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Inhibition of Antiskin Allograft Immunity Induced by Infusions with *P*hotoinactivated *E*ffector *T* Lymphocytes (PET Cells)

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Induction of tolerance for skin allotransplantation requires selective suppression of the host response to foreign histocompatibility antigens. This report describes a new approach which employs pre-treatment with 8-methoxypsoralen (8-MOP) and ultraviolet A light (UVA) to render the effector cells of graft rejection immunogenic for the syngeneic recipient. Eight days after BALB/c mice received CBA/j skin grafts, their splenocytes were treated with 100 ng/ml 8-MOP and 1 J/cm^2 UVA prior to reinfusion into naive BALB/c recipients. Recipient mice were tested for tolerance to alloantigens in mixed leukocyte culture (MLC), cytotoxicity (CTL), delayed-type hypersensitivity assays (DTH), and challenge with a fresh CBA/j graft.

Splenocytes from BALB/c recipients of photoinactivated splenocytes containing the effector cells of CBA/j alloantigen rejection proliferated poorly in MLC and generated lower cytotoxic T-cell responses to CBA/j alloantigens in comparison with sensitized and naive controls and suppressed the MLC and CTL response to alloantigen from sensitized and naive BALB/c mice. *In vivo,* the DTH response was specifically suppressed to the relevant alloantigen in comparison with controls. BALB/c mice treated in this fashion retained a CBA/j skin graft for up to 42 days post-transplantation without visual evidence of rejection. These results showed that reinfusion of photoinactivated effector cells resulted in an immunosuppressive host response which specifically inhibited *in vitro* and *in vivo* responses that correlate with allograft rejection and permitted prolonged retention of histoincompatible skin grafts.

The capacity selectively to down-regulate aberrant T-cell populations is an important goal of immunotherapy. Efforts in that direction have been made in a laboratory model of autoreactive disease in which cloned anti-myelin basic protein T cells induce experimental autoimmune encephalomyelitis (EAE) in susceptible strains of rodents [1]. Cohen et al. [2,3] have shown that, if these pathogenic cloned T cells are extracorporeally inactivated with methods that alter membrane fluidity (such as hydrostatic pressure) and then intravenously reinfused, they can immunoprotect syngeneic animals in a clonotypic manner against the same autoreactive disease.

Among the obstacles that currently preclude application of that approach to

Abbreviations: AMLC: autologous mixed leukocyte culture Con A: concanavalin A CTCL: cutaneous T-cell lymphoma CTL: cytotoxicity assay DTH: delayed-type hypersensitivity response EAE: experimental autoimmune encephalomyelitis 8-MOP: 8-methoxypsoralen MLC: mixed leukocyte culture PBS: phosphate saline buffer PET: photoinactivated effector T cells Tc: effector T cells of cytotoxic response TCR: T-cell receptor Tdth: effector T cells of delayed-type hypersensitivity response UVA: ultraviolet A light

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treatment of human diseases reflecting increased numbers or activity of pathogenic T cells are the difficulty in isolation, *in vitro* expansion, and in identifying such aberrant clones. Finally, it would be necessary that the mode of cell treatment be less systemically toxic than standard therapies currently available.

Recently, we have reported that cutaneous T-cell lymphoma (CTCL) patients who received intravenous reinfusion of mononuclear cells which have been extracorporeally exposed to 8-methoxypsoralen in the presence of ultraviolet A light (UVA), demonstrated a profound and prolonged clinical response which appears to be immunologically mediated [4]. It is noteworthy that systemic toxicity was quite limited. If it can be demonstrated that this clinically practical therapy leads to a specific regulatory T-cell response, a potentially significant therapeutic principle may have been recognized: unfractionated populations of autologous mononuclear cells containing an expanded clone(s) of pathogenic T cells can immunize against the activity of the same clone(s) if extracorporeally altered in an appropriate fashion.

After the introduction of a histoincompatible tissue transplant, populations of delayed-type hypersensitivity (Tdth) and cytotoxic effector T cells (Tc) are expanded, antibody directed against the graft antigens is produced. This immune response leads to the destruction and rejection of the transplanted tissue [5]. Class I and Class II cell-surface molecules encoded by genes within the major histocompatibility complex serve as the main targets of allogeneic responses between genetically unrelated individuals [6]. Tdth responses are largely directed at Class II antigens [7], and Tc are mainly directed at Class I molecules [8]. The antigen-binding receptor for target Class I and II molecules is the relevant T-cell receptor (TCR) [9].

