1 or C3H/Law spleen cells, respectively. Lower part: C57BL/6 mice were used as donors of either C57BL/6-anti-(bm12 x bm1)F1 immune lymph node cells or C57BL/6-anti-bm13 immune lymph node cells 5 days after s.c. immunization with (bm12 x bm1)F1 spleen cells or bm13 spleen cells, respectively. In both experiments the anti-kappa binding fraction was isolated from cultures of suppressed or control spleen cells as described in the Materials and Methods section. For evaluation of suppression 5 x 10^6 immune lymph node cells, suspended in 50 ul of the kappa positive fraction (0.5 mg/ml), were s.c. injected into the dorsum of the right hind foot of the relevant recipients. As a control all mice were s.c. injected with 5 x 10^6 normal lymph node cells, suspended in 50 ul of the same kappa positive fraction, into the left hind foot. Each bar represents the mean specific ILT response ± 1 SEM (n=5). The degree of suppression was calculated as the percentage decrease of specific ILT response as compared to the relevant ILT control group.

kappa$^+$ cells completely prevented the transfer of suppression, while the B cell enriched adherent cell population clearly caused suppression of DTH in the recipient mice (Fig 5, upper part, lines 6 and 8).

Also when tested in the ILT-assay for evaluating the effect on the expression phase of DTH, the suppression appeared to be mediated by B cells (Fig 5, lower part, line 4). This indicates that the simultaneous treatment with LPS and allogeneic spleen cells activates B cells to mediate a suppressive effect on DTH.

Mechanism of action of the LPS induced suppressor cells

Having established the B cell nature of the LPS-induced suppressor cells we investigated their mechanism of action. Therefore, four days after treatment of (C57BL x CBA)F1 mice with LPS and BALB/c spleen cells, or LPS only, their spleen cells were cultured in vitro as described in the Materials and Methods section. Three days later the culture supernatants were obtained and the anti-kappa binding fraction was isolated by affinity chromatography.

This immunoglobulin (Ig) fraction was concentrated and tested for its suppressive activity in the ILT-assay. As shown in Fig 6, line 2, this preparation suppressed the ILT-reactivity of DBA/2-anti-(C57BL x CBA)F1 immune lymphocytes after simultaneous injection into the right hind foot of (C57BL x CBA)F1 recipient mice. The suppressive activity proved to be antigen-specific because the ILT-reactivity of DBA/2-anti-C3H/Law immune lymphocytes in C3H/Law mice could not be suppressed (Fig 6, line 4). Similar results were obtained when the Ig preparations from C57BL/6 mice that had been treated with LPS and H-2K plus H-2I incompatible (bm12 x bm1)F1 spleen cells, or LPS only. The latter was evaluated in the ILT-assay for suppression of the activity of C57BL/6-anti-(bm12 x bm1)F1 immune lymphocytes (Fig 6, line 6) and C57BL/6-anti-bm13 immune lymphocytes (Fig. 6, line 8).

Binding specificity of the suppressive Ig

The suppressive Ig molecules might exert their effect either by binding to the relevant alloantigens or by binding to the antigen receptors that are expressed by the immune lymphocytes. To discriminate between these possibilities, control and suppressive Ig preparations obtained after three days of culture of spleen cells from DBA/2 mice that had been treated with LPS and (C57BL x CBA)F1 spleen cells or LPS only were adsorbed to either (C57BL x CBA)F1 lymphocytes, DBA/2-anti-(C57BL x CBA)F1 immune lymphocytes or DBA/2-anti-C3H/Law immune lymphocytes. After adsorbtion the supernatant was tested for its suppressive activity in the ILT-assay. As shown in Fig 7, lines 4 and 8, neither adsorbtion to (C57BL x CBA)F1 lymphocytes which express the specific alloantigens, nor adsorbtion to DBA/2-anti-C3H/Law immune lymphocytes, which express antigen receptors with third party specificity, could adsorb the suppressive activity. In contrast, adsorbtion to DBA/2-anti-(C57BL x CBA)F1 immune lymphocytes, which express the specific antigen receptors, completely adsorbed the suppressive activity (Fig 7, line 6). This indicates that the suppressive activity of the LPS-induced suppressive Ig is directed towards the antigen receptor of the DTH reactive T cells.

DISCUSSION

This report deals with the requirements for the induction of the LPS-induced suppression of DTH to H-antigens in mice, the characterization of the suppressor cells involved and the mechanism of suppression. Remarkably, the suppressor cells involved in the transfer of suppression were no longer detectable in the spleen at 15 days after LPS-treatment. On the other hand, in the actively suppressed mice themselves the state of suppression was observed even at 55 days after LPS-injection (Fig 1). A similar time course has been reported for the transfer of suppression of efferent DTH to Herpes Simplex Virus (HSV-1) by LPS-induced Ts cells (14) and for the transfer of suppression of DTH to alloantigens by Ts cells induced by prior i.v. tolerization of mice with a high dose of irradiated allogeneic spleen cells (2). Bril et al. (15) and Bianchi et al. (16) extended the latter studies and showed that after the disappearance of the functionally active Ts cells

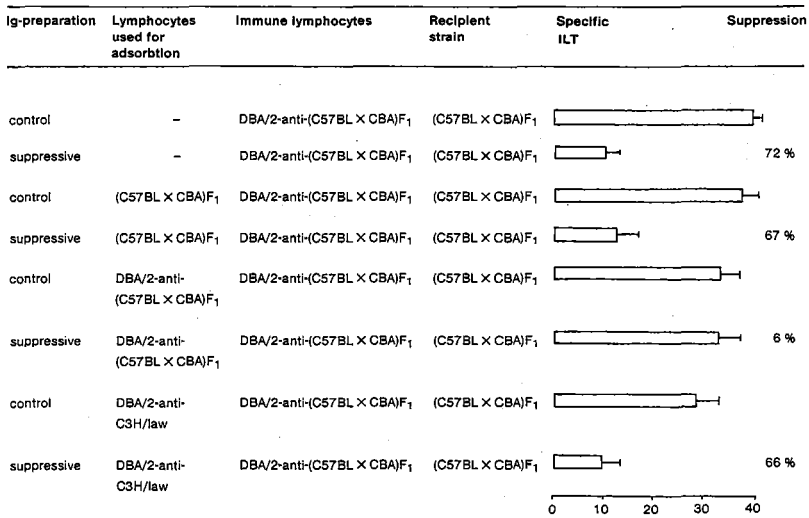

Fig. 7. Binding specificity of the suppressive Ig. DBA/2 mice were injected with LPS and (C57BL x CBA)F1 spleen cells or LPS only. Four days later their spleen cells were obtained. After three days of in vitro culture of these cells the culture supernatants were obtained and the kappa positive fractions were purified and concentrated as described in the Materials and Methods section. A volume of 300 ul (0.5 mg/ml) of control and suppressive Ig was adsorbed to a total number of 6 x 10^7 (C57BL x CBA)F1 lymph node cells, DBA/2-anti-(C57BL x CBA)F1 immune lymph node cells or DBA/2-anti-C3H/Law immune lymph node cells. After adsorbtion the residual Ig in the supernatant was tested for suppression of the ILT reactivity of DBA/2-anti-(C57BL x CBA)F1 immune lymphocytes. Each bar represents the mean specific ILT response $\pm$ 1 SEM (n=5). The degree of suppression was calculated as the percentage decrease of specific ILT response as compared to the relevant ILT control group.

from the spleen specific memory Ts cells can be found in the thoracic duct lymph, which indicates that these cells can recirculate. Similar memory-type cells might account for the long-lasting suppression of DTH in mice actively suppressed by treatment with LPS and allogeneic spleen cells.

The lipid A component of LPS, which accounts for the mitogenic signal of LPS to B cells, appeared to be required for the induction of suppression by LPS (Fig 2). This was also reported by Koenig and Hoffmann (17) for the LPS-induced suppression of antibody production in vitro, and by Colizzi (18) for the LPS-induced suppression of contact-hypersensitivity to oxazolone in mice.

Moreover, suppression of DTH could not be induced in mice of the LPS low responder strains CBA/N and B10.ScCr (Fig 3). These strains have been reported to possess a genetic defect, which renders them unresponsive to the mitogenic activity of LPS (12,13). This finding emphasizes the importance of the lipid A component for the induction of suppression by LPS.

The adoptive transfer experiments in which T cells and macrophages were eliminated from the cell suspensions to be transferred (Fig 4) clearly show that these cell types are not required for the transfer of suppression. The supposition that B cells were therefore the most likely candidates to account for the LPS-induced suppression was proven to be correct by our finding that depletion of B cells from the cell suspensions to be transferred completely abrogated the adoptive transfer of suppression, while transfer of B cell enriched cell suspensions did cause suppression of the subsequently induced DTH reactivity (Fig 5). This conclusion was further supported by our finding that intraperitoneal (i.p.) treatment of mice with Cyclophosphamide (200 mg/kg), which preferentially affects rapidly proliferating cells, completely prevented the induction of suppressor B cells as well as their suppressive activity (data not shown).

Suppressive activity of B cells on various cell-mediated immune responses has been described after administration of LPS (17-20) and after infection with whole microorganisms (21,22). For instance, Colizzi (18,19) reported that contact sensitivity to oxazolone can be inhibited by LPS-induced suppressor B cells. Vallera et al. (20) reported that LPS-treatment induces a population of B cells that suppress cell-mediated cytotoxicity in mice through the release of a, yet unidentified, soluble factor. Benedettini et al. (22) reported that S. aureus bacteria activate suppressor B cells which inhibit contact hypersensitivity to oxazolone. Campa et al. (23) reported that BCG-infection induces idiotypic B cells which interact with anti-idiotypic B cells which, in turn, activate antigen specific Ts cells. These Ts cells would eventually inhibit the efferent phase of DTH to BCG. Noteworthy in this respect are the studies of Hausmann et al. (24). They reported that a population of anti-idiotypic B cells is required for the activation of Ts effector cells which suppress the plaque-forming cell (PFC) response in the 4-hydroxy-3-nitrophenylacetyl (NP) system.

Altmann and Blyth (14) reported that suppression of the efferent phase of DTH to HSV-1 after LPS-treatment was mediated by nylon wool adherent, Thy-1$^+$ suppressor cells. We found, however, that the suppression of the efferent phase of DTH to H antigens, as measured in the ILT assay, was mediated by B cells (Fig 5, lower part), in an antigen-specific manner (data not shown). Furthermore, an anti-kappa binding fraction in the supernatant of cultured suppressed spleen cells accounted for suppression of the efferent phase of DTH (Fig 6). The specificity of the suppressive effect is in agreement with the notion that the suppression is mediated by antibodies. The adsorbtion studies (Fig 7) clearly demonstrate that the antibo-

dies bind to the alloantigen receptor on the DTH reactive T lymphocytes and can therefore be regarded as anti-idiotypic.

Colizzi et al. (25) reported that anti-idiotypic antibodies can mediate antigen-specific suppression of DTH to PPD. These antibodies were obtained from serum from BCG infected mice. Both in our studies and in those of Colizzi et al. the possibility cannot be ruled out that these antibodies activate Ts cells to inhibit the DTH effector T cells, just like suggested by Campa et al. (23). This possibility becomes even more likely in view of our previously reported finding that, in spite of the apparent antigen specificity of the LPS-induced suppression, also the DTH response to third party alloantigens could be suppressed (8). This phenomenon of bystander suppression has so far only been attributed to Ts cells (26,27). However, if so, the precursors of these Ts cells should occur in the lymph node cell suspension used as a source of DTH effector T cells. This possibility is currently under investigation.

In contrast, Gill and Liew (28) could not detect any suppressive activity in the supernatant of cultured spleen cells containing LPS-induced suppressor cells that suppressed DTH to sheep red blood cells. This might be due to the fact that they investigated the activity of unpurified culture supernatant only, and not the activity of a purified, highly concentrated preparation. Furthermore, they injected the culture supernatant i.v. or s.c. during antigen priming, but did not use the ILT-assay. The advantage of the ILT-assay, in this respect, is that the simultaneous injection of immune lymphocytes and the suppressive preparation eliminates the need for the inhibitory molecules to diffuse over longer distances to reach the specifically sensitized immunocompetent cells. Thus, in the ILT-assay a much higher effective concentration of inhibitory molecules can be achieved. This might also explain why the LPS-induced state of suppression could not be transferred with serum from LPS-treated mice (8).

In conclusion, simultaneous treatment of mice with LPS and allogeneic spleen cells induces a state of B cell dependent suppression of alloantigen-specific DTH. The anti-idiotypic nature of the suppression suggests that the B cells are activated by the antigen-specific DTH reactive T cells. This B cell activation can be anticipated to occur in the absence of LPS injection as well. However, without the simultaneous stimulation by the mitogenic moiety of LPS the activation of anti-idiotypic B cells does not lead to a state of suppression. Apparently, the mitogenic signal of LPS accounts for further clonal expansion of the activated anti-idiotypic B cells, to such a level that they effectively inhibit the DTH-reactive T cells.

ACKNOWLEDGMENTS

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CHAPTER IV.3.

LIPOPOLYSACCHARIDE-INDUCED SUPPRESSION OF GRAFT-VERSUS-HOST REACTIVITY IN MICE

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SUMMARY

Reconstitution of lethally irradiated mice with spleen cells from donors that had been treated with lipopolysaccharide (LPS) intravenously and allogeneic spleen cells subcutaneously leads to a suppressed anti-host delayed-type hypersensitivity (DTH). Either donor injection alone proved to be ineffective. The state of suppression appeared to be antigen-specific, but, depending on the experimental conditions, also anti-host DTH to third party alloantigens could be suppressed. The suppression was mediated by a population of Thy-1$^-$ suppressor cells that could also be induced in athymic nude mice. The suppressor cells specifically adhered to anti-kappa-coated plastic plates, but were not adsorbed by passage through a Sephadex G-10 column. Thus, it appears that the combined donor treatment with LPS and allogeneic spleen cells induces a population of B cells that can suppress anti-host immune reactivity.

INTRODUCTION

Delayed-type hypersensitivity (DTH) to histocompatibility (H) antigens is a T cell dependent phenomenon, which can develop under host-versus-graft (HvG) conditions as well as under graft-versus-host (GvH) conditions. HvG-related DTH can be induced by skin transplantation (1), and by subcutaneous (s.c.) or intravenous (i.v.) immunization (2,3) of mice with an immunogenic dose of allogeneic spleen cells. DTH responses can be elicited some days later by s.c. injection of a challenge dose of the relevant allogeneic spleen cells into a hind foot. The resultant hind foot swelling due to DTH peaks 24 to 48 hrs later and can be used as a measure for the strength of the DTH response (1-3).

GvH reactivity can be induced a.o. by reconstitution of lethally irradiated hosts with allogeneic spleen cells or bone marrow cells. The assay to measure GvH-related DTH (anti-host DTH) in mice was originally described by Wolters and Benner (4). It consists of an i.v. transfer of spleen and/or lymph node cells from the irradiated, reconstituted hosts to naive secondary recipients that are syngeneic to the original spleen or bone marrow donor, followed by a s.c. hind foot challenge of these secondary recipients with spleen cells from mice that are syngeneic to the irradiated hosts. The anti-host DTH response appears several hours later as a measurable swelling of the injected hind foot and peaks 24 to 48 hrs after challenge. In contrast to the induction of HvG-related DTH, the induction of GvH-related DTH requires that donor and irradiated recipient are at least incompatible for the H-2I-A locus, and/or a stimulating Mls locus (5,6). However, after prolonged stimulation of the GvH-reactive T cells it was found that a single H-2K disparity can also account for the development of an anti-host DTH reaction after allogeneic spleen cell transplantation (7).

Previous studies have shown that the T cells accounting for HvG-related DTH and GvH-related DTH are highly susceptible for the activity of antigen-specific suppressor T (Ts) cells (3,8-11).

Recently we reported that the combined treatment of mice with lipopolysaccharides (LPS) i.v. and allogeneic spleen cells s.c. induces an antigen-specific state of suppression of HvG-related DTH, mediated by B cells through the release of anti-idiotypic antibodies (12,13). In view of the similarities in T cells mediating HvG-related DTH and GvH-related DTH we investigated the effect of combined treatment of donor mice with LPS and allogeneic spleen cells on the development of anti-host DTH.

MATERIALS AND METHODS

Mice

(C57BL/Rij x CBA/Rij)F1 ($H\text{-}2^{b/q}$) female mice, 4 weeks of age, were purchased from the Radiobiological Institute TNO, Rijswijk, The Netherlands. DBA/2 ($H\text{-}2^{d}$), C57BL/6 ($H\text{-}2^{b}$), B10.BR ($H\text{-}2^{k}$), A.TH ($H\text{-}2^{t2}$) and C57BL/10 nude female mice, 4 to 6 weeks of age, were purchased from HARLAN OLAC Ltd., Bicester, Oxon, U.K. B6.C-$H\text{-}2^{bml2}$ ($H\text{-}2^{bml2}$), B6.C-$H\text{-}2^{bml}$ ($H\text{-}2^{bml}$), (B6.C-$H\text{-}2^{bml2}$ x B6.C-$H\text{-}2^{bml}$)F1 ($H\text{-}2^{bml2/bml}$), B10.A ($H\text{-}2^{a}$), B10.AQR ($H\text{-}2^{y1}$), B10.T(6R) ($H\text{-}2^{y2}$) and (B10.A x B10.T(6R))F1 ($H\text{-}2^{a/y2}$) female mice were bred and raised at our department. All mice were kept in well-ventilated, light cycled rooms and had access to pelleted food (Hope Farms, Woerden, The Netherlands) and acidified water ad libitum. The animals' age at the start of each experiment ranged from 10 to 16 weeks.

Preparation of cell suspensions

Mice were killed by carbon dioxide exposure and their spleens and/or inguinal, axillary and mesenteric lymph nodes were removed and placed in a balanced salt solution (BSS). After gentle mincing with scissors the organ particles were squeezed through a nylon gauze filter to get a single cell suspension. Cell numbers were counted with a Coulter counter model BZI (Coulter Electronics Ltd., Harpenden, Herts, U.K.). Cell viability always exceeded 95%, as determined with the trypan blue exclusion method.

Irradiation

Recipient mice received 9.0 Gy of whole body X-irradiation. Irradiation was performed in a Philips Müller MG 300 X-ray machine, whose characteristics have been described elsewhere (4). Radiation control mice died within 20 days of irradiation.

Induction of GvH-reactivity

GvH reactions were induced in lethally irradiated mice by i.v. injection of 1×10^7 nucleated allogeneic spleen cells within four hours after irradiation.

Induction of suppression

Donor mice were i.v. injected with 100 ug LPS (Salmonella typhosa 0901; Difco Laboratories Ltd., Detroit, MI, U.S.A.) dissolved in 0.5 ml BSS. Within two hours after LPS injection the mice were s.c. injected in the inguinal areas with 1×10^7 allogeneic spleen cells suspended in 100 ul BSS, equally distributed over both areas.

Cell separation techniques

The methods for selective depletion of various spleen cell subsets have extensively been described (13-15). Briefly, T cells were lysed by treatment with anti-Thy-1.2 monoclonal antibodies and complement (13). B cells were depleted by panning on plastic tissue culture dishes coated with rat-anti-mouse kappa monoclonal antibodies (13,14). Macrophages and monocytes were depleted by passage of spleen cells through Sephadex-G10 columns (13,15). The effectiveness of these procedures were evaluated by FACS-analysis (FACS II, Becton Dickinson, Sunnyvale, CA, U.S.A.) employing the relevant monoclonal antibodies (anti-Thy-1.2 for T cells, anti kappa for B cells and F4/80 for monocytes/macrophages). Each recipient received 1×10^7 suppressed spleen cells, which had been depleted of the relevant leucocyte subset, only or together with 1×10^7 spleen cells from syngeneic donors.

Assay for DTH

Five days after induction of GvH reactivity the irradiated recipients' spleen and mesenteric and peripheral lymph nodes were removed, pooled and prepared to single cell suspensions. These cells were i.v. transferred to naive secondary recipients syngeneic to the donors of the spleen cells that had induced the GvH reaction. To prevent embolism the secondary recipients were intraperitoneally (i.p.) injected with 15 U heparin (Liquémine, Hoffmann-La Roche and Co., Basel, Switzerland) 30 min before transfer. The secondary recipients were challenged by s.c. injection of 2×10^7 spleen cells, syngeneic to the irradiated recipients, into the dorsum of the right hind foot. DTH responses were measured 24 hrs later with a foot thickness meter with 0.05 mm accuracy. The specific increase in foot thickness due to DTH was calculated as the relative increase in foot thickness of the secon-

dary recipients minus the relative increase in foot thickness of control mice which only received the heparin and the challenge injection. The increase of foot thickness of the control mice ranged from 15 to 25%.

RESULTS

Treatment of donors with LPS and allogeneic spleen cells induces suppression of anti-host DTH

To investigate whether the LPS treatment for induction of suppression of DTH under HvG conditions can also induce suppression of anti-host DTH during GvH, DBA/2 mice were i.v. injected with LPS and s.c. injected with (C57BL x CBA)F1 spleen cells. Control mice received either LPS OR BSS i.v. and either syngeneic or allogeneic spleen cells s.c. Four days later their spleen cells were obtained and used to reconstitute lethally irradiated (C57BL x CBA)F1 recipients. Another five days later the spleens and mesenteric and peripheral lymph nodes of these irradiated recipients were obtained and i.v. transferred to naive DBA/2 recipients. Within 2 hrs after transfer these secondary recipients were challenged with (C57BL x CBA)F1 spleen cells to assay for anti-host DTH. As shown in Fig. 1, line 4, anti-host DTH hardly developed in the irradiated hosts reconstituted with spleen cells from donor mice that had been treated with LPS as well as allogeneic

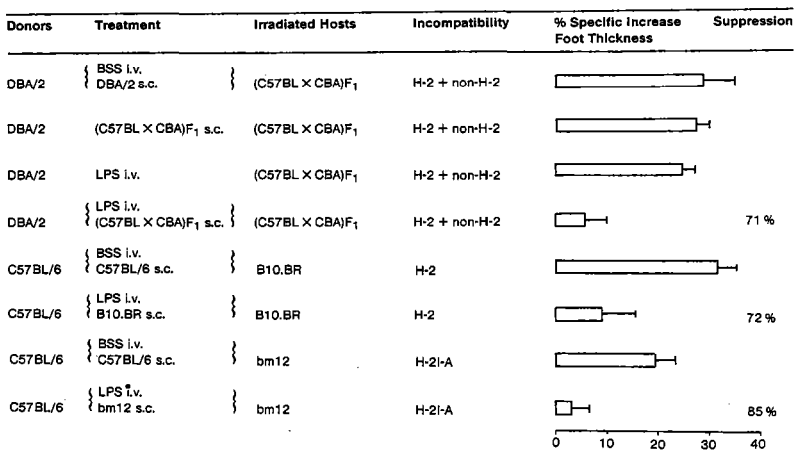

Fig. 1. LPS treatment of spleen cell donors induces suppression of antihost DTH. Groups of DBA/2 and C57BL/6 donor mice were i.v. injected with LPS and s.c. injected with either (C57BL x CBA)F1, B10.BR or bm12 spleen cells. Control mice received BSS and syngeneic spleen cells. One group of DBA/2 mice received LPS only, whereas another group of DBA/2 mice received (C57BL x CBA)F1 spleen cells only. Four days later their spleen cells were obtained and used to reconstitute lethally irradiated (C57BL x CBA)F1, B10.BR or bm12 recipients, respectively. Another five days later the anti-host DTH was evaluated. Each bar represents the mean anti-host DTH $\pm$ 1 SEM (n=5). The percentage suppression was calculated as the degree of decrease of DTH as compared to the relevant DTH control group.

spleen cells. Either injection alone proved ineffective to induce suppression (Fig. 1, lines 1 and 2). Suppression was also found with the H-2 incompatible but non-H-2 compatible donor-host combination C57BL/6 - B10.BR (Fig. 1, line 6), and the H-2I-A incompatible donor-host combination C57BL/6 - B6.C-H-2^{bm12} (Fig. 1, line 8).

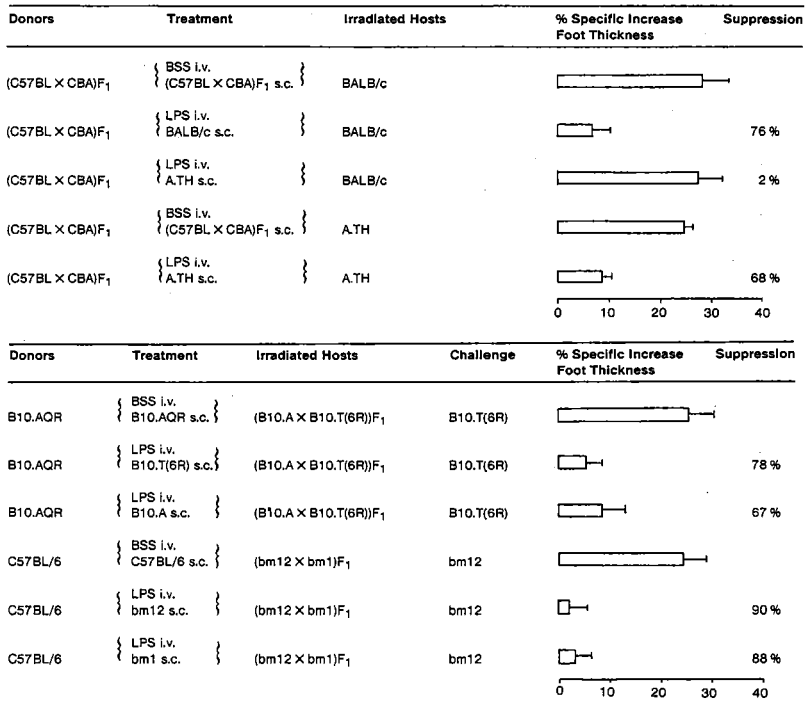

Fig. 2. Specificity of the LPS-induced suppression of anti-host DTH. Upper part: Groups of (C57BL x CBA)F1 donor mice were i.v. injected with LPS and s.c. injected with either BALB/c or A.TH spleen cells. Control mice received BSS and syngeneic spleen cells. Four days later their spleen cells were used to reconstitute lethally irradiated BALB/c and A.TH recipients. Another five days later the anti-host DTH was evaluated. Each bar represents the mean anti-host DTH $\pm$ 1 SEM (n=5). The percentage suppression was calculated as the degree of decrease of DTH as compared to the relevant DTH control group. Lower part: Groups of B10.AQR and C57BL/6 donor mice were i.v. injected with LPS and s.c. injected with B10.T(6R), B10.A, bm1 or bm12 spleen cells. Control mice received BSS and syngeneic spleen cells. Four days later their spleen cells were obtained and used to reconstitute lethally irradiated (B10.A x B10.T(6R)F1 and (bm12 x bm1)F1 recipients. Another five days later the anti-host DTH was evaluated. Each bar represents the mean anti-host DTH $\pm$ 1 SEM (n=5). The percentage suppression was calculated as the degree of decrease of DTH as compared to the relevant DTH control group.

Specificity of suppression of anti-host DTH induced by donor treatment with LPS and allogeneic spleen cells

For investigation of the specificity of the LPS-induced suppression of anti-host DTH, (C57BL x CBA)F1 donor mice were i.v. injected with LPS and s.c. injected with either BALB/c or A.TH spleen cells. Four days later their spleen cells were used to reconstitute lethally irradiated BALB/c recipients. Another five days later the anti-host DTH in the recipients' spleens and lymph nodes was assayed. As shown in Fig. 2, upper part, line 2, the combined treatment of the donors with LPS and BALB/c spleen cells suppressed the anti-host DTH in the recipient BALB/c mice. However, combined treatment of the donors with LPS and A.TH spleen cells could not suppress the anti-host DTH in the recipient BALB/c mice (Fig. 2, upper part, line 3), although the combined treatment of donor mice with LPS and A.TH spleen cells did suppress the development of anti-host DTH in A.TH mice (Fig. 2, line 5). Thus, we conclude that the LPS-induced suppression of anti-host DTH is specific for the alloantigens that were injected together with the LPS.

To investigate whether donor-treatment with LPS and allogeneic spleen cells can also suppress anti-host DTH to third party alloantigens, B10.AQR donor mice were i.v. injected with LPS and B10.A spleen cells. Four days later their spleen cells were used to reconstitute irradiated (B10.A x B10.T(6R))F1 recipients. Five days later their spleen and lymph node cells were i.v. transferred to naive B10.AQR recipients, which were subsequently challenged with B10.T(6R) spleen cells to assay for anti-host DTH. As shown in Fig. 2, lower part, line 3, anti-host DTH to the third party B10.T(6R) alloantigens expressed in the (B10.A x B10.T(6R))F1 recipients, was suppressed after treatment of the B10.AQR donor mice with LPS and B10.A spleen cells. Similar results were obtained when irradiated (bm12 x bm1)F1 recipients were reconstituted with spleen cells from C57BL/6 donor mice treated with LPS and bm1 spleen cells (Fig. 2, lower part, line 6). Thus, the combined treatment with LPS and allogeneic spleen cells can also suppress anti-host DTH to third party alloantigens, provided the latter are inherited by the irradiated recipients together with the alloantigens that, together with LPS, had induced the suppression.

LPS-induced suppression of anti-host DTH is not mediated by T cells

Two approaches were used to investigate the possibility that Ts cells are involved in the observed suppression. Firstly, spleen cells from T cell deficient B10 nude mice that had been treated with LPS and B10.BR spleen cells were injected into irradiated B10.BR recipients together with spleen cells from naive C57BL/6 donors. As shown in Fig. 3, upper part, line 4, spleen cells from nude mice treated with LPS and allogeneic spleen cells suppressed anti-host DTH by simultaneously injected naive spleen cells. Secondly, spleen cells from DBA/2 mice that had been injected with LPS i.v. and (C57BL x CBA)F1 spleen cells s.c. four days earlier were treated with anti-Thy-1.2 monoclonal antibodies and complement to eliminate T cells. The residual cells were i.v. injected into irradiated (C57BL x CBA)F1 recipients together with spleen cells from naive DBA/2 donors. As shown in Fig. 3, lower part, line 3, elimination of T cells could not abrogate the suppressive activity of LPS-induced suppressor cells. Thus, it appears that the suppression is a cell mediated, dominant phenomenon, which is T cell independent.

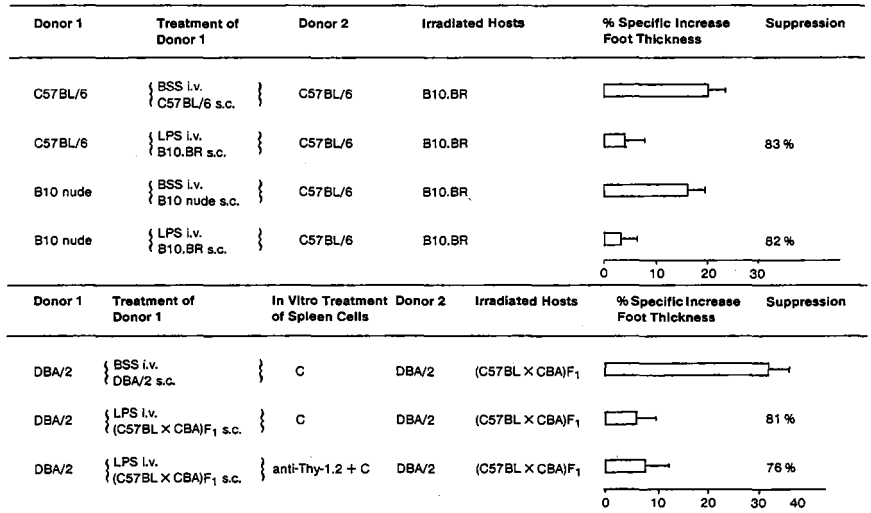

Fig. 3. LPS-induced suppression of anti-host DTH is not mediated by Ts cells. Upper part: Groups of C57BL/6 and B10 nude donor mice were i.v. injected with LPS and s.c. injected with B10.BR spleen cells. Control mice received BSS and syngeneic spleen cells. Four days later their spleen cells were obtained and mixed with an equal number of spleen cells from naive C57BL/6 donors. This inoculum was used to reconstitute lethally irradiated B10.BR recipients. Five days later the anti-host DTH was evaluated. Each bar represents the mean anti-host DTH ± 1 SEM (n=5). Lower part: Groups of DBA/2 donor mice were i.v. injected with LPS and s.c. injected with (C57BL x CBA)F1 spleen cells. Control mice received BSS and syngeneic spleen cells. Four days later their spleen cells were obtained and treated with anti-Thy-1.2 monoclonal antibodies and complement or complement only. The residual cells were mixed with an equal number of spleen cells from naive DBA/2 donors and this inoculum was used to reconstitute lethally irradiated (C57BL x CBA)F1 recipients. Five days later the anti-host DTH was evaluated. Each bar represents the mean anti-host DTH ± 1 SEM (n=5). The percentage suppression was calculated as the degree of decrease of DTH as compared to the DTH control group.