Prolonged survival of skin allografts is difficult to achieve due to the particularly vigorous host immune response to histoincompatible antigens associated with this organ. Therefore, the efficacy of protocols intended to inhibit the effector T cells mediating rejection of skin allografts can be demonstrated in this sensitive system. Andersson, Binz, and Wigzell [10] have reported that specific immune unresponsiveness can be induced *in vivo* in rodents, by immunizing the animal with autologous antigen-specific lymphoblasts obtained after *in vitro* sensitization, fractionation, and emulsification in Freund's complete adjuvant. Thus, autoimmunization against clonotypic determinants, presumably of the TCR for a given potent antigen, may constitute an efficient way of producing specific immune tolerance in immunocompetent adult animals.

To inhibit selectively the response to alloantigen, we exploited the observation that the antigen receptor of effector T cells can be immunogenic and that specific reactions against TCR can suppress its function [11]. In order to inhibit the proliferation of the effector cells of allogeneic responses, we selected a pharmacologic method of photoinactivation by treating the cells with 8-methoxypsoralen (8-MOP) and ultraviolet A light (UVA), which would permit retention of an intact cell membrane. 8-MOP, a naturally occurring furocoumarin, has the remarkable capacity of being activated by UVA from a biologically inert form to one capable of forming covalent bonds with pyrimidine bases of DNA [12]. The DNA effect induced by 8-MOP is analogous to the chemical impact of a bifunctional alkylating agent like mitomycin C. Psoralen photoaddition can be more exquisitely titrated, however, by altering both drug level and UVA intensity. We have previously established conditions for UVA-/8-MOP-induced DNA cross-links to inhibit mitosis by murine mononuclear cells maximally while simultaneously sparing the lymphocytes' capacity to serve as stimulators [13], in

MLC. This procedure requires an irradiance of 1 joule/cm^2 UVA in the presence of 100 ng/ml of 8-MOP.

The following experiments were conducted to determine whether pre-exposure of BALB/c mice immune system to splenocytes obtained from other BALB/c mice, undergoing rejection of CBA/j skin grafts and inactivated with 8-MOP photoactivated by UVA light, would render the syngeneic recipients unresponsive to the CBA/j alloantigens as tested by *in vitro* and *in vivo* assays, which correlate with allograft rejection. In this report, we show that pre-exposure of the mouse immune system to cells generated during skin transplant rejection and photoinactivated with 8-MOP and UVA can be one way to render the secondary syngeneic recipients relatively tolerant to the alloantigen carried by the original skin transplant.

MATERIALS AND METHODS

Mice

BALB/cBYJ (H-2^d, Mls^b), CBA/j (H-2^k, Mls^d), and C57BL/10 (H-2^b, Mls^b) mice were purchased from The Jackson Laboratory, Bar Harbor, ME. All mice were maintained in a specific pathogen-free facility. Female CBA/j mice four to six weeks of age were used as skin transplant donors. Female BALB/c mice four to six weeks of age were the skin transplant recipients. C57BL/10 (H-2^b, Mls^b) female mice were the donors of splenocytes used as the irrelevant alloantigen control in *in vitro* and *in vivo* experiments.

Skin Transplantation and Preparation of Cell Suspension

The skin grafting was performed following the procedure of Billigham and Medawar [14]. The truncal skin of donor mice (CBA/j) was shaved and the animals were sacrificed. Full-thickness skin was obtained by sharp dissection of truncal skin, followed by removal of fat and panniculus carnosus by blunt dissection. Skin graft recipient mice (BALB/c) were shaved and anesthetized intramuscularly with .01 ml of Ketamine hydrochloride (100 mg/ml), diluted 10:1 with Xylasine (20 mg/ml). A circular piece of skin measuring approximately 1 × 1 cm was removed from each recipient mouse down to fascia from the grafting site. The grafts were applied to the fascia and sutured with Vycril 3-0 suture (Ethicon, Johnson & Johnson Co., Somerville, NJ). Rejection was allowed to take place. Eight, 12, and 15 days after transplant, spleens were removed from the mice undergoing allograft rejection. Single-cell suspensions were prepared, and red cells were lysed with 0.83 percent NH_4Cl. After two washings, cells were adjusted to the appropriate concentration in phosphate saline buffer (PBS) for injections (see immunization protocol) or media (culture media consisting of RPMI 1640 supplemented with 20 mM Hepes buffer, 2 mM glutamine, 5 percent heat-inactivated fetal calf serum (FCS) (all from Gibco, Grand Island, NY), 5×10^{-5} M 2-mercaptoethanol, 100 μg/ml streptomycin, and 100 U/ml penicillin for assays (see MLC, Cytotoxicity Assay, and so on). The cell suspension was 89 percent viable as determined by trypan blue exclusion. Mean spleen cell yield was calculated for all splenocytes obtained from BALB/c mice undergoing CBA/j skin allograft rejection, and cytotoxicity assays were performed at the different time intervals mentioned (see cytotoxicity assay). Degree of rejection was defined as the product of mean cell yield and the highest cytotoxic response obtained. By this definition, the peak of rejection was considered to be eight days post-transplant.

Splenocytes obtained from these transplanted (sensitized) mice served as a source of the immunizing population of cells.

Photoinactivation

Optimal conditions for murine splenocyte photoinactivation, as determined by their proliferative response to nonspecific mitogens, has been reported elsewhere [13]. 8-MOP (Elder Pharmaceutical, Costa Mesa, CA) was dissolved in 100 percent ethanol and subsequently in PBS. Splenocytes from BALB/c mice rejecting CBA/j skin graft were incubated in 100 ng/ml of 8-MOP in shielded tubes for 20 minutes. The cell suspension was dispensed into Petri dishes (Falcon, Becton Dickinson & Co., Oxnard, CA) and exposed to 1 J/cm^2 of UVA. The irradiation unit consisted of six black light bulb fluorescent tubes (40 Sylvania), emitting broad-band UVA energy (320–400 nm). Emission in the UVB range was filtered out by a sheet of window glass. Light dose was monitored by a photometer (International Light, IL 700A), equipped with a UVA probe. The cell suspension was aseptically collected and washed twice with PBS to remove the excess 8-MOP.

Immunization Protocol

Recipient mice were injected intravenously in the tail vein with 200 μl of PBS containing 30–50 $\times$ 10^6 cells. Among these cells was the *in vivo* expanded population of *p*hotoinactivated *e*ffector *T* (PET) cells mediating CBA/j skin allograft rejection. BALB/c recipients were injected once every week for three months. Recipient mice were then tested for T-cell unresponsiveness to CBA/j alloantigens in mixed leukocyte culture (MLC), cytotoxicity (CTL), and delayed-type hypersensitivity (DTH) assays and challenged with fresh CBA/j skin transplants.

MLC

MLC responses were performed following modifications of the methods of Rock et al. [15] and Kruisbeek et al. [16] and represent the *in vitro* proliferative response to histoincompatible Class II alloantigens [17]. Erythrocyte-free X-irradiated (1,800 rads) skin donor stimulator splenocytes (CBA/j) were suspended in media and aliquoted at a concentration of 2 $\times$ 10^5 cells/well in a volume of 50 μl. Increasing numbers of responder cells (1, 2, 4, and 8 $\times$ 10^5) from either naive, sensitized, or PET-treated BALB/c mice were cultured in triplicate with 2 $\times$ 10^5 irradiated stimulator cells at a final volume of 200 μl in wells of round-bottomed microtiter plates (Corning, Corning, NY). Plates were incubated at 37°C, in humidified atmosphere containing 5 percent CO_2. After four days in culture [18], the wells were pulsed with 1 μCi of ^{3}[H] thymidine for the final 18 hours of incubation. Cultures were harvested with automated culture harvester (MASH, Cambridge). Incorporation of radioactivity was measured by liquid scintillation counting. Data were presented as delta cpm, which represents the allogeneic stimulation response minus the response to autologous irradiated cells. Mean values of six experiments in triplicates are presented with standard error (SE). In the three-party inhibition experiments, increasing numbers of responder cells (1, 2, 4, and 5 $\times$ 10^5) from either naive, sensitized, or PET-treated BALB/c mice were suspended in media and co-cultured with stimulator cells. An equivalent amount of cells from either PET-treated or sensitized mice was the third

population of cells added to the wells. Cultures were incubated and harvested as previously described. Data were presented as delta cpm, which represents the allogeneic stimulation response minus the value obtained from the addition of the response to autologous irradiated cells from both populations of responder cells. Mean values of two to three experiments done in triplicate are given plus or minus SE. Percentage of inhibition was calculated by the formula: %Inhibition = [1 − (delta cpm of sensitized or naive + PET-treated responder cells/delta cpm of sensitized + naive responder cells)] × 100.