LPS-induced suppression of anti-host DTH is mediated by B cells

Next we investigated whether the LPS-induced suppression of anti-host DTH is mediated by B cells. Spleen cells from (C57BL x CBA)F1 mice treated with LPS and DBA/2 spleen cells were separated into kappa-negative and kappa-positive fractions by panning on plastic dishes coated with anti-mouse-kappa monoclonal antibodies. Subsequently each fraction was injected into irradiated DBA/2 mice, together with naive (C57BL x CBA)F1 spleen cells. As shown in Fig. 4, upper part, line 4, suppression was mediated by kappa-positive spleen cells. Moreover, anti-host DTH developed normally in the DBA/2 recipients reconstituted only with spleen cells depleted of kappa-positive cells (Fig. 4, line 6).

To dissociate between the activity of kappa-positive B cells and that of macrophages that carry Fc receptor-bound Ig molecules, spleen cells from (C57BL x CBA)F1 mice treated with LPS and DBA/2 spleen cells were filtered

through Sephadex-G10 columns to eliminate macrophages and monocytes. As shown in Fig. 4, lower part, line 4, the LPS-induced suppressor cells did not adhere to Sephadex-G10. Thus, we conclude that the LPS-induced suppressor cells are B cells.

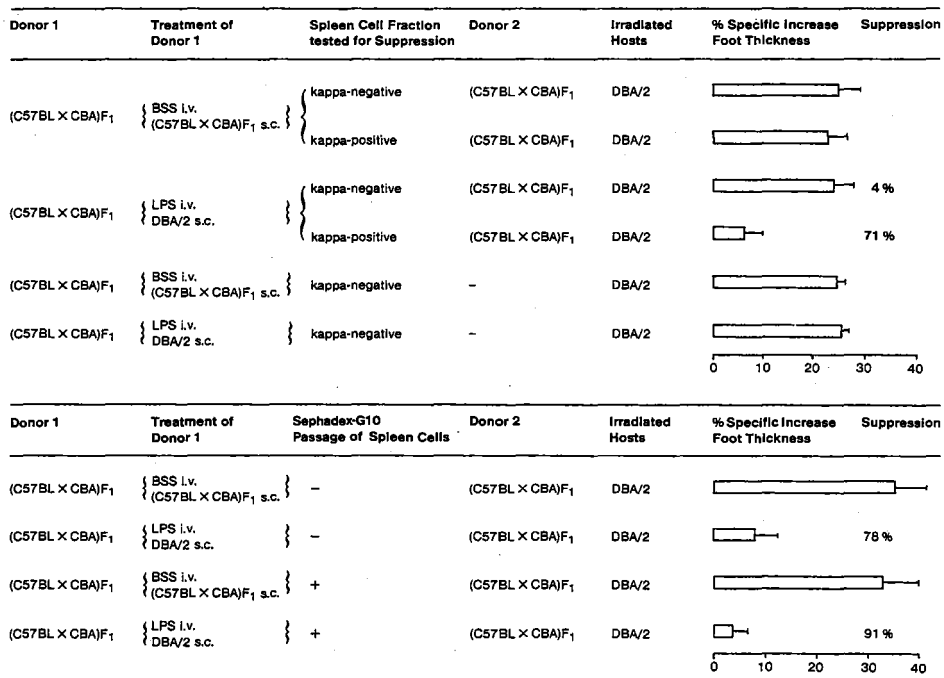

Figure 4. LPS-induced suppression of anti-host DTH is mediated by B cells. Upper part: Groups of (C57BL x CBA)F1 donor mice were i.v. injected with LPS and s.c. injected with DBA/2 spleen cells. Control mice received BSS and syngeneic spleen cells. Four days later their spleen cells were obtained and separated into kappa-negative and kappa-positive fractions. These fractions were mixed with an equal number of spleen cells from naive syngeneic mice and this inoculum was used to reconstitute lethally irradiated recipients. One group of recipients received cells of the kappa negative fraction only. Five days later the anti-host DTH was evaluated. Each bar represents the mean anti-host DTH ± 1 SEM (n=5). The percentage suppression was calculated as the degree of decrease of DTH as compared to the relevant DTH control group. Lower part: In a similar donor-recipient combination suppressed donor spleen cells were obtained and the splenic leukocytes were filtered through a Sephadex-G10 column. The adherent and nonadherent cells were mixed with an equal number of spleen cells from naive (C57BL x CBA)F1 donors. This inoculum was used to reconstitute lethally irradiated DBA/2 recipients. Five days later the anti-host DTH was evaluated. Each bar represents the mean anti-host DTH ± 1 SEM (n=5). The percentage suppression was calculated as the degree of decrease of DTH as compared to the relevant DTH control group.

DISCUSSION

To our knowledge this paper is the first to report about antigen-specific, LPS-induced suppression of GvH-reactivity. The data shown in Fig. 1 clearly indicate that the development of DTH reactivity under GvH conditions can be suppressed by the combined treatment of donor mice with LPS and allogeneic spleen cells.

Injection of the donors with allogeneic spleen cells only or LPS only proved to be ineffective to induce suppression (Fig. 1). The latter finding is in contrast with the findings of Rose et al. (16). They reported that repeated intraperitoneal (i.p.) administration of LPS to donor mice and subsequent reconstitution of lethally irradiated allogeneic recipients with donor spleen cells considerably delayed mortality from GvH disease. They attributed the reduced GvH-reactivity to the relative reduction of spleen lymphocyte number by the LPS-induced proliferation of erythroid and myeloid splenic elements rather than to the activation of some suppressor mechanism. In our studies on the LPS-induced suppression of DTH under HvG-conditions we found that i.p. injection as well as i.v. injection of LPS, together with a s.c. injection of allogeneic spleen cells, induces a state of suppression (Molendijk et al., unpublished).

As shown in Fig. 2, upper part, the suppression of anti-host DTH by the combined treatment of donor mice with LPS and allogeneic spleen cells is antigen-specific. Bril et al. (11) reported that i.v. tolerization of donor mice with a high dose of irradiated allogeneic spleen cells induces a state of suppression of anti-host DTH which also appeared to be antigen-specific. Suppression of HvG-related DTH by i.v. treatment of the responder mice with LPS in combination with s.c. immunization with allogeneic spleen cells was also found to be antigen-specific (11).

In spite of the observed antigen-specificity of the suppression cells, the anti-host DTH response to third-party alloantigens could be suppressed by the combined treatment of donor mice with LPS and allogeneic spleen cells (Fig. 2, lower part). Such suppression of anti-host DTH to third party alloantigens was also reported by Bril et al. (11), for suppression exerted by i.v. tolerized donor spleen cells. Apparently in both systems a non-specific suppressive mechanism is activated. In both situations, however, suppression of anti-host DTH to third party alloantigens was only observed when the irradiated recipients had inherited both the third party alloantigens and the alloantigens used to induce suppression in the donor mice.

Wolters et al. (3) reported that the induction of anti-host DTH required at least an H-2I-A or Mls incompatibility between donor and irradiated host. Obviously such an incompatibility is not required for the induction of suppression by LPS and allogeneic spleen cells (Fig. 2, lower part).

Two mechanisms may account for the LPS-induced suppression. Firstly, a clonal deletion mechanism might eliminate the specific DTH-reactive T cells. Secondly, the suppression might be due to the activity of antigen-specific suppressor cells. In view of our finding that anti-host DTH normally developed after elimination of B cells from the spleens of donor mice subjected to the suppressive protocol, the former mechanism appears very unlikely (Fig. 4, upper part). Furthermore, the second possibility is supported by the observation that B cells from suppressed donors were able to suppress the anti-host DTH response by spleen cells from naive mice as well (Figs 3 and 4).

The LPS-induced suppression of anti-host DTH was found to be mediated by B cells, just like the LPS-induced suppression of DTH under HvG condi-

tions (13). A purified immunoglobulin preparation from the supernatant of cultured spleen cells from suppressed mice appeared to suppress the immune-lymphocyte-transfer (ILT) reactivity antigen-specifically. Adsorption studies revealed that the activity of the suppressive Ig was directed to the alloantigen receptor on the immune lymphocytes. Thus, an anti-idiotypic antibody is involved in the suppression of HvG-related DTH to alloantigens. Campa et al. (17) reported that BCG injection of mice induced idiotypic B cells which activated anti-idiotypic B cells which, in turn activated a population of idiotypic Ts cells. These Ts cells suppressed the efferent phase of DTH to BCG. In view of the observed suppression of anti-host DTH to third party alloantigens, which has so far only been attributed to Ts cells, the possibility cannot be excluded that a similar mechanism is operating in our model.

Thompson and Jutila (18) reported that a spleen cell population of plastic adherent cells induced by repeated LPS injection, markedly reduced mortality due to GvH when these cells were injected together with naive spleen cells into allogeneic neonates. A relatively large number of lymphocytes were found to adhere to the plastic adherent cells (19). In view of their observation that a small volume of serum from LPS injected mice also inhibited GvH-reactivity, it might well be that the suppression in their system was in fact mediated by B cells.

In conclusion, donor immunization with LPS and allogeneic spleen cells activates a population of B cells which suppresses the development of DTH under GvH conditions. Future investigations are aimed at revealing the mechanism of action of these B cells.

ACKNOWLEDGMENTS

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CHAPTER V

INDUCTION OF SUPPRESSION BY ULTRAVIOLET IRRADIATION

CHAPTER V.1.

SUPPRESSION OF DELAYED-TYPE HYPERSENSITIVITY TO HISTOCOMPATIBILITY ANTIGENS BY ULTRAVIOLET IRRADIATION

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Immunology 62: 299, 1987.

SUMMARY

Delayed-type hypersensitivity (DTH) reactions to allogeneic histocompatibility antigens in mice could be systemically suppressed by a single exposure to UV-B irradiation. The extent of suppression reached its maximum four days after irradiation, gradually waned thereafter and disappeared at day 21. Reexposure of these mice to UV-B after waning reinduced the state of suppression. The suppression could be transferred to naive mice by means of splenic T lymphocytes. The suppressor T (Ts) cells suppressed the proliferative activity in the lymph nodes draining the site of immunization, but not the activity of already activated DTH reactive T cells. Phenotypical analysis of these Ts cells revealed that two subpopulations of T cells are involved: one with the Lyt-$1^{+}2^{-}$ phenotype, the other with the Lyt-$1^{-}2^{+}$ phenotype.

INTRODUCTION

Subcutaneous (s.c.) injection of mice with an immunizing dose of murine allogeneic spleen cells induces a state of delayed-type hypersensitivity (DTH) directed against histocompatibility (H) antigens that are expressed on the injected spleen cells (Van der Kwast and Benner, 1978). Some days after immunization a DTH response can be elicited in such mice by a s.c. injection of the relevant allogeneic spleen cells into a hind foot. The DTH response is characterized by a swelling of the injected hind foot which peaks 24 hours after the eliciting injection. This in vivo assay for cell-mediated immunity against allogeneic H antigens in mice closely resembles the footpad swelling assay originally described by Gray and Jennings (1955) and has extensively been used in our laboratory to monitor anti-graft and Graft-versus-Host (GvH) immunity (Bianchi et al., 1981; Van der Kwast et al., 1981; Bril et al., 1984). The DTH reactive T cells probably predominantly represent helper T (Th) cells since Bianchi et al. (1981) reported that cloned helper T cells effectively mediated DTH in mice. Depending on the experimental conditions DTH reactivity to H antigens has been shown to be regulated by antigen-specific suppressor T (Ts) cells (Bianchi et al., 1984) or antigen-specific B cells (Molendijk et al., submitted). Other forms of cell-mediated immunity have also been shown to be sensitive to the regulatory activity of suppressor cells. For instance, GvH reactivity (Bril et al., 1985), contact-hypersensitivity (Zembala and Asherson, 1974), and mixed-lymphocyte reactions (MLR) (Rich and Rich, 1974) have been shown to be inhibitable by Ts cells.

The induction of suppressor cells usually involves the administration or presentation of antigens in such a way that suppressive mechanisms are selectively activated. However, suppressive mechanisms can also be activated using methods that do not imply the administration of antigens. Exposure of mice to UV-B radiation (Fisher and Kripke, 1977, 1978) has been reported to induce Ts cells that suppress the immune response against simultaneously induced skin tumours. Also contact-hypersensitivity responses have been shown to be suppressed in mice after application of a contact sensitizer several days after exposure to UV-B radiation (Noonan, DeFabo and Kripke, 1981a). The UV-induced Ts cells were identified as antigen-specific Lyt-1^+2^- T cells (Ullrich and Kripke, 1984) that exerted their effect on the proliferation phase of the immune response (Ullrich, 1985). Recently Ullrich (1986) reported that spleen cells from UV-B exposed mice immunized with alloantigens inhibited MLR reactivity to similar alloantigens antigen-specifically. Furthermore, DTH to these alloantigens was suppressed in the UV-B exposed mice.

The aim of the present study was to investigate in more detail the effects of UV-B exposure on the induction and development of DTH against H antigens, and to identify the putative suppressor cells and their mode of action.

MATERIALS AND METHODS

Mice

(C57BL/Rij x CBA/Rij)F_1 ($H\text{-}2^{b/q}$) female mice, 4 weeks of age, were purchased from the Radiobiological Institute TNO, Rijswijk, The Nether-

lands. BALB/c (H-2^d), DBA/2 (H-2^d), B10.D2 (H-2^d) and C57BL/6 (H-2^b) female mice, 4 weeks of age, were purchased from HARLAN OLAC Ltd., Bicester, U.K. and from Bomholtgard, Ry, Denmark. All mice were kept in light-cycled rooms and had access to food and acidified water at libitum. The age of the animals during the experiments ranged from 10 to 16 weeks.

Preparation of cell suspensions

Donor mice were rapidly killed by carbon-dioxide exposure and their spleen and/or lymph nodes were removed and placed in a balanced salt solution (BSS). The organs were minced with scissors and squeezed through a nylon gauze filter to get single-cell suspensions. Cells were counted with a Coulter Counter model BZI (Coulter Electronics, Harpenden, U.K.). This procedure resulted in cell suspensions with at least 95% viability as tested by the trypan blue exclusion test.

Induction of suppression by UV-irradiation

A modification of the method described by DeFabo and Kripke (1979) was used. Briefly, the dorsal fur of responder mice was shaved using electrical hair clippers. The mice were then exposed to a single dose of 10^4 J/m^2 UV-B (280-320 nm) from a bank of standard Philips TL 12 UV lamps. Control mice were treated similarly but were not exposed to UV-B irradiation.

Cell transfer

For adoptive transfer single cell suspensions were prepared as described and transferred to the recipient mice through a tail vein. The cells to be transferred were suspended in a volume of 1.0 ml BSS.

Induction of DTH to alloantigens

DTH to allogeneic H antigens was induced by s.c. immunization of responder mice with 1 x 10^7 allogeneic spleen cells suspended in a volume of 100 ul. Fifty ul of this suspension was injected into each inguinal area.

Estimation of cell proliferation in the draining lymph nodes

Incorporation of tritiated thymidine (3H-TdR) was used to measure DNA synthesis in the draining lymph nodes, according to the method described by Moorhead (1976). Briefly, quintuple cultures of 1 x 10^6 cells/ml in tissue culture medium (RPMI 1640 supplemented with 5 to 10% fetal calf serum) from each group were pulsed for 5 hr in vitro with 1 uCi of 3H-TdR (spec. act. 5Ci/mM). At the end of the culture period the cells were harvested automatically using a cell harvester (Cryoson, Midden Beemster, The Netherlands) and radioactivity was counted in a liquid scintillation counter (Packard model 3375). Radioactivity was corrected for background and quenching and expressed as cpm.

Selective elimination of Thy-1.2, Lyt-1.2 and Lyt-2.2 positive cells

Monoclonal IgM anti-Thy-1.2 antibodies (clone F7D5) were purchased from HARLAN OLAC Ltd., Bicester, U.K. Monoclonal IgG2a anti-Lyt-1.2 (clone CG16) and monoclonal IgG2a anti-Lyt-2.2 (clone AD4(15)) were purchased from Cedarlane Laboratories Ltd., Hornby, Ontario, Canada. Cell suspensions were treated for 30 min at 4°C with either anti-Thy-1.2, anti-Lyt-1.2, or anti-Lyt-2.2 antibodies. After incubation the cells were centrifuged, resuspended in BSS and incubated with guinea pig complement (Behringwerke, Marburg-Lahn, F.R.G.) for 15 min at 37°C. The cells were then washed three times, resuspended in BSS and adoptively transferred. The applied proce-

dure eliminated at least 90% of the viable lymphocytes that were positive for the marker detected by the monoclonal antibody used.

Assay for delayed-type hypersensitivity

The DTH assay for measuring immune reactivity to allogeneic H antigens has been described in detail elsewhere (Van der Kwast and Benner, 1978). DTH responses were elicited by a s.c. 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 s.c. 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 immunized mice minus the relative increase in foot thickness of control mice which received the challenge only. The hind foot swelling of these control mice varied between 16 and 25%.