Cytotoxicity Assay

Cytotoxicity assays were performed to detect the presence of alloreactive cells primarily responsive to Class I alloantigen disparity [8]. Skin graft recipient, naive, and PET-treated BALB/c mice were sacrificed, and a mononuclear cell suspension of spleen cells was prepared. Splenocytes from graft recipient mice were restimulated *in vitro*, following the methods of Buracoff et al. [19] and Granstein et al. [20]. Briefly, 8×10^6 responder spleen cells were co-cultured with 3×10^6 erythrocyte-free 8-MOP-/UVA-inactivated skin donor strain (CBA/j) splenocytes in 24-well, flat-bottomed microtiter plates (Falcon). Cultures were incubated for five days at 37°C in a humidified atmosphere containing 5 percent CO_2. Cytotoxicity of effector cells was assessed by testing concanavalin A-(Con A) stimulated splenocytes from skin donor mice (CBA/j) or syngeneic mice (BALB/c) as targets. Con A blasts were prepared by two-day culture of 3×10^7 spleen cells in the presence of 2.5 μg/ml of Con A (Sigma Chemical Co., St. Louis, MO). Con A blasts were separated from non-viable cells by Lympholyte M (Accurate Chemical, Westbury, NY) gradient flotation. Viable Con A blasts were washed twice and used for ^{51}chromium labeling. Labeling of the blast cells was performed by incubating 3×10^7 recovered cells with 200 μl of $Na^{51}CrO_4$ (5 mCi/ml; Amersham Corp., Arlington Heights, IL) for 90 minutes, shaking the suspension every 15 minutes.

The chromium release assay was performed in flat-bottomed microtiter plates (Falcon, Becton Dickinson & Company, Oxnard, CA) by the addition of 1×10^4 ^{51}Cr-labeled target cells to 1, 2, 4, and 8×10^5 effector cells in a total volume of 200 μl of media. The plates were centrifuged (300 *g*, one minute) at the beginning of the assay, incubated for four to five hours at 37°C, and centrifuged again (500 *g*, 15°C, ten minutes). A volume of 100 μl of media was removed from each well and dissolved in 3 cc of Biocount (Research Products International Corp., Mt. Prospect, IL) for liquid scintillation counting. Percentage of specific ^{51}Cr release was calculated according to the formula $E\text{-}S/M\text{-}S \times 100$, in which E is the isotope release in the presence of effector cells, S is the spontaneous isotope release in the presence of medium, and M is the maximal release obtained by adding to target cells an equal volume of 1 percent Triton X-100. The results are reported as the mean percentage of specific release of three experiments performed in triplicate with spontaneous release of the isotope not higher than 20 percent. Inhibition experiments were performed by culturing 4×10^6 splenocytes from either naive or sensitized mice mixed with 4×10^6 splenocytes from PET-treated mice in the presence of 3×10^6 8-MOP/UVA-inactivated splenocytes from skin donor mice (CBA/j). Chromium-labeled targets were prepared as described above. Determination of the ^{51}Cr specific release was performed and reported as previously described.

TABLE 1
Autologous Mixed Leukocyte Response

Number of Responder Cells × 10^{5b} (H-2^d, Mls^b)	H-2 and Mls Haplotype[c] Stimulator Cells 2×10^5	CPM [^{3}H] thymidine (X + SE)[a] Naive BALB/c Mice $n = 3$	Sensitized BALB/c $n = 3$	PET tx BALB/c $n = 6$
1	H-2^d Mls^b	250 + 73	281 + 32	4,907 + 620
2	H-2^d Mls^b	806 + 39	1,630 + 293	32,045 + 3,811
4	H-2^d Mls^b	5,577 + 1,044	7,767 + 715	46,820 + 2,339
8	H-2^d Mls^b	14,980 + 787	15,087 + 627	45,147 + 5,044

[a]These results are the background values for the experiments presented in Fig. 1.
[b]The responder cell population (naive, sensitized, or PET × BALB/c splenocytes) were aliquoted at increasing concentrations of cells/well in triplicate and tested for [^{3}H] thymidine incorporation.
[c]X-irradiated splenocytes from naive BALB/c mice were aliquoted as stimulator cells at 2×10^5 cells/well.