Assay for immune-lymphocyte-transfer reactivity

The immune-lymphocyte-transfer (ILT) reactivity by lymph node cells from immunized donors, directed against particular H antigens, was determined by s.c. injection of 5 x 10^6 of these lymph node cells into the dorsum of the right hind foot of recipients expressing the relevant H antigens. A control group of mice, syngeneic to the recipients of immune lymph node cells, was included. These mice received a s.c. injection of 5 x 10^6 lymph node cells from non-immune mice syngeneic with the immunized donor mice, a so-called normal lymphocyte transfer (NLT). Twenty-four hours later the thickness of the hind feet was measured as in the DTH assay. Specific ILT reactivity was calculated as (ILT - NLT) and was expressed in 10^{-2} mm.

RESULTS

Kinetics of UV-B induced suppression of DTH

To investigate whether UV-B irradiation could inhibit DTH reactivity against H antigens (C57BL x CBA)F_1 responder mice were exposed to 10^4 J/m^2 of UV-B irradiation. Four days later these mice and a group of unexposed control mice were s.c. immunized with H-2 and non-H-2 incompatible BALB/c spleen cells and another five days later DTH responses were elicited by a s.c. injection of a challenge dose of BALB/c spleen cells in the right hind feet of these mice. As shown in Fig. 1, line 2 the group of mice that had been exposed to the UV-B irradiation showed a poor DTH response, whereas the group of unexposed control mice showed a good DTH response (Fig. 1, line 1). Moreover, UV-B exposed BALB/c mice immunized and challenged with H-2 compatible but non-H-2 incompatible DBA/2 spleen cells and UV-B exposed C57BL/6 mice immunized and challenged with H-2 incompatible but non-H-2 compatible B10.D2 spleen cells showed a poor DTH response (Fig. 1, lines 4 and 6).

Subsequently we investigated the optimal interval between the UV-B exposure and the induction of DTH for suppression to become obvious, and the longevity of the state of suppression. Thus DTH against BALB/c H antigens was induced in (C57BL x CBA)F_1 mice at varying intervals after exposure to UV-B. As can be seen in Fig. 2, suppression gradually increased from day 2 after UV-B exposure and reached its maximum at day 4. Thereafter the suppression gradually waned and completely disappeared by day 21 after

UV-B irradiation. However, the state of suppression could be reinduced by reexposure of these mice to a similar dose of UV-B irradiation on day 21 (Fig. 2).

Characterization of the cells accounting for the UV-B induced suppression

The underlying cause of the UV-B induced suppression was investigated in transfer experiments. Therefore, BALB/c donor mice were exposed to UV-B irradiation. Four days later the mice were sacrificed, their spleens were removed and a single cell suspension was prepared. A part of the spleen cells was directly tranferred to syngeneic recipient mice who received one spleen equivalent each. Another part of the spleen cells was treated with monoclonal anti-Thy-1.2 antibodies and complement to eliminate T lymphocytes. After treatment one spleen equivalent of the T cell depleted suspension was infused per syngeneic recipient. As a control, a third part of the spleen cells was exposed to complement only and otherwise treated similarly. Within four hours after transfer all groups were s.c. immunized with (C57BL x CBA)F_1 spleen cells. Five days later they were challenged to assay for DTH reactivity. The results show that the state of suppression can be adoptively transferred with spleen cells (Fig. 3A, line 2). The transfer of suppression was completely abrogated, however, by the selective elimination of the T cells by anti-Thy-1.2 antibodies and complement in vitro (Fig. 3A, line 4). Apparently, UV-B irradiation induces Ts cells that suppress DTH to H antigens.

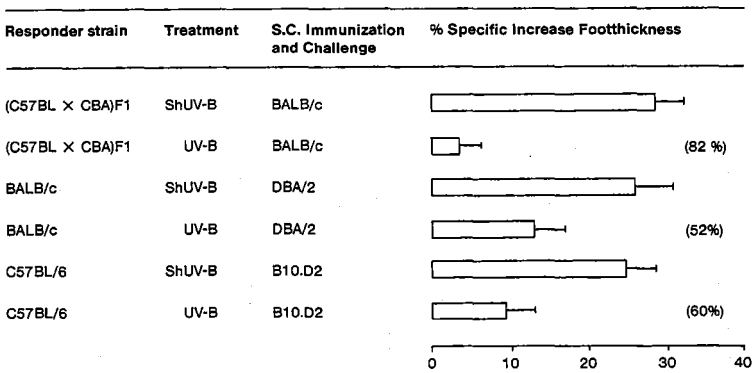

Fig. 1. Effect of UV-B exposure on DTH to alloantigens. Groups of (C57BL x CBA)F_1 mice, BALB/c mice and C57BL/6 mice received a dose of 10^4 J/m^2 of UV-B irradiation on their shaven backs. Control mice were treated similarly but were not irradiated, indicated by ShUV-B. Four days later all groups were s.c. immunized with 1 x 10^7 spleen cells from BALB/c, DBA/2 or B10.D2 donor mice. Another five days later all mice received a s.c. challenge of 2 x 10^7 similar allogeneic spleen cells in the right hind feet. Hind foot thickness was measured 24 hours later and the percentage specific increase of foot thickness was calculated as described in the Materials and Methods section. Each bar represents the mean response $\pm$ 1 SEM (n = 5). The percentage suppression of the response by the UV-B treatment is indicated between brackets.

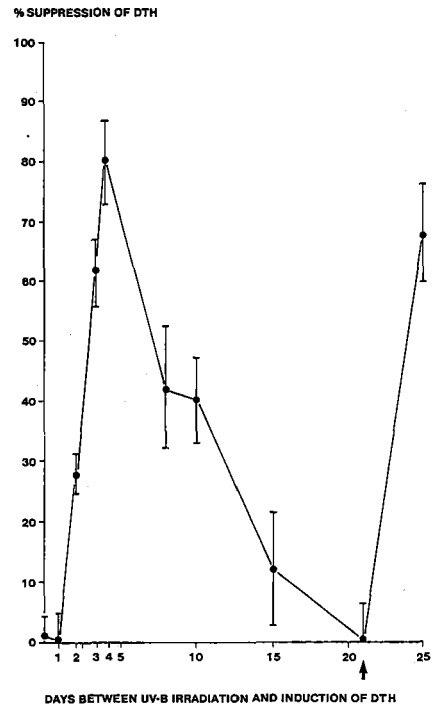

Fig. 2. Kinetics of the UV-B induced suppression. Groups of (C57BL x CBA)F_1 mice received 10^4 J/m^2 of UV-B irradiation on their shaven backs at various intervals before or on the same day as a s.c. immunization with 1 x 10^7 BALB/c spleen cells. One group of (C57BL x CBA)F_1 mice was reexposed to UV-B (10^4 J/m^2) 21 days after the first exposure to UV-B (arrow) and s.c. immunized with BALB/c spleen cells 4 days later. A control group of (C57BL x CBA)F_1 mice was immunized only. Five days later in all groups DTH responses were elicited and measured another 24 hours later. The percentage suppression of DTH was calculated as:

$$\frac{\text{specific DTH response of the suppressed group}}{\text{specific DTH response of the control group}} \times 100\%$$

Each experimental point represents the calculated percentage suppression of DTH of one group of mice (n=5) $\pm$ 1 SEM.

Subsequently, we investigated the phenotype of the Ts cells. Therefore, spleen cells from UV-B irradiated (C57BL x CBA)F_1 mice were treated with anti-Lyt-1.2 monoclonal antibody and complement or anti-Lyt-2.2 monoclonal antibody and complement to deplete subsets of T cells. The depleted cell suspensions were adoptively transferred to syngeneic recipient mice. One group of recipient mice received a mixture of both suspensions. Within 4 hours after transfer all mice were s.c. immunized with BALB/c spleen cells. Five days later all mice were challenged to assay for DTH to BALB/c H

antigens. As can be seen in Fig. 3B, lines 3 and 4, transfer of suppression was abrogated by depletion of Lyt-1^+ cells as well as by depletion of Lyt-2^+ cells. However, suppression of DTH is obvious in the recipients of a mixture of the differently treated cell suspensions (Fig. 3, line 5). Apparently, transfer of the state of suppression requires two populations of T lymphocytes, one expressing the Lyt-1^+2^- phenotype, the other expressing the Lyt-1^-2^+ phenotype.

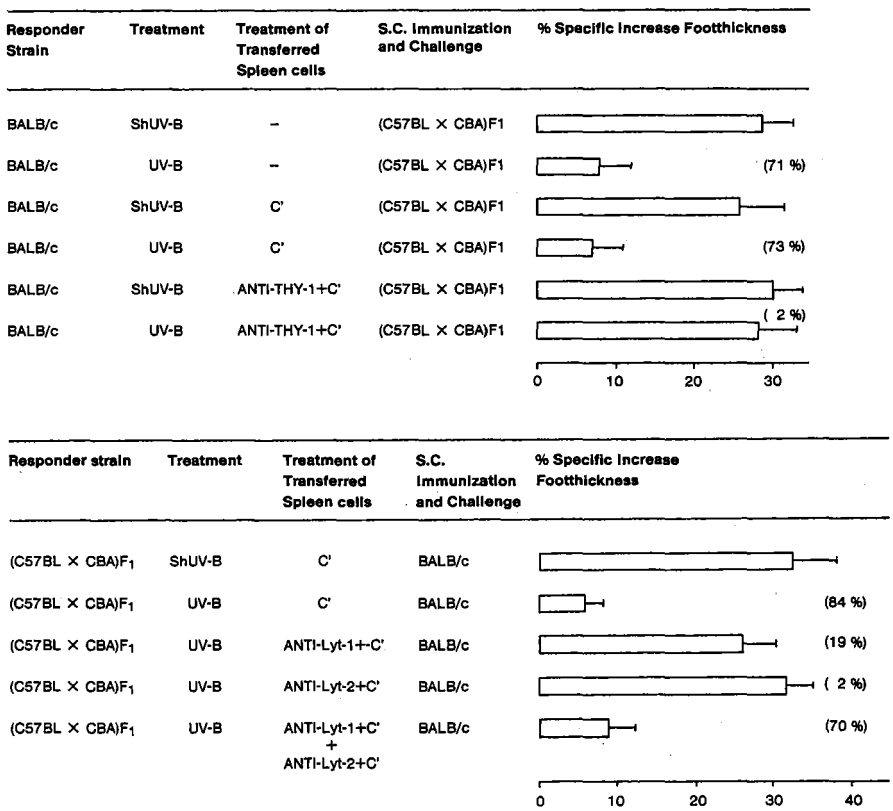

Fig. 3. Characterization of the UV-B induced suppressor cells. Groups of BALB/c mice were i.v. infused with spleen cells from syngeneic mice which had been exposed to UV-B irradiation ($10^4 J/m^2$) 4 days earlier or with spleen cells with spleen cells from syngeneic control mice. The cells to be transferred were treated either with anti-Thy-1.2 and complement (Fig. 3A), with anti-Lyt-1.2 and complement or with anti-Lyt-2.2 and complement (Fig. 3B). Within four hours after transfer all recipient mice were s.c. immunized with 1 x 10^7 (C57BL x CBA)F_1 spleen cells. Five days later DTH responses were elicited. Each bar represents the mean response $\pm$ 1 SEM (n=5). The percentage suppression of the response by the UV-B treatment is indicated between brackets.

Target cells of the UV-B induced suppressor cells

Ts cells might suppress the activation and/or proliferation of the DTH reactive T cells (induction phase of DTH), the activity of DTH effector T cells after challenge (expression phase of DTH) or both. The effect on the induction phase was examined by determining the proliferative activity in the draining lymph nodes of (C57BL x CBA)F_1 recipients of spleen cells from syngeneic UV-B irradiated mice. This was done 5 days after transfer of the suppressor cells and subsequent s.c. immunization with DBA/2 spleen cells. As shown in Fig. 4, upper part, line 2, the immunization-induced proliferative activity in the draining lymph nodes of these mice was significantly reduced. Moreover, in vitro treatment of the spleen cells to be transferred with anti-Thy-1.2 monoclonal antibody and complement completely eliminated the suppression of proliferation (Fig. 4, lower part, line 4). This indicates that the UV-B induced Ts cells inhibit the induction phase of DTH.

The effect on the expression phase of DTH was investigated using the immune lymphocyte transfer (ILT) assay. Thus using the immune lymphocyte transfer (ILT) assay. Thus, a group of (C57BL x CBA)F_1 mice was s.c. immunized with H-2 and non-H-2 incompatible DBA/2 spleen cells. Five days later their draining lymph nodes were removed and used as source of DTH effector T cells. These DTH effector T cells were mixed with spleen cells from another group of (C57BL x CBA)F_1 mice which had been exposed to UV-B irradiation 4 days earlier. The mixed cell populations were injected into the right hind feet of DBA/2 mice, to determine the suppressive activity of the UV-B induced suppressor cells. A control group of DBA/2 mice received an injection of (C57BL x CBA)F_1-anti-DBA/2 DTH effector T cells mixed with spleen cells from (C57BL x CBA)F_1 mice not exposed to UV-B. As shown in Fig. 5, line 2, the UV-B induced Ts cells did not inhibit the DTH reactivity of the already activated DTH effector T cells. Similar results were obtained in a H-2 compatible, non-H-2 incompatible donor-recipient combination using DBA/2 mice receiving BALB/c-anti-DBA/2 DTH effector T cells (Fig. 5, line 4) and in a H-2 incompatible, non-H-2 compatible donor-recipient combination using B10.D2 mice receiving C57BL/6-anti-B10.D2 DTH effector T cells (Fig. 5, line 6). Thus, the UV-B induced Ts cells are unable to suppress the expression phase of DTH to H antigens. In control experiments using Ts cells induced by i.v. injection of the donor mice with allogeneic spleen cells (van der Kwast et al., 1981), ILT responses were suppressed (data not shown).

DISCUSSION

Several studies have shown that irradiation of experimental animals and humans with UV-B usually causes immunosuppression, as reviewed by Kripke (1984). For instance, DeFabo and Kripke (1979) reported that UV-B irradiation most effectively inhibited tumor rejection in mice. This required a dose of 2 x 10^5 J/m^2 of UV-B. Furthermore, Toews et al. (1980), Austad and Mork (1982), Elmets et al. (1983) and Kripke (1984) showed that contact-hypersensitivity reactions to various contact sensitizing agents were suppressed in mice exposed to 1 to 4 x 10^4 J/m^2 of UV-B. Apparently, the inhibition of tumor rejection requires much higher doses of UV-B than the inhibition of contact-hypersensitivity and DTH (Noonan, DeFabo and Kripke; 1981a).

With regard to the effect of UV-B-irradiation on the immune response to H-antigens the literature data are quite contradictory, yet. Morison and

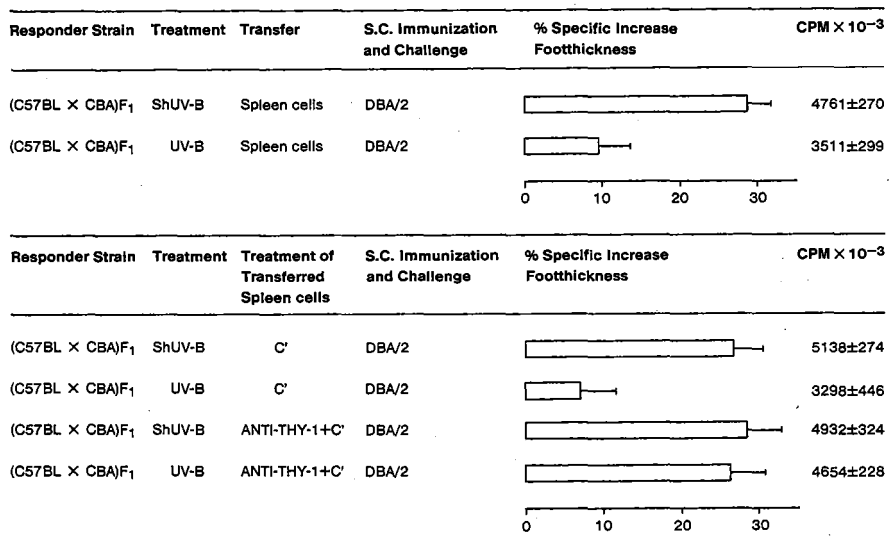

Fig. 4. Effect of UV-B induced suppressor cells on the induction phase of DTH. Groups of 8 (C57BL x CBA)F_1 mice were i.v. infused with spleen cells from syngeneic donor mice which had been exposed to UV-B irradiation (1 x 10^4 J/m^2) four days earlier or with spleen cells from syngeneic control mice. Within four hours all recipient mice were s.c. immunized with 1 x 10^7 DBA/2 spleen cells in the inguinal areas. Five days later three animals of each group were sacrificed and the proliferative activity in the inguinal lymph nodes was assessed. The remaining animals in each group were challenged to assay for DTH (upper part of figure). In a separate, but otherwise similar experiment spleen cells from UV-B irradiated donor mice and spleen cells from unirradiated donor mice were treated *in vitro* with anti-Thy-1.2 and complement or complement only to eliminate T lymphocytes from the spleen cell suspensions to be infused (lower part of figure). Each bar represents the mean response ± 1 SEM (n = 5). The proliferative activity is expressed in cpm ± 1 SEM.