Delayed-Type Hypersensitivity Response

DTH assay was performed following the methods of Granstein et al. [20] and Van der Kwast et al. [21] and represent *in vivo* allogenic responses to histoincompatible Class II alloantigens [7]. Spleen cell suspension from CBA/j mice ($30–50 \times 10^6$ X-irradiated splenocytes) were injected in the flank of five naive BALB/c mice (positive control group), five PET-treated BALB/c mice (experimental group 1), and five BALB/c mice treated with photoinactivated naive splenocytes (experimental group 2). For elicitation, the previously immunized groups of mice as well as five naive BALB/c mice (negative control group) received 10×10^6 CBA/j splenocytes injected subcutaneously in the right hind footpad in 0.20 ml of PBS. After 24 hours, both hind footpads were measured for thickness with a dial caliper (Monostat, Swiss-made Fisher Scientific, Springfield, NJ). The thickness of the left footpad (noninjected) was subtracted from the right (injected) footpad to measure the DTH response. Percentage of suppression was calculated by the formula: $1 - (E\text{-}N/P\text{-}N) \times 100$, where E is the measurement obtained from the experimental group of mice, N is the negative control, and P is the positive control. Specific suppression of the DTH response was determined by immunizing and challenging analogous groups of mice with splenocytes from C57Bl/10 mice (H-2^b).

Statistical Analysis

Statistical significance was determined according to student's t test. A p value of less than 0.05 was considered significant.

RESULTS

Specific Suppression of MLC

BALB/c female mice, immunized with syngeneic PET cells, demonstrated a highly significant ($p < 0.001$) autologous response (Table 1) at all numbers of responder cells tested and also showed a highly significant hyporesponsiveness to H-2^k, Mls^d alloantigen in MLC, when compared to the proliferative response of splenocytes obtained from either naive (Fig. 1a) or sensitized BALB/c mice. When splenocytes from

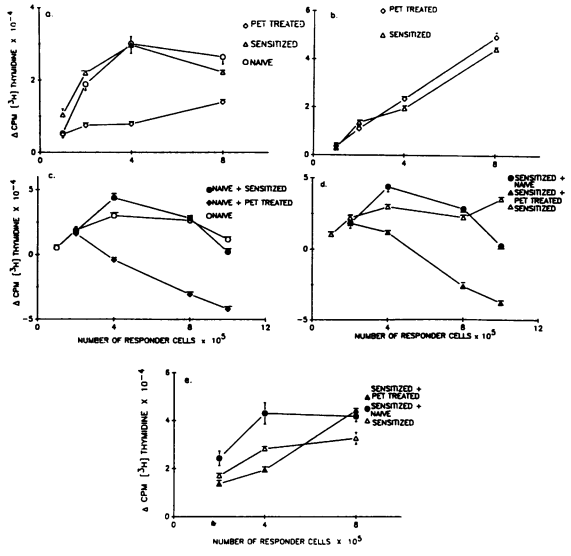

FIG. 1. Proliferative response in mixed leukocyte culture of splenocytes from BALB/c mice. The response of: **a,** PET-treated BALB/c splenocytes (◇) to H-2^k, Mls^d alloantigen expressed on CBA/j spleen cells compared to the proliferative response of naive (○) and sensitized (△) BALB/c spleen cells. **b,** PET-treated BALB/c splenocytes (◇) to an irrelevant alloantigen H-2^b, Mls^b, expressed on B10 stimulator splenocytes compared to sensitized BALB/c splenocytes (△). **c,** Naive BALB/c mice (○) to H-2^k, Mls^d alloantigen compared to splenocytes from naive BALB/c mice added in a 1:1 ratio to either sensitized (●) BALB/c splenocytes or PET-treated (◆) BALB/c splenocytes. **d,** Sensitized BALB/c splenocytes (△) to H-2^k, Mls^d compared to sensitized BALB/c splenocytes added 1:1 to either naive (●) or PET-treated (▲) BALB/c splenocytes. **e,** Sensitized BALB/c splenocytes (△) to an irrelevant alloantigen H-2^b, Mls^b compared to sensitized BALB/c splenocytes added in a 1:1 ratio to either naive (●) or PET-treated (▲) BALB/c splenocytes.

PET-treated BALB/c mice were exposed in MLC to H-2^b, Mls^b, however, an irrelevant alloantigen, the proliferative response was comparable to that of splenocytes from sensitized BALB/c mice (Fig. 1b). Therefore, specific hypoproliferative response in MLC to the relevant MHC antigens could be induced by immunization with PET cells. Thus, this specific hyporesponsiveness could be the result of deletion of the effector cell population mediating the recognition of the target alloantigen or inhibition of the effector cells mediated by a suppressive cell population in the spleens of PET-treated mice. Therefore, a three-party MLC was performed to determine whether the MLC hyporesponsiveness resulted from a deletion of the effector cell population or whether an inhibitory cell population had been generated (Fig. 1c, 1d). As anticipated, when splenocytes from sensitized BALB/c mice were admixed equally (1:1) with splenocytes from naive BALB/c mice and then stimulated with CBA/j, an augmented proliferative response, over that of naive BALB/c cells alone, was obtained. When splenocytes from mice pre-treated with PET cells were similarly admixed with

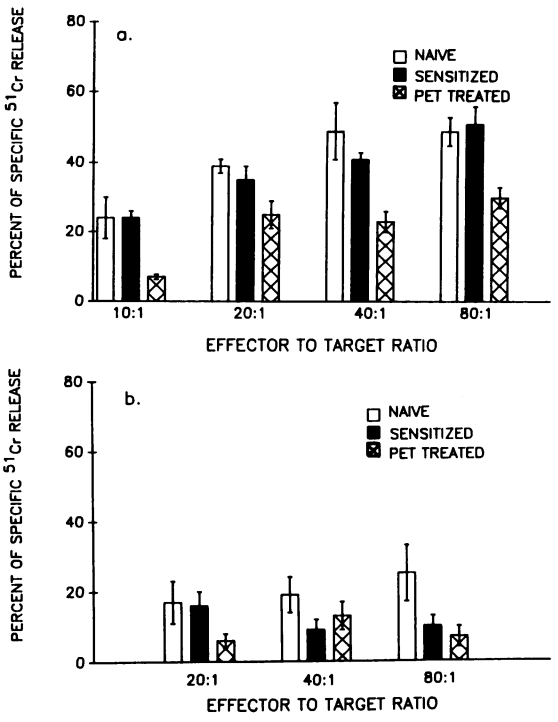

FIG. 2. Cytotoxic response of splenocytes from BALB/c mice. The generation of cytotoxic effector cells in splenocytes from: **a**, Sensitized BALB/c mice were re-exposed *in vitro* to H-2^k, Mls^d alloantigen. Naive BALB/c and PET-treated mice splenocytes were exposed *in vitro*, to H-2^k, Mls^d alloantigen and all splenocytes tested for lysis of ^{51}Cr-labeled CBA/j or **b.** BALB/c targets at increasing effector-to-target ratio.

cells from naive mice, however, the proliferative response was markedly suppressed (>100 percent) ($p < 0.001$) (Fig. 1c). Similar results were obtained after admixing the splenocytes from PET-treated mice with the cells from sensitized mice ($p < 0.001$) (Fig. 1d). When splenocytes from sensitized and PET-treated mice were admixed and exposed to X-irradiated cells bearing the irrelevant antigen H-2^b, Mls^b, however, some suppression was observed (lowest p value, <0.02) but not to the extent of the suppression demonstrated to the H-2^k, Mls^d alloantigen ($p < 0.001$) (Fig. 1e). Therefore, the diminution of MLC reactivity against donor antigens in splenocytes from PET-treated mice resulted from the presence of a specific inhibitory cell population.