Pike (1985), for instance, showed that GvH-reactivity as measured in the popliteal lymph node (PLN) assay was suppressed after exposure of the recipient mice to a dose of 2 x 10^4 J/m^2 UV-B irradiation, whereas Kripke et al. (1977) showed that neither GvH-reactivity in the PLN assay nor skin graft rejection was inhibited by chronic UV-B irradiation (3 to 4 x 10^5 J/m^2) of the recipient mice. Spellman, Woodward and Daynes (1977) reported that MLR responses to murine allogeneic cells were unaffected after exposure of the responder cell donors to 5 x 10^4 J/m^2 of UV-B.

This is in clear contrast to the effects of UV-B on the DTH-reactivity to H-antigens described by Ullrich (1986) and reported here. Our results indicate that exposure of mice to a relatively low dose (1 x 10^4 J/m^2) of UV-B causes a systemic suppression of DTH to H-antigens (Fig. 1). The suppression reached its maximum at day 4 after UV-B irradiation but gradually waned and completely disappeared by day 21 (Fig. 2). Similar results for suppression of contact-hypersensitivity have been reported by Noonan et

DTH Effector Cells[1]	Incompatibility	Donor of UV-B Induced Suppressor Cells[2]	Recipient Strain	ILT-NLT ($\times 10^{-2}$ mm)[3]
(C57BL × CBA)F_1-anti-DBA/2	H-2 + non-H-2	–	DBA/2	
(C57BL × CBA)F_1-anti-DBA/2	H-2 + non-H-2	(C57BL ×CBA)F_1	DBA/2	
BALB/c-anti-DBA/2	non-H-2	–	DBA/2	
BALB/c-anti-DBA/2	non-H-2	BALB/c	DBA/2	
C57BL/6-anti-B10.D2	H-2	–	B10.D2	
C57BL/6-anti-B10.D2	H-2	C57BL/6	B10.D2	

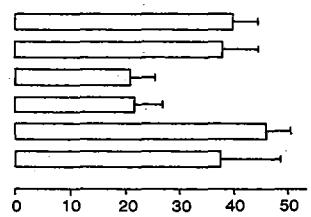

Fig. 5. Effect of UV-B induced suppressor cells on the expression phase of DTH, as tested in the ILT - NLT assay.

1) DTH effector T cells were induced by s.c. immunization of (C57BL x CBA)F_1, BALB/c and C57BL/6 mice with either 1 x 10^7 DBA/2 or B10.D2 spleen cells.
2) Suppressor cells were induced by UV-B irradiation (10^4 J/m^2) of (C57BL x CBA)F_1, BALB/c and C57BL/6 mice four days prior to using the mice as donors of putative suppressor cells for the ILT-NLT assay. Control groups of non-suppressed prospective donor mice were not exposed to UV-B (indicated by a dash).
3) ILT responses were elicited into the right hind feet of DBA/2 and B10.D2 mice by s.c. injection of 5 x 10^6 immune lymphocytes activated by recipient-type H antigens, together with 1 x 10^7 naive spleen cells that were syngeneic to the donor of the immune lymphocytes. NLT responses were elicited using 5 x 10^6 nonimmune lymphocytes together with 1 x 10^7 syngeneic naive spleen cells.
Suppression of ILT responses by UV-B induced suppressor cells was assayed by injecting 5 x 10^6 immune lymphocytes ('DTH effector cells') together with 1 x 10^7 spleen cells from UV-B irradiated mice ('suppressor cells') that were syngeneic to the donor of the immune lymphocytes into the right hind feet of the relevant recipient mice.

al. (1981b). The state of suppression of DTH to H antigens could easily be reinduced by reexposure of the responder animals to UV-B (Fig. 2).

The suppression was due to the activity of Ts cells that could be obtained from the spleen of UV-B irradiated mice four days after exposure (Fig. 3). Two populations of T cells appeared to be required for the adoptive transfer of suppression. One expresses the Lyt-1^+2^- phenotype, whereas the other expresses the Lyt-1^-2^+ phenotype (Fig. 3). Similar results were reported by Howie et al. (1986) for UV-B induced Ts cells that suppressed DTH to HSV-1. In contrast, transfer of suppression of tumor rejection required only one type of T cell, namely Lyt-1^+2^- T cells (Ullrich and Kripke, 1984). In view of the difference in dose-requirement and suppressor cell phenotype UV-B induced suppression of tumor rejection and UV-B induced suppression of DTH to cell-bound H-antigens are obviously due to different mechanisms.

The UV-B induced Ts cells inhibited the induction phase of DTH as judged by the suppressed proliferative response in the draining lymph nodes of the UV-B irradiated mice (Fig. 4). Bianchi et al. (1984) also reported

suppression of the induction phase of DTH to H antigens by Ts cells. These cells were induced in mice by the i.v. injection of a relatively high dose of irradiated allogeneic spleen cells and expressed the Lyt-1^+2^+ phenotype. Apparently, T cells of different phenotypes can exert their effect during the induction phase of DTH to H antigens. Ullrich (1985) has shown antigen-specific suppression of the proliferation of dinitrofluorobenzene (DNFB)-specific T cell clones *in vitro* by splenic T lymphocytes from mice that had received 5 x 10^4 J/m^2 of UV-B and had been painted with DNFB at an unexposed site five days later.

Ullrich (1986) also reported antigen-specific suppression of allogeneic MLR by Ts cells induced after UV-B irradiation of spleen cell donors and subsequent s.c. immunization with allogeneic spleen cells. Spleen cells from mice exposed to UV-B but not sensitized with allogeneic spleen cells were incapable of suppressing MLR. These data would indicate that UV-B irradiation alone is not sufficient for the induction of Ts cells and that it is the combination of UV-B irradiation and s.c. immunization that accounts for the generation of suppressor cells. The transfer studies reported in this paper (Figs 3 and 4), however, clearly show that UV-B irradiation alone does induce antigen-nonspecific Lyt-1^+2^- and Lyt-1^-2^+ Ts cells. Subsequent antigenic stimulation might account for a further clonal expansion of the specific Ts cells. Our present studies are aimed to verify this hypothesis.

The ILT-NLT transfer experiments show that the Ts cells induced by UV-B irradiation alone are unable to suppress already activated DTH effector T cells (Fig. 5). In contrast, Bianchi et al. (1984, 1986) reported that the Ts cells induced by their protocol suppress not only the induction of DTH reactive T cells, but also the functional activity of already activated DTH effector T cells. This indicates that the mode of action of Ts cells might be dictated by the procedure used for their induction.

The mechanism by which skin-exposure to UV-B leads to splenic Ts cells and systemic immunosuppression is still unclear. An effect on Langerhans cells has been proposed by Noonan et al. (1981b). These skin associated antigen presenting cells play a pivotal role in the development of contact-hypersensitivity. Modulation of their number and functional activity, which has been reported in mice after UV-B irradiation (Fox et al, 1981; Lynch et al., 1983) could lead to passing of the applied antigen through the skin barrier without being presented to the immune system in an immunogenic manner, thereby leading to the preferential induction of Ts cells. This, however, does not explain the occurrence of non-specific suppressor cells in the spleen of UV-B irradiated mice as shown by transfer experiments (Figs 3 and 4). Also the observation of Noonan et al. (1984) that the effects of UV-B-irradiation on Langerhans cells required another wavelength (i.e. 270-290 nm) than UV-B induced suppression of contact-hypersensitivity (i.e. 320 nm) does not support their original hypothesis.

Another mechanism that has been proposed for UV-induced systemic immuno-suppression is the release of a factor from the skin that induces splenic Ts cells. Evidence for such a factor has been presented by DeFabo and Noonan (1983), Swartz (1984) and more recently also by Harriott-Smith and Halliday (1986).

The effect of UV-B-irradiation on the immune system might not only be dependent on the dose and wavelength of the irradiation, but also on the genetic constitution of the recipients. Freeman, Bergstresser and Streilein (1982) reported that BALB/c, A/J and DBA/2 mice did not show local

immunosuppression after exposure to low dose (8×10^2 J/m^2) UV-B-irradiation, whereas this effect could easily be detected in C3H, C57BL/6 and B10.S mice. In our studies all strains tested (BALB/c, (C57BL x CBA)F_1 and C57BL/6) showed systemic inhibition of DTH-responsiveness to H-antigens after UV-B exposure. This might well be due to the ten fold higher dose of UV-B applied by us. Lower doses of UV-B irradiation failed to induce systemic suppression of DTH to H antigens in all strains tested (data not shown). However, we did not evaluate the occurrence of local immunosuppression at the irradiated site after exposure to these lower doses of UV-B irradiation.

In conclusion, our data and the literature cited indicate that UV-B irradiation profoundly suppresses DTH to H antigens by the induction of Ts cells. Without deliberate immunization these Ts cells are non-specific. Studies are in progress to establish the specificity and restriction pattern of these Ts cells after immunization, the possible role of skin-derived factors in their development and their influence on allograft survival.

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CHAPTER V.2.

SUPPRESSION OF DELAYED-TYPE HYPERSENSITIVITY TO HISTOCOMPATIBILITY ANTIGENS BY ULTRAVIOLET IRRADIATION

ROLE FOR SKIN-DERIVED FACTOR(S) IN THE INDUCTION OF SUPPRESSOR T CELLS

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Submitted for publication.

SUMMARY

We have previously shown that UV-B irradiation of mice induces two types of suppressor T (Ts) cells that suppress delayed-type hypersensitivity (DTH) to allogeneic histocompatibility (H) antigens. This paper reports that these Ts cells are induced by serum factor(s) that can be detected as early as 3 hrs after irradiation, but not at later time points. These serum factor(s) could induce suppressor cells in H-2 incompatible recipients. It was found that one type of Ts cell in addition to the Lyt-1 cell surface marker expressed the L3T4 cell surface marker, whereas the other type of Ts cell appeared L3T4-negative. In contrast to the UV-B induced serum factor(s), Ts cells from UV-B irradiated mice could mediate suppression in H-2 compatible recipients only.

INTRODUCTION

In a previous paper (Molendijk et al., 1987a) we reported that exposure of mice to UV-B irradiation induces a T cell mediated state of suppression of delayed type hypersensitivity (DTH) to histocompatibility (H) antigens. The degree of suppression reached its maximum four days after irradiation, gradually waned thereafter and disappeared at Day 21. Reexposure of these mice to a similar dose of UV-B reinduced the state of suppression. Two populations of suppressor T (Ts) cells appeared to be involved: one with the Lyt-1^+2^- phenotype and the other with the Lyt-1^-2^+ phenotype. These cells suppressed the induction phase of DTH (i.e. the proliferative activity of draining lymph node cells in response to subcutaneous (s.c.) immunization with allogeneic spleen cells), but not the expression phase of DTH (i.e. the activity of already activated DTH effector T cells).

Other cell mediated immune responses can also be suppressed by UV-B irradiation. For instance, Fisher and Kripke (1977, 1978) reported that UV-B irradiation induced Ts cells that suppressed the rejection of simultaneously induced skin tumours. Noonan, De Fabo and Kripke (1981) reported that contact hypersensitivity (CHS) to dinitrofluorobenzene (DNFB) was suppressed after UV-B irradiation. More recently, Ullrich (1986) reported about suppression of allogeneic mixed-lymphocyte-reaction (MLR) by UV-B induced Ts cells.

A major question has been the relationship between UV-B irradiation of the skin, which penetrates only superficially (Kripke, 1984), and the appearance of Ts cells in the spleen of the irradiated mice. Several investigators demonstrated involvement of serum factor(s) in the induction of suppression (Swartz, 1984; Harriot-Smith and Halliday, 1986). In view of the probability that UV-B irradiation induces an immunoregulatory pathway which controls multiple immune responses (Ullrich, Yee and Kripke, 1986), we investigated whether serum factor(s) are involved in the development of UV-B induced Ts cells that regulate DTH against H antigens.

MATERIALS AND METHODS

Mice

(C57BL/Rij x CBA/Rij)F1 ($H\text{-}2^{b/q}$) female mice, 4 to 6 weeks of age, were purchased from the Radiobiological Institute TNO, Rijswijk, The Netherlands. DBA/2 ($H\text{-}2^d$) and BALB/c ($H\text{-}2^d$) female mice, 4 to 6 weeks of age, were purchased from Bomholtgart, Ry, Denmark. BALB.K ($H\text{-}2^k$) female mice 4 to 6 weeks of age were purchased from HARLAN, OLAC Ltd., Bicester, Oxon, U.K. Strain 129 ($H\text{-}2^b$) female mice were bred and raised at our department. All mice were kept in light-cycled rooms and had access to pelleted food (Hope Farms, Woerden, The Netherlands) and acidified water ad libitum. The animals age at the start of each experiment ranged from 10 to 14 weeks.

Preparation of cell suspensions and serum

Donor mice were killed by carbondioxide exposure. Their spleens were removed, placed in a balanced salt solution (BSS), minced with scissors and squeezed through a nylon gauze filter to get a single cell suspension. Cell numbers were counted with a Coulter Counter model BZI (Coulter Electronics, Harpenden, Herts, U.K.). Cell viability always exceeded 95%, as determined by trypan blue exclusion. Blood was obtained by cardiac puncture and allowed to clot for 1 hr at 37°C. After centrifugation (20 min, 600 g) the serum

was collected and, when not immediately used, stored in aliquots of 1 ml at -20°C.

UV-B irradiation

The method for induction of suppression by UV-B irradiation has been described (Molendijk et al., 1987a). Briefly, the dorsal fur of responder mice was shaven using electrical hair clippers. The mice were then exposed to a single dose of 1 x 10^4 J/m^2 UV-B from a bank of standard Philips TL12 UV lamps. Control mice were treated similarly but were not exposed to UV-B irradiation.

Adoptive transfer of cells and serum

For adoptive transfer spleen cell suspensions were transferred to the recipients through a tail vein. Each recipient received one whole spleen equivalent of cells suspended in a volume of 1.0 ml BSS. Serum was also transferred through a tail vein. Unless stated otherwise, recipient mice received 0.75 ml of serum.

Separation of Lyt-2 positive and Lyt-2 negative spleen cell fractions

Spleen cell suspensions were fractionated into Lyt-2 positive and Lyt-2 negative fractions by panning on plastic plates coated with anti-Lyt-2 monoclonal antibodies, as has been described (Mage et al., 1977; Molendijk et al., 1987b).

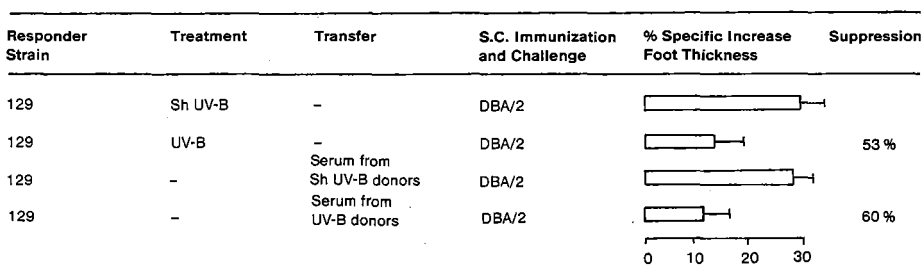

Fig. 1. UV-B irradiation of mice induces immunosuppressive factor(s). Groups of strain 129 mice were either exposed to 10^4 J/m^2 of UV-B irradiation or sham-irradiated. Three hours later the mice were sacrificed and their serum was prepared. A volume of 0.75 ml of either preparation was i.v. injected into syngeneic recipients. Four days later these recipients as well as a group of irradiated control mice and a group of sham-irradiated control mice were s.c. immunized with DBA/2 spleen cells. Another five days later all mice were challenged with DBA/2 spleen cells. Each bar represents the mean percentage specific DTH response $\pm$ 1 SEM (n=5). The percentage suppression of DTH was calculated as the specific DTH response of the suppressed group divided by the specific DTH response of the control group x 100%.

Depletion of L3T4 positive spleen cells

Spleen cell suspensions were treated with IgG2b anti-L3T4 (clone GK 1.5, American Type Culture Collection, Rockville, MD, U.S.A.) for 30 min at 4°C. After two washings the cells were incubated with Rabbit Low Tox "M" complement (Cedarlane Laboratories Ltd., Hornby, Ontario, Canada) for 25 min at 37°C. After three washings the cells were suspended in BSS and adoptively transferred.

Induction of DTH

DTH was induced by s.c. immunization of responder mice with 1×10^7 allogeneic spleen cells suspended in a volume of 100 ul, equally spread over each inguinal area.

Assay for DTH

DTH responses were elicited by s.c. injection of a challenge dose of 2×10^7 of the relevant allogeneic spleen cells, suspended in a volume of 50 ul BSS, into the dorsum of the right hind foot five days after s.c. immunization. The DTH response was measured 24 hr later as the difference in thickness of the hind feet. The specific increase in foot thickness was calculated as the relative increase in foot thickness of the immunized mice minus the relative increase in foot thickness of control mice which received the challenge injection only. The increase of foot thickness of these control mice varied between 15% and 25%.

RESULTS

Serum from UV-B treated mice exerts immunosuppressive activity

To investigate whether suppressive activity could be detected in serum from UV-B exposed mice, strain 129 mice were exposed to 1×10^4 J/m^2 of UV-B irradiation. Three hours later 10 mice were sacrificed and their blood was obtained of which the serum was prepared. Serum was also prepared from blood of sham-irradiated mice. A volume of 0.75 ml of either serum was i.v. injected into naive syngeneic recipients. Four days later all recipient mice as well as groups of irradiated and sham-irradiated responder mice were s.c. immunized with DBA/2 spleen cells to induce DTH. Another five days later DTH responses were elicited. As shown in Fig. 1, line 4, DTH responses were suppressed not only in the irradiated responder mice, but also in the recipients of serum from UV-B irradiated donors.

Kinetics of suppressive activity in the serum of UV-B treated donors

To investigate the kinetics of the suppressive activity exerted by serum from UV-B treated donors, BALB/c mice were exposed to UV-B irradiation. At 3 hours, 1 day and 4 days after irradiation groups of irradiated and sham-irradiated mice were sacrificed and their blood was obtained from which the serum was prepared. A volume of 0.75 ml of either preparation was i.v. injected into naive syngeneic recipients. Four days after irradiation all recipients were s.c. immunized with (C57BL x CBA)F1 spleen cells to induce DTH. Another five days later DTH responses were elicited. As shown in Fig. 2, line 2, suppressive activity appeared in the serum as early as 3 hours after irradiation. In contrast, suppressive activity could not be detected in serum prepared 1 or 4 days after irradiation (Fig. 2, lines 4 and 6).

Donor Strain	Treatment	Transfer	Time Point	S.C. Immunization and Challenge	% Specific Increase Foot Thickness	Suppression
BALB/c	Sh UV-B	Serum	3 hrs	(C57BL × CBA)F_1		
BALB/c	UV-B	Serum	3 hrs	(C57BL × CBA)F_1		65 %
BALB/c	Sh UV-B	Serum	1 day	(C57BL × CBA)F_1		
BALB/c	UV-B	Serum	1 day	(C57BL × CBA)F_1		0 %
BALB/c	Sh UV-B	Serum	4 days	(C57BL × CBA)F_1		
BALB/c	UV-B	Serum	4 days	(C57BL × CBA)F_1		35 %

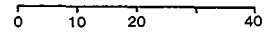

Fig. 2. Kinetics of suppressive activity in the serum of UV-B treated mice. Groups of BALB/c mice were either exposed to UV-B irradiation or sham-irradiated. At 3 hrs, 1 day and 4 days after irradiation one group of UV-B irradiated mice and one group of sham-irradiated mice were sacrificed and serum was prepared. A volume of 0.75 ml of either preparation was i.v. injected into syngeneic recipients. Four days later all recipients were s.c. immunized with (C57BL x CBA)F1 recipients. Another five days later DTH responses were elicited. Each bar represents the mean percentage specific DTH response ± 1 SEM (n=5).

Donor Strain	Treatment	Primary Transfer	Secondary Transfer	S.C. Immunization and Challenge	% Specific Increase Foot Thickness	Suppression
BALB/c	Sh UV-B	-	Spleen Cells	(C57BL × CBA)F_1		
BALB/c	UV-B	-	Spleen Cells	(C57BL × CBA)F_1		68 %
BALB/c	Sh UV-B	Serum	-	(C57BL × CBA)F_1		
BALB/c	UV-B	Serum	-	(C57BL × CBA)F_1		59 %
BALB/c	Sh UV-B	Serum	Spleen Cells	(C57BL × CBA)F_1		
BALB/c	UV-B	Serum	Spleen Cells	(C57BL × CBA)F_1		73 %

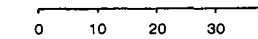

Fig. 3. Serum from UV-B exposed mice induces splenic suppressor cells. Three groups of BALB/c mice were exposed to UV-B irradiation and three other groups were sham-irradiated. Three hours later the serum of two groups of UV-B exposed mice and two groups of sham-irradiated mice was i.v. injected into syngeneic recipients. Four days later spleen cells from the third group of UV-B exposed mice and of the third group of sham-irradiated mice, and spleen cells from a group of recipients of serum from UV-B exposed and a group of recipients of serum from sham-irradiated mice were i.v. injected into syngeneic recipients. Within two hrs after transfer all recipients were s.c. immunized with (C57BL x CBA)F1 spleen cells. Five days later DTH responses were elicited. Each bar represents the mean percentage specific DTH response ± 1 SEM (n=5).

Donor Strain	Treatment	Spleen Cell Fraction Used for Depletion	In Vitro Treatment of Spleen Cell Fraction	Spleen Cell Fraction Added	S.C. Immunization and Challenge	% Specific Increase Foot Thickness	Suppression
(C57BL × CBA)F_1	Sh UV-B	Lyt-2$^-$	C	Lyt-2$^+$	DBA/2		
(C57BL × CBA)F_1	UV-B	Lyt-2$^-$	C	Lyt-2$^+$	DBA/2		73 %
(C57BL × CBA)F_1	Sh UV-B	Lyt-2$^-$	anti-L_3T_4 + C	Lyt-2$^+$	DBA/2		
(C57BL × CBA)F_1	UV-B	Lyt-2$^-$	anti-L_3T_4 + C	Lyt-2$^+$	DBA/2		4 %
(C57BL × CBA)F_1	Sh UV-B	Lyt-2$^+$	C	Lyt-2$^-$	DBA/2		
(C57BL × CBA)F_1	UV-B	Lyt-2$^+$	C	Lyt-2$^-$	DBA/2		71 %
(C57BL × CBA)F_1	Sh UV-B	Lyt-2$^+$	anti-L_3T_4 + C	Lyt-2$^-$	DBA/2		
(C57BL × CBA)F_1	UV-B	Lyt-2$^+$	anti-L_3T_4 + C	Lyt-2$^-$	DBA/2		66 %

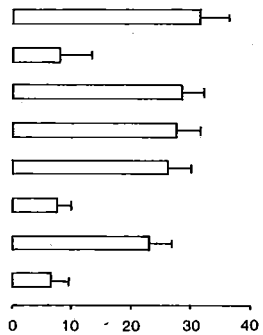

Fig. 4. L3T4 phenotype of UV-B induced Ts cells. Groups of (C57BL x CBA)F1 mice were either exposed to UV-B or sham-irradiated. Four days later their spleens were obtained and separated into Lyt-2^+ and Lyt-2^- cells by panning. Subsequently half of each fraction was treated with anti-L3T4 monoclonal antibodies and complement or complement only. The residual viable cells were mixed with the untreated half of each opposite fraction and the mixtures were i.v. injected into syngeneic recipients, which were s.c. immunized with DBA/2 spleen cells within two hrs after transfer. Five days later DTH responses were elicited. Each bar represents the mean percentage specific DTH response $\pm$ 1 SEM (n=5).

Donor Strain	Treatment	Transfer	Recipient Strain	S.C. Immunization and Challenge	% Specific Increase Foot Thickness	Suppression
BALB/c	Sh UV-B	Serum	BALB/c	(C57BL × CBA)F_1		
BALB/c	UV-B	Serum	BALB/c	(C57BL × CBA)F_1		58 %
BALB/c	Sh UV-B	Serum	BALB.K	(C57BL × CBA)F_1		
BALB/c	UV-B	Serum	BALB.K	(C57BL × CBA)F_1		64 %

0 10 20 30 40

Donor Strain	Treatment	Transfer	Recipient Strain	S.C. Immunization and Challenge	% Specific Increase Foot Thickness	Suppression
BALB/c	Sh UV-B	Spleen Cells	BALB/c	(C57BL × CBA)F_1		
BALB/c	UV-B	Spleen Cells	BALB/c	(C57BL × CBA)F_1		55 %
BALB/c	Sh UV-B	Spleen Cells	BALB.K	(C57BL × CBA)F_1		
BALB/c	UV-B	Spleen Cells	BALB.K	(C57BL × CBA)F_1		0 %

0 10 20 30 40

Fig. 5. MHC-restriction of UV-B induced suppressive serum and Ts cells. Upper part: Groups of BALB/c mice were either exposed to UV-B irradiation or sham-irradiated. Three hours later their serum was prepared and i.v. injected into either BALB/c or BALB.K recipients. Four days later all recipients were s.c. immunized with (C57BL x CBA)F1 spleen cells. Another five days later DTH responses were elicited. Each bar represents the mean percentage specific DTH response ± 1 SEM (n=5). Lower part: In an otherwise similar experiment spleen cells from UV-B or sham-irradiated BALB/c mice were i.v. transferred to either BALB/c or BALB/K recipients four days after irradiation. Within two hrs after transfer all recipients were s.c. immunized with (C57BL x CBA)F1 spleen cells. Five days later DTH responses were elicited. Each bar represents the mean percentage specific DTH response ± 1 SEM (n=5).

Serum from UV-B treated mice induces splenic suppressor cells

To investigate the mechanism whereby serum from UV-B exposed donors induces a state of suppression of DTH in recipient mice, serum from UV-B exposed BALB/c mice obtained 3 hrs after irradiation, was i.v. injected into naive, syngeneic recipients. Four days later the recipients were killed and their spleen cells were i.v. transferred to secondary BALB/c recipients, which were subsequently s.c. immunized with (C57BL x CBA)F1 spleen cells. Five days later DTH responses were elicited. As shown in Fig. 3, line 6, DTH responses were suppressed in the recipients of spleen cells from donors that had been injected with serum from UV-B exposed mice.

L3T4 phenotype of UV-B induced Ts cells

We previously reported that two populations of Ts cells are involved in the suppression of DTH after UV-B irradiation. One expresses the Lyt-1^+2^- phenotype and the other the Lyt-1^-2^+ phenotype (Molendijk et al. 1987a). To investigate the L3T4 phenotype of the former Ts cell type, (C57BL x CBA)F1 mice were exposed to UV-B. Four days later their spleen cells were obtained and divided into a Lyt-2^+ and a Lyt-2^- cell fraction by panning. After fractionation the Lyt-2^- fraction was treated with anti-L3T4 monoclonal antibodies and complement or complement only. Thereafter the residual cells were mixed with the Lyt-2^+ cell fraction and i.v. injected into naive syngeneic recipients. Within two hours after transfer the recipients were s.c. immunized with DBA/2 spleen cells. Five days later DTH responses were elicited. As shown in Fig. 4, upper part, line 4, treatment of the Lyt-2^- fraction (containing the Lyt-1^+2^- Ts cells) with anti L3T4 and complement completely abrogated the transfer of suppression. In contrast, similar treatment of the Lyt-2^+ fraction (containing the Lyt-1^-2^+ Ts cells) prior to mixing with the Lyt-2^- fraction did not affect the transfer of suppression (Fig. 4, lower part, line 4). Thus, one of the UV-B induced subpopulations of Ts cells expresses the L3T4$^+$,Lyt-1^+2^- phenotype, whereas the other expresses the L3T4$^-$,Lyt-1^-2^+ phenotype.

MHC restriction of UV-B induced suppressive serum and Ts cells

To investigate whether UV-B induced suppressive serum could induce a state of suppression in major histocompatibility complex (MHC) incompatible recipients, BALB.K mice received serum from UV-B exposed BALB/c mice. Four days later DTH to (C57BL x CBA)F1 alloantigens was induced by s.c. immunization. Another five days later DTH responses were elicited. As shown in Fig. 5, upper part, lines 2 and 4, DTH responses in the BALB.K recipients of serum from UV-B exposed BALB/c mice were suppressed to the same extent as DTH responses in the BALB/c recipients. In contrast, when BALB.K mice received spleen cells from UV-B exposed BALB/c mice four days after irradiation, DTH reactivity to (C57BL x CBA)F1 alloantigens was not suppressed (Fig. 5, lower part, line 4).

DISCUSSION

The present study indicates that in the serum of UV-B irradiated mice soluble factor(s) occur which, after transfer, induce a state of suppression in the recipient mice (Fig. 1). Suppression appeared after subsequent induction of DTH four days after injection of serum. Study of the kinetics of the suppressive activity showed that the suppressive activity occurs 3 hrs after irradiation and is of transient nature (Fig. 2). Swartz (1984) reported that suppressive activity in serum from UV-B irradiated mice could be detected 2 to 6 hrs after irradiation, but not at later time points. In his experiments suppression appeared after induction of contact hypersensitivity to 2-chloro-1,3,5-trinitrobenzene (TNCB), 5 days after serum injection. They found that at least 0.4 ml of serum had to be injected to induce suppression. We injected 0.75 ml of serum in order to induce suppression, although injection of 0.5 ml of serum already induced some suppression (data not shown).

Harriott-Smith and Halliday (1986) reported that serum from UV-B irradiated and sensitized mice suppressed the *in vitro* leucocyte adherence inhibition (LAI) assay antigen-nonspecifically. They also reported that 3

to 5 days after UV-B irradiation alone serum from irradiated, non-sensitized mice possesses suppressive properties. However, we could only detect suppressive activity in the serum of UV-B irradiated donors at 3 hrs after irradiation.

The data shown in Fig. 3 clearly demonstrate that after the injection of serum from UV-B irradiated donors suppressor cells can be found in the recipients' spleen which, after adoptive transfer, suppress DTH against H antigens. This was also reported by Swartz (1984). However, he investigated the presence of suppressor cells in the spleen of serum recipients after sensitization. In contrast, we could detect suppressor cells without prior immunization of the recipients, indicating that the serum factor(s) itself induced the suppressor cells. Preliminary results indicate that these suppressor cells indeed belong to the T cell lineage (data not shown).

We previously reported that the population of UV-B induced Ts cells actually consists of two Ts cell types, one with the Lyt-1^+2^- phenotype and the other with the Lyt-1^-2^+ phenotype. The data in Fig. 4 show that the former cell type also expresses the L3T4 cell surface marker, whereas the latter cell does not.

We recently found that Ts inducer cells induced by i.v. immunization of mice with a high dose of irradiated allogeneic spleen cells also display the L3T4^+,Lyt-1^+2^- phenotype (Molendijk et al., 1987c; Molendijk et al., submitted). These Ts inducer cells activated a population of L3T4^-,Lyt-1^+2^+ Ts effector cells which actually suppressed the DTH response. Whether the UV-B induced L3T4^+,Lyt-1^+2^- Ts cells also exert an inducer function with regard to the other Ts cell subpopulation remains to be elucidated.

As shown in Fig. 5, upper part, serum from UV-B irradiated BALB/c donors could induce a state of suppression in MHC-incompatible BALB.K recipients. Harriott-Smith and Halliday (1986) found that serum from UV-B irradiated donors could at least partially inhibit allogeneic cells in the LAI-assay. In contrast, Ts cells from UV-B treated BALB/c donors could not exert their effect in H-2 incompatible BALB.K recipients (Fig. 5, lower part). Thus, it appears that the UV-B induced serum factor(s) are MHC-unrestricted, whereas the UV-B induced Ts cells, which are induced by the serum factor(s), are MHC-restricted. The Ts inducer cells and the Ts effector cells induced by i.v. immunization with allogeneic spleen cells, on the other hand, could exert their effect in MHC incompatible recipients as well. Apparently, DTH to H antigens can be regulated by different immunoregulatory T cell pathways.

With regard to the nature of the UV-B induced soluble suppressor factor several possibilities have been proposed. For instance, prostaglandins (PG) and leukotriens might account for the UV-B induced immunosuppression (Kripke, 1984). However, we did not find any difference in PG concentration of serum from UV-B irradiated and sham-irradiated mice.

Recently, Schwarz et al. (1986; 1987) elegantly demonstrated the production of a soluble inhibitor (MW 40 kD) of interleukin 1 (IL-1) activity by UV-B irradiated epidermal cells and a keratinocyte cell line. Most interestingly this factor could suppress the induction phase of CHS to DNFB when CHS was induced five days after injection, but not the expression phase of CHS. This is in agreement with our previously reported finding that the UV-B induced Ts cells can suppress the induction phase of DTH, but not the expression phase of DTH (Molendijk et al., 1987a). It would be very interesting to investigate whether the factor described by Schwarz et al. induces a population of Ts cells which actually suppresses CHS.

Ross et al. (1986; 1987) recently reported evidence for the involvement of urocanic acid in the UV-B induced suppression of DTH to HSV. We are currently investigating whether this substance is involved in the induction of suppression of DTH to alloantigens by UV-B irradiation.

In conclusion, the data presented here indicate that UV-B irradiation induces the release of soluble factor(s) from the skin, which induces two subpopulations of cooperating Ts cells in the spleen of the irradiated mice. The factor(s) is not MHC restricted in its capacity to induce suppression of DTH, in contrast to the Ts cells generated.

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CHAPTER VI

GENERAL DISCUSSION

GENERAL DISCUSSION

Two types of T cells play a pivotal role in the regulation of cell mediated immune responses: Th cells and Ts cells. Th cells provide help for other cells mediating immune responses, e.g. B cells and Tc cells. DTH is believed to be mediated by a specialized subset of Th cells (Mosmann and Coffman, 1987) that are susceptible for the regulatory activity of Ts cells.

Several studies indicate that two or three mutually interacting subsets of Ts cells are involved in suppression of DTH to haptens (Dorf and Benacerraf, 1984). A Ts ind cell, usually expressing the Lyt-1^+2^- phenotype, activates a Lyt-1^-2^+ Ts eff cell, either directly or via an interaction with a Lyt-1^+2^+ Ts trans cell.

Previous work from our laboratory has shown that systemic (i.v.) immunization of mice with a relatively high dose of irradiated allogeneic spleen cells induces an antigen-specific state of suppression of DTH to alloantigens (Van der Kwast et al., 1981). The suppression was mediated by a population of Lyt-1^+2^+ Ts cells that suppressed the induction phase as well as the expression phase of DTH (Bianchi et al., 1984). Moreover, these Ts eff cells could exert their activity in recipients that were incompatible for MHC as well as Igh loci (Bianchi et al., 1986). In spite of their specificity, these Ts eff cells could also suppress DTH to unrelated third party alloantigens (Bianchi et al., 1983). This, however, only occurred when the third party alloantigens were administered together with the alloantigens that had induced the suppression.

In addition to the above described Ts eff cells we found evidence for the involvement of a radiation-sensitive Ts ind cell in the regulation of DTH to alloantigens (Chapter III, sections 1 and 2). These cells occurred in the spleen of i.v. tolerized mice one day after tolerization, expressed the L3T4$^+$,Lyt-1^+2^- phenotype, but could not themselves exert a suppressive effect on DTH. Rather, they were found to activate precursors of L3T4$^-$, Lyt-1^+2^+ Ts eff cells, which ultimately suppressed DTH. In adoptive transfer experiments (Chapter III, section 2) it was found that the Ts ind cells preferentially interact with precursors of Ts eff cells of recipient origin. However, when precursors of Ts eff cells were not available in the recipient mice, for instance in nude mice, the Ts ind cells could interact with Lyt-2^+ precursors of Ts eff cells of donor origin that were simultaneously transferred with the Ts ind cells. The Ts ind cells could exert their activity in MHC and Igh incompatible recipients (Chapter III, sections 1 and 2). This also occurred after elimination of the Lyt-2^+ cells from the transferred inoculum. Thus it appears that the Ts ind cells and the Ts eff cells do not need to be compatible for MHC and/or Igh in order to interact effectively.

Liew et al. (1982) also found two populations of Ts cells in the regulation of DTH to alloantigens. He found that after i.v. tolerization with a very high dose of irradiated allogeneic spleen cells Lyt-1^+2^- Ts cells were activated that could suppress DTH to alloantigens. After an i.p. booster immunization with the relevant alloantigens he found Lyt-1^+2^+ Ts cells. In view of our own data it may well be that Liew's Lyt-1^+2^- Ts cells actually represent Ts ind cells which did not themselves suppress DTH, but

activated a population of Lyt-1^+2^+ Ts eff cells. It is unclear whether or not the latter cells expressed the L3T4 marker and thus might correspond to the L3T4$^-$Lyt-1^+2^+ Ts eff cells identified in our studies.

Liew also found that in order to obtain suppression in his protocol donor and responder mice should be at least incompatible for the putative I-J locus. However, Bianchi et al. (1984) found suppression in every possible allogeneic donor-responder combination, including I-J compatible combinations. In an attempt to identify the reason for this discrepancy we tried to reproduce Liew's findings but repeatedly failed to do so. As shown in Fig. 1, applying Liew's own protocol for the induction of suppression we found suppresion in combinations that are either H-2, non-H-2, H-Y, H-2K, H-2I, H-2S,D or H-2K,I-A incompatible. Thus, in our hands, the concept of "allo I-J restricted" Ts cells appeared invalid. Indeed, in an earlier paper Liew (1982b) himself showed suppression in a H-2 compatible, non-H-2 incompatible donor-responder combination.

Bianchi et al. (1984) showed that i.v. immunization, but not local (s.c.) immunization could induce suppression. They attempted to induce suppression by s.c. immunization in the inguinal areas. Instead of suppression it was found that, depending on the alloantigenic differences, some time after the attempted tolerization secondary type DTH responses could be elicited (Bianchi et al., 1985). However, as described in Chapter III, section 3, s.c. immunization in the hind feet (HFI) of mice did induce a state of suppression of DTH. The latter became obvious when the immunized mice were s.c. immunized in the inguinal areas to induce DTH. The suppression appeared to be mediated by antigen-specific Lyt-1^-2^+ Ts eff cells which suppressed the induction phase but not the expression phase of DTH. These cells also suppressed DTH to third party alloantigens, provided the latter were administered during the induction phase of DTH, together with the alloantigens that had induced the suppression. Thus, it appears that suppression of DTH to third party alloantigens after specific restimulation is a general characteristic of Ts cells specific for alloantigens.

In contrast to the systemically induced Ts cells, the activaty of HFI-induced Ts cells appeared H-2 restricted. This confirms reports on the *in vitro* regulation of MLR by HFI-induced Ts cells (Rich, 1975). Also in such *in vitro* studies a Ts ind cell/Ts eff cell pathway was detected (Chaouat et al., 1982). So far, the existence of such a suppressor pathway has not been investigated for the HFI-induced suppression of DTH *in vivo*.

From these and other studies it is obvious that there is not a single phenotype characteristic for Ts cells. Instead the different Ts cell phenotypes found support the notion that suppression can be mediated by one or more Ts cell circuits involving different Ts cell subpopulations.

In spite of the failure to induce suppression of DTH by s.c. immunization with alloantigens in the inguinal areas only, we could induce suppression when the s.c. immunization in the inguinal areas was combined with an i.v. injection with bacterial lipopolysaccharide (LPS) (Chapter IV). Both the induction phase and the expression phase of DTH could be suppressed by this treatment. The induction of suppression depended on the mitogenic activity of LPS. The state of suppression appeared antigen-specific, but again, also DTH to third party alloantigens could be suppressed. In view of our findings in Chapter III and those of Bianchi et al. (1983) we initially hypothesized that the suppression was mediated by Ts cells. Surprisingly, however, we found that B cells rather than Ts cells were the mediators of suppression. Moreover, a highly purified immunoglobulin preparation prepared from the supernatant of cultured LPS-suppressed spleen cells suppres-

Responder Strain	I.V. Immunization + I.P. Immunization	S.C. Immunization and Challenge	Incompatibility	% Specific Increase Foot Thickness	Suppression
C57BL/6	C57BL/6	DBA/2	H-2 + non-H-2		
C57BL/6	DBA/2	DBA/2	H-2 + non-H-2		72 %
BALB.B	BALB.B	C57BL/6	non-H-2		
BALB.B	C57BL/6	C57BL/6	non-H-2		75 %
C57BL/6	C57BL/6	B10.D2	H-2		
C57BL/6	B10.D2	B10.D2	H-2		80 %
B10.D2 ♀	B10.D2 ♀	B10.D2 ♂	H-Y		
B10.D2 ♀	B10.D2 ♂	B10.D2 ♂	H-Y		79 %
B10.A	B10.A	B10.AQR	H-2K		
B10.A	B10.AQR	B10.AQR	H-2K		87 %
B10.AQR	B10.AQR	B10.T(6R)	H-2I		
B10.AQR	B10.T(6R)	B10.T(6R)	H-2I		77 %
B10.A	B10.A	B10.BR	H-2S,D		
B10.A	B10.BR	B10.BR	H-2S,D		86 %
B10.A	B10.A	B10.A(5R)	H-2K,I-A		
B10.A	B10.A(5R)	B10.A(5R)	H-2K,I-A		78 %

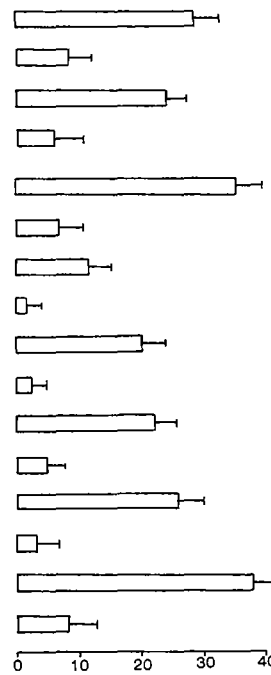

Fig. 1. Induction of suppression of DTH to alloantigens using Lie's protocol. Groups of mice were i.v. injected with 1.5 x 10^8 iradiated (20 Gy) allogeneic spleen cels as indicated. Control mice received syngeneic spleen cells. Six days later allo mice were i.p. injected with 2.0 x 10^8 of the relevant, irradiated spleen cells. Another four days later all mice were i.p. injected with Cy (100 mg/kg). Two days later all mice were s.c. injected with 1 x 10^8 of the relevant, irradiated allogeneic spleen cells to induce DTH. Another five days later all mice were s.c. injected into the right hind foot with 1 x 10^7 of the relevant unirradiated allogeneic spleen cells. Each bar represents the mean percentage specific increase of foot-thickness $\pm$ 1 SEM (n=5). The percentage suppression was calculated as the percentage decrease of foot-thickness as compared to the relevant DTH control group.

sed the ILT reactivity antigen specifically (Chapter IV.2). The suppressive activity of the Ig preparation was adsorbed by incubation with syngeneic antigen-specific immune lymphocytes. This indicates that the antibody is directed towards the antigen receptor of DTH reactive T cells. Such antibodies are called anti-idiotypic.

B cell mediated suppression of murine DTH has been described by other investigators as well. For instance, Colizzi et al. (1983) reported that anti-idiotypic antibodies from the serum of BCG infected mice suppressed

DTH to PPD. Benedettini et al. (1984) showed that suppressor B cells induced by S. aureus inhibited CHS to oxazolone. Campa et al. (1986) reported that BCG infection induced idiotypic B cells, whose interaction with anti-idiotypic B cells resulted in the activation of antigen specific Ts cells that suppressed DTH to BCG. In view of the observed suppression of DTH to third party alloantigens Campa's model might be applicable to ours. This remains to be investigated.

In Chapter IV, section 3 we describe that the development of DTH under GvH conditions can also be suppressed by LPS-induced suppressor cells. The suppression was found to be antigen-specific, but again anti-host DTH to third party alloantigens could be suppressed as well. This occurred when the third party alloantigens were inherited and expressed by the irradiated host together with the alloantigens that, in combination with LPS, had induced the suppression. Suppression after i.v. injection of donor mice with allogeneic spleen cells induced a Ts cell-mediated suppression of anti-host DTH (Bril et al., 1985). However, LPS-induced suppression of anti-host DTH was mediated by B cells, just like LPS-induced suppression of DTH under HvG conditions. Thompson et al. (1978) reported that the induction of GvH-disease by infusion of lymphoid cells into allogeneic neonatal mice could be suppressed by the injection of serum obtained from LPS-treated mice that were syngeneic to the donors of the GvH-inducing lymphoid cells. This might suggest a role of antibody in the LPS-induced suppression of anti-host DTH, perhaps similar to the role of antibody we found in the LPS-induced suppression of DTH under HvG conditions.

From the studies described in Chapter IV the following model for the LPS-induced suppression of DTH was developed. The stimulation of anti-idiotypic B cells by the receptors of the DTH reactive T cells and the mitogenic moiety of LPS accounts for clonal expansion of the activated anti-idiotypic B cells. These B cells subsequently secrete the anti-idiotypic antibodies that suppress the DTH reactive T cells antigen-specifically.

In the chapters discussed so far, suppression was induced by protocols involving some kind of immunization. In Chapter V, section 1, we describe that exposure of mice to ultraviolet-B (UV-B) irradiation can also induce suppression of DTH. The suppression rapidly developed after UV-B irradiation, reached its maximum at day 4, and thereafter gradually waned. Reexposure of the mice could easily reinduce the state of suppression.

In adoptive transfer experiments it was found that the UV-B induced suppression is mediated by two populations of splenic Ts cells: one expressing the $L3T4^{+}$,$Lyt\text{-}1^{+}2^{-}$ phenotype, the other expressing the $L3T4^{-}$,$Lyt\text{-}1^{-}2^{+}$ phenotype. Together, they suppressed the induction phase but not the expression phase of DTH. Possibly, the former cell type acts as a Ts ind cell that activates the latter cell type. Since these Ts cells were induced without any immunization they are obviously antigen non-specific. Ullrich (1985; 1986) described suppression of MLR as well as suppression of hapten-specific lymphoproliferation in vitro by UV-B induced Ts cells. Others described systemic suppression of CHS of mice to certain chemicals by UV-B irradiation (Noonan et al., 1981; Kripke, 1984). It might well be that in our studies antigen-specific Ts cells are generated subsequently to the s.c. immunization of the UV-B irradiated mice with allogeneic spleen cells for inducing a state of DTH. Such antigen-specific Ts cells might ultimately suppress the development of DTH reactive T cells.

A major question in this research area has been the relationship between the UV-B irradiation of the skin and the appearance of Ts cells in the spleen. Effects on skin-associated antigen-presenting-cells (Langerhans

cells) have been suggested (Fox, 1983). However, Noonan et al. (1984) observed a discrepancy between the wave length dependency of the induction of suppression of CHS and of the effects of UV-B on Langerhans cells. Another possibility, which we explored in Chapter V, section 2, is the release of soluble factor(s) from the skin that reach the spleen via the bloodstream and there might activate Ts cells. Indeed, we found evidence for such factor(s). Shortly (3 hrs) after UV-B irradiation we could detect suppressive activity in the serum of the irradiated mice. Infusion of 0.75 ml of such serum induced splenic suppressor cells that suppressed a subsequently induced DTH response. The serum factor(s) could induce a state of suppression in MHC-incompatible recipients. In contrast, the UV-B induced Ts cells did not exert their activity across a MHC barrier.

With regard to the i.v. and the UV-B-induced suppression of DTH clinical data have been reported suggesting their effectiveness in humans. For instance, allogeneic blood transfusion is generally accepted to be beneficial to the outcome of kidney allograft survival in man (Opelz et al., 1973; 1983; Persijn et al., 1979).

Studies on the effects of UV-B irradiation on human skin revealed that after UV-B irradiation the number of Langerhans cells at the irradiated site is decreased (Aberer et al., 1981). Moreover, Scheibner et al. (1983) found that skin from sun-exposed parts of the body contained significantly less Langerhans cells than the skin from protected parts of the body. Hersey et al. (1983) reported that healthy volunteers, exposed to a 2 week course of exposure to UV-A and UV-B solarium irradiation, showed impaired CHS responses to DNCB. Whether this impaired immune responsiveness and the effects on Langerhans cells are related remains to be investigated.

To my knowledge, clinical data on the effects of LPS on immune responses in humans have not been reported yet.

In conclusion, the studies described in this thesis indicate that DTH is a valuable model to monitor the activity of various types of suppressor cells. Moreover, it is clear that DTH to H antigens is highly susceptible for suppressor cell activity. Future research should be aimed at veryfying the effect of these suppressor cells on the activity of Tc cells and the outcome of transplantations.

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SUMMARY

In this thesis three modes of induction of suppression of delayed-type hypersensitivity (DTH) to histocompatibility (H) antigens in mice are described, namely (1) intravenous (i.v.) immunization or hind-foot-immunization with allogeneic spleen cells; (2) simultaneous i.v. immunization with bacterial lipopolysaccharide (LPS) and s.c. immunization with allogeneic spleen cells; and (3) exposure to ultraviolet-B (UV-B) irradiation.

In Chapter III the induction of suppression by immunization with alloantigens is described. I.v. immunization via a tail vein as well as s.c. immunization in the hind feet are shown to induce suppression of DTH. Section 1 of Chapter III presents evidence that several T suppressor (Ts) subpopulations are involved in the suppression of DTH to H antigens, namely, a population of Ts inducer (Ts ind) cells and a population of Ts effector (Ts eff) cells. Ts ind cells can be obtained from the spleen of mice one day after i.v. immunization with 5×10^7 irradiated allogeneic spleen cells. Their activity becomes obvious when recipient mice are s.c. immunized with the relevant allogeneic spleen cells to induce DTH three days after receiving a Ts ind cell-containing spleen cell suspension. Ts ind cells express the Lyt-1^+2^- phenotype and can induce a state of suppression in MHC and Igh incompatible recipients.

These studies are extended in Chapter III, section 2. It is shown that the Ts ind cells are antigen-specific and express the L3T4 cell surface marker. In contrast, Ts eff cells typed L3T4$^-$. Adoptively transferred Ts ind cells preferentially interact with recipient-derived precursors of Ts eff cells. When such precursors are not available (e.g., when Ts ind cells are adoptively transferred into nude mice) they can activate simultaneously transferred donor-derived Lyt-2^+ T cells, which results in suppression of DTH. Elimination of Lyt-2^+ cells from the Ts ind cell-containing inoculum prior to adoptive transfer to MHC and Igh incompatible recipients did not prevent induction of a state of suppression in these recipients. Thus, Ts ind cells can interact with precursors of Ts eff cells in a MHC and Igh unrestricted manner.

In Chapter III, section 3, it is shown that s.c. immunization of mice into the hind feet (hind-foot-immunization: HFI) with an immunogenic dose of allogeneic spleen cells induces a Ts cell mediated state of suppression of DTH. The Ts cells express the Lyt-1^-2^+ phenotype and suppress the induction phase but not the expression phase of DTH. They are antigen-specific, but depending on the experimental conditions they can also suppress DTH to third party alloantigens. This occurs when the third party alloantigens are administered during the induction phase, but not the expression phase of DTH, together with the alloantigens that had induced the suppression. The HFI-induced Ts cells appear MHC restricted in their activity. After adoptive transfer they can exert their effect in allogeneic recipients, provided these recipients share the H-2D locus with the donors of the HFI-induced Ts cells.

Chapter IV describes the characteristics of the suppression of DTH induced by simultaneous immunization with LPS and allogeneic spleen cells. The optimal protocol for the induction of this form of suppression is 100 ug LPS injected i.v., followed within 2 hours by a s.c. immunization with

1 x 10^7 allogeneic spleen cells (Chapter IV, section 1). The state of suppression lasts at least 60 days and affects both phases of DTH. The LPS-induced suppression is cell-mediated and antigen-specific. However, the DTH response to third party alloantigens can be suppressed as well. This occurs when the latter antigens are administered during either phase of DTH together with the alloantigens that, together with LPS, had induced the suppression.

In Chapter IV, section 2, it is shown that in spite of the long-lasting nature of the suppression, suppressor cells cannot be detected in the spleen later than 12 days after LPS treatment. Moreover, it is shown that the induction of suppression is dependent on the mitogenic activity of LPS delivered by its lipid A part. The LPS-induced suppression is mediated by B cells. These B cells secrete immunoglobulins (Ig) that suppress immune-lymphocyte-transfer reactivity. The immunosuppressive activity of the Ig preparation is greatly reduced after adsorbtion to antigen-specific DTH reactive T cells, but not after adsorbtion to the relevant alloantigens or to DTH reactive T cells with third party specificity. This indicates that the suppressive Ig specifically binds to the antigen receptor of DTH reactive T cells.

B cells activated by simultaneous treatment of donor mice with LPS and allogeneic spleen cells can also suppress anti-host DTH which normally develops under graft-versus-host conditions (Chapter IV, section 3). This B cell mediated suppression of anti-host DTH is antigen-specific. However, anti-host DTH to third party alloantigens can be suppressed as well. This occurs when the latter alloantigens are inherited and expressed by the irradiated recipients together with the alloantigens that, together with LPS, had induced the suppression.

The above described methods to induce suppression of DTH both employ some form of immunization. In Chapter V the induction of non-specific suppression by a method not involving immunization is described, namely exposure of the skin of mice to UV-B irradiation. This treatment induces a Ts cell mediated state of suppression, which is maximal at day 4 after UV-B exposure (Chapter IV, section 1). Thereafter the suppression gradually wanes and has disappeared at day 21. Reexposure of the mice to UV-B rapidly reactivates the suppression. The Ts cell population mediating the UV-B induced suppression of DTH is heterogeneous, since both $L3T4^+$,$Lyt\text{-}1^+2^-$ Ts cells and $L3T4^-$,$Lyt\text{-}1^-2^+$ Ts cells are involved. These Ts cells suppress the induction phase but not the expression phase of DTH to H antigens.

In Chapter V, section 2, it is shown that the UV-B induced Ts cells are activated by soluble serum factor(s), released from the skin in response to UV-B exposure. These factor(s) can be detected at 3 hours after UV-B exposure, but not at later time points. They can induce a state of suppression in MHC incompatible recipients. The UV-B induced Ts cells, on the other hand, cannot exert their activity in MHC-incompatible recipients.

In conclusion, these investigations show that DTH to H antigens can be regulated by several populations of suppressor cells that can be induced by various modalities. These suppressor cell populations may differ with regard to their characteristics and their mode of action.

SAMENVATTING

In dit proefschrift worden drie methoden voor de inductie van suppressie van vertraagd-type overgevoeligheid (VTO; in het Engels: DTH) tegen histocompatibiliteitsantigenen bij de muis beschreven, namelijk (1) intraveneuze (i.v.) of subcutane (s.c.) immunisatie met allogene miltcellen; (2) gelijktijdige i.v. immunisatie met bacterieel lipopolysaccharide (LPS) en s.c. immunisatie met allogene miltcellen; en (3) blootstelling aan licht van de ultraviolet-B (UV-B) golflengte.

In hoofdstuk III wordt de inductie van suppressie door immunisatie met alloantigenen beschreven. Zowel i.v. immunisatie via een staartvene als s.c. immunisatie in de achterpoten blijkt suppressie van DTH te induceren. Sectie 1 van hoofdstuk III beschrijft experimenten die aantonen dat meerdere T suppressor (Ts) subpopulaties betrokken zijn bij de suppressie van DTH, namelijk, een populatie Ts inducer (Ts ind) cellen en een populatie Ts effector (Ts eff) cellen. Ts ind cellen zijn één dag na i.v. immunisatie van muizen met 5×10^7 bestraalde allogene miltcellen aantoonbaar in de milt. De activiteit van deze cel wordt duidelijk wanneer zulke recipiënten s.c. geïmmuniseerd worden met de relevante allogene miltcellen drie dagen na de infusie van de Ts ind cel bevattende miltcelsuspensie. Ts ind cellen hebben het $Lyt\text{-}1^+2^-$ fenotype en kunnen een staat van suppressie induceren in recipiënten die niet identiek zijn t.a.v. hun MHC- en/of Igh loci.

Uitgebreider onderzoek naar deze Ts ind cel staat beschreven in hoofdstuk III, sectie 2. Hierin wordt aangetoond dat de Ts ind cel antigeen-specifiek is en de L3T4 membraanmarker tot expressie brengt. Daarentegen zijn Ts eff cellen $L3T4^-$. Getransfereerde Ts ind cellen gaan bij voorkeur een interactie aan met voorlopers van Ts eff cellen die van de recipiënt afkomstig zijn. In het geval dat zulke voorlopers niet beschikbaar zijn (bijv. als de Ts ind cellen bij nude muizen worden ingespoten) kunnen de Ts ind cellen tegelijkertijd getransfereerde $Lyt\text{-}2^+$ cellen van donor origine activeren, wat uiteindelijk leidt tot suppressie van DTH. Eliminatie van $Lyt\text{-}2^+$ cellen uit een miltcelsuspensie met daarin Ts ind cellen kan de inductie van suppressie in MHC en Igh incompatibele recipiënten niet voorkomen. Dus de interactie van Ts ind cellen met Ts eff cellen is niet onderhevig aan MHC of Igh restrictie.

Hoofdstuk III, sectie 3, beschrijft dat een s.c. immunisatie van muizen in hun achterpoten (HFI) met een immunogene dosis (1×10^7) allogene miltcellen een door Ts cellen gemedieerde staat van suppressie van DTH induceert. Deze Ts cellen hebben het $Lyt\text{-}1^-2^+$ fenotype en onderdrukken de inductiefase, maar niet de expressiefase van DTH. Zij zijn antigeen-specifiek, maar kunnen ook de DTH reactie tegen 'third party' alloantigenen onderdrukken. Dit gebeurt wanneer de 'third party' alloantigenen toegediend worden tijdens de inductiefase, maar niet tijdens de expressiefase, van DTH tezamen met de alloantigenen waarmee de suppressie geïnduceerd was. De door HFI geïnduceerde Ts cellen zijn MHC gerestricteerd. Na transfer kunnen zij hun effect in allogene recipiënten alleen uitoefenen, als deze een H-2D locus bezitten dat identiek is aan dat van de donor van de Ts cellen.

In hoofdstuk IV worden de karakteristieken beschreven van de suppressie van DTH die geïnduceerd wordt door gelijktijdige immunisatie met LPS en allogene miltcellen. Het optimale protocol voor de inductie van deze vorm van suppressie is een i.v. injectie van 100 ug LPS, binnen twee uur gevolgd

door een s.c. immunisatie met 1 x 10^7 allogene miltcellen (hoofdstuk IV, sectie 1). De staat van suppressie duurt ten minste 60 dagen en grijpt op beide fasen van DTH aan. De door LPS geïnduceerde suppressie wordt door cellen gemedieerd en is antigeen-specifiek. Echter, de DTH response tegen 'third party' alloantigenen kan eveneens onderdrukt worden, mits deze worden toegediend tijdens de inductiefase of de expressiefase van DTH tezamen met de alloantigenen waarmee, tezamen met LPS, de suppressie geïnduceerd was.

Hoofdstuk IV, sectie 2, beschrijft dat, hoewel de door LPS geïnduceerde suppressie langdurig van aard is, de suppressorcellen toch niet langer dan 12 dagen na LPS-behandeling in de milt aantoonbaar zijn. Verder blijkt de inductie van suppressie afhankelijk te zijn van de mitogene activiteit van LPS, die door het lipid A gedeelte van het LPS veroorzaakt wordt. De door LPS geïnduceerde suppressor cellen zijn B cellen. Deze B cellen scheiden immunoglobulinen (Ig) uit die de 'immune-lymphocyte-transfer' reactiviteit van DTH reactieve T cellen kunnen onderdrukken. De immuunsupprimerende activiteit van deze Ig is verminderd na adsorbtie aan antigeen-specifieke DTH reactieve T cellen, maar niet na adsorbtie aan de betreffende alloantigenen of aan DTH reactieve T cellen specifiek voor 'third party' alloantigenen. Dit toont aan dat het supprimerende Ig specifiek is voor de antigeenreceptor van de DTH reactieve T cellen.

Hoofdstuk IV, sectie 3, beschrijft dat B cellen, die door de gelijktijdige behandeling van donor muizen met LPS en allogene miltcellen geactiveerd worden, ook de anti-gastheer DTH respons onderdrukken die normaliter optreedt na infusie van immuuncompetente miltcellen in letaal bestraalde allogene recipiënten. De suppressie van de anti-gastheer DTH is antigeen-specifiek, terwijl eveneens de anti-gastheer DTH tegen 'third party' alloantigenen onderdrukt kan worden. Dit gebeurt alleen wanneer deze door de bestraalde recipienten geërfd worden en tot expressie worden gebracht tezamen met de alloantigenen die, tezamen met LPS, de B cellen geactiveerd hadden.

De hierboven beschreven methoden om suppressie van DTH te induceren maken beide gebruik van één of andere vorm van immunisatie. In hoofdstuk V wordt een methode voor de inductie van niet-specifieke suppressie beschreven waarbij niet van immunisatie gebruik wordt gemaakt, namelijk blootstelling van de huid van muizen aan licht van de UV-B golflengte. Deze behandeling induceert een door Ts cellen gemedieerde suppressie, die optimaal is op dag 4 na bestraling (hoofdstuk V, sectie 1). Daarna neemt de intensiteit van de suppressie geleidelijk af. De suppressie en is niet meer aantoonbaar op dag 21. Hernieuwde blootstelling aan UV-B induceert opnieuw suppressie. De Ts celpopulatie die door UV-B bestraling geïnduceerd wordt is heterogeen. Zowel $L3T4^+$,$Lyt\text{-}1^+2^-$ Ts cellen als $L3T4^-$,$Lyt\text{-}1^-2^+$ Ts cellen spelen een rol. Deze cellen onderdrukken de inductiefase, maar niet de expressiefase, van DTH tegen H alloantigenen.

In hoofdstuk V, sectie 2, wordt beschreven dat de door UV-B geïnduceerde Ts cellen geactiveerd worden door oplosbare serumfactoren die door cellen in de huid uitgescheiden worden als reactie op blootstelling aan UV-B. Deze factoren komen drie uur na bestraling in het serum van bestraalde muizen voor, maar niet op latere tijdstippen, en kunnen een staat van suppressie induceren in MHC incompatibele recipiënten. In tegenstelling tot de serumfactor(en) kunnen de door UV-B geïnduceerde Ts cellen hun effect niét uitoefenen in MHC incompatibele recipiënten.

Geconcludeerd kan worden dat DTH tegen alloantigenen gereguleerd kan worden door meerdere populaties suppressorcellen die langs verschillende wegen geïnduceerd kunnen worden. Zowel de eigenschappen als het aangrijpingspunt van deze suppressorcel populaties kunnen verschillen.

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CURRICULUM VITAE

Schrijver dezes werd op 14 april 1961 geboren te Rotterdam. Een voorspoedige kleuter- en basisschool-carrière (respectievelijk Prinses Margrietschool en Dr. de Visserschool) te Pernis werd gevolgd door een even voorspoedige middelbare schooltijd aan de Christelijke Scholengemeenschap 'Maarten Luther' te Rotterdam. Na het behalen van het VWO-diploma (juni 1979) en een gelukkige loting werd in augustus 1979 begonnen met de studie Geneeskunde aan de Erasmus Universiteit te Rotterdam. Het Propedeutisch examen werd in augustus 1980 behaald. Dit werd in juli 1982 gevolgd door het Kandidaatsexamen. Als onderdeel van dit examen werd van februari 1982 tot en met juni 1982 onderzoek verricht naar de herstelcapaciteit van sporters na anaërobe inspanning, onder leiding van Dr. David L. Costill, Human Performance Laboratory, Ball State University, Muncie, Ind, U.S.A. Tijdens dit onderzoek werden ook de eerste schreden op het (aërobe) marathonpad gezet. Na het behalen van het Doctoraalexamen (augustus 1984) werd in september 1984 een aanstelling als onderzoekmedewerker van de Nederlandse Organisatie voor Zuiver-Wetenschappelijk Onderzoek verkregen. In deze hoedanigheid werd van september 1984 tot en met september 1987 onder leiding van Prof. Dr. R. Benner op de afdeling Celbiologie, Immunologie en Genetica van de Erasmus Universiteit te Rotterdam het experimentele werk verricht dat in dit proefschrift beschreven staat.

ABBREVIATIONS

ABA	azobenzene arsonate
BCG	Bacilles-Calmette-Guerin
BSS	balanced salt solution
C	complement
CHS	contact hypersensitivity
cpm	counts per minute
^{51}Cr	^{51}Cromium
Cy	cyclophosphamide
DNCB	dinitrochlorobenzene
DNFB	dinitrofluorobenzene
DTH	delayed-type hypersensitivity
FCS	fetal calf serum
GvH	graft-versus-host
H	histocompatibility
HFI	hind-foot-immunization
HSV	Herpes Simplex Virus
^{3}H-TdR	tritiated thymidine
HvG	host-versus-graft
Ig	immunoglobulin
ILT	immune-lymphocyte-transfer
^{125}I-UrD	^{125}iodinated uridine
i.p.	intraperitoneal
i.v.	intravenous
LPS	lipopolysaccharide
MHC	major histocompatibility complex
MLC	mixed-lymphocyte-culture
MLR	mixed-lymphocyte-reactivity
NLT	normal-lymphocyte-transfer
NP	4-hydroxy-3-nitrophenylacetyl
PBS	phosphate-buffered saline
PFC	plaque-forming cell
PLN	popliteal lymph node
PPD	purified protein derivative
RT	room temperature
ShUV-B	sham ultraviolet-B
SRBC	sheep red blood cells
s.c.	subcutaneous
Tc	T cytotoxic
Th	T helper
Tk	T killer
TNCB	trinitrochlorobenzene
Ts	T suppressor
Ts ind	T suppressor inducer
Ts eff	T suppressor effector
Ts trans	T suppressor transducer
UV-B	ultraviolet-B