Suppression of Cytotoxicity

To evaluate the capacity to generate cytotoxic T cells from the spleens of PET-treated BALB/c mice, ^{51}Cr-labeled CBA/j or BALB/c lymphoblasts were added to an established BALB/c versus CBA/j MLC assay (Fig. 2). Although a maximal specific cytotoxic T-cell response of 30 percent was obtained at 80:1 E:T ratio from PET-treated BALB/c mice to ^{51}Cr-labeled CBA/j targets, this response was significantly less than that observed in naive BALB/c splenocytes at 20, 40, and 80:1 E:T ratio ($p < 0.02$, <0.005, and <0.005, respectively) to identical ^{51}Cr-labeled CBA/j targets. Also, the specific cytotoxic T-cell response observed in spleen cells from PET-treated BALB/c mice was significantly less than that of sensitized BALB/c mice at 10, 20, and 80:1 E:T ratio ($p < 0.005$, <0.001, and <0.001, respectively) (Fig. 2a). There was less than 25 percent cytotoxic response elicited from the spleens of naive, sensitized, or PET-treated mice to BALB/c ^{51}Cr-labeled target cells (Fig. 2b). Therefore, specific hyporesponsive cytotoxic T-cell response to the original alloantigen

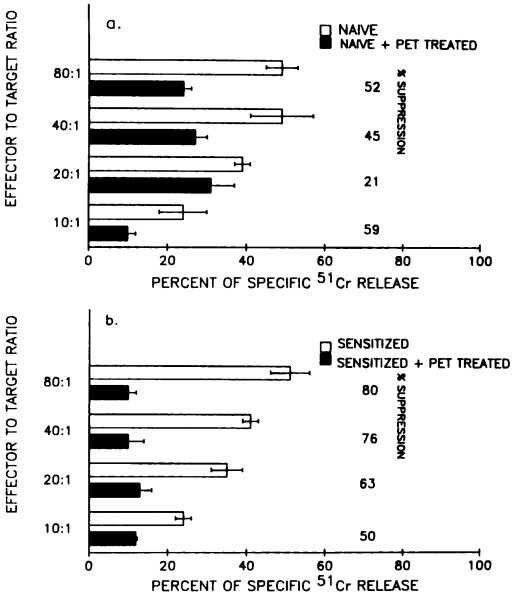

FIG. 3. Inhibition of the specific cytotoxic T-cell response. **a.** Naive BALB/c mice splenocytes or **b.** sensitized BALB/c splenocytes exposed to H-2^k, Mls^d alloantigens *in vitro* and tested for lysis of CBA/j ^{51}Cr-labeled targets at increasing effector-to-target ratios and compared to lysis elicited when cells from PET-treated mice were admixed 1:1 to (*a*) naive BALB/c or (*b*) sensitized BALB/c mice splenocytes.

presented by the skin graft can be observed in the spleen cells of PET-treated mice. Thus, a three-party ^{51}Cr-release assay was performed to determine whether the diminished cytotoxic response to CBA/j alloantigen from the spleens of PET-treated BALB/c mice was the result of an inhibitory cell population or the deletion of the effector T-cell population (Fig. 3). First, splenocytes from naive BALB/c mice were admixed at a 1:1 ratio with cells from the PET-treated BALB/c mice and then incubated with CBA/j stimulators. The subsequent addition of ^{51}Cr-labeled CBA/j target lymphoblasts revealed a significant suppression at 40 and 80:1 E:T ratio ($p < 0.02$ and <0.002, respectively) of the normally pronounced cytotoxic T-cell response of naive BALB/c mice splenocytes to the CBA/j alloantigen (Fig. 3a). Highly significant suppression ($p < 0.005$, <0.005, <0.001, and <0.001), at all E:T ratios tested, of the sensitized BALB/c cytotoxic T-cell response to CBA/j alloantigen was obtained when cells from the sensitized BALB/c mice were admixed with splenocytes from the PET-treated BALB/c mice in the presence of inactivated CBA/j splenocytes (Fig. 3b). The minimal autologous cytotoxic response of 25 percent observed at 80:1 E:T ratio in splenocytes from naive BALB/c mice was also significantly suppressed ($p < 0.002$) by the addition of cells from PET-treated mice (Fig. 4a). The autologous cytotoxic T-cell response observed in splenocytes from sensitized BALB/c mice was also suppressed (Fig. 4b) but not to a significant level. Therefore, pre-exposure of the immune system to PET cells resulted in augmented cell-mediated inhibition of the cytotoxic T-cell response to alloantigen.

Specific Inhibition of DTH

DTH experiments were performed to demonstrate *in vivo* inhibition of the response to alloantigen (Fig. 5). When PET-treated mice were primed on the flank and seven days after the footpad challenged with H-2^k, Mls^d alloantigen bearing lymphocytes, an 86 percent suppression of the DTH response (footpad swelling) was demonstrated as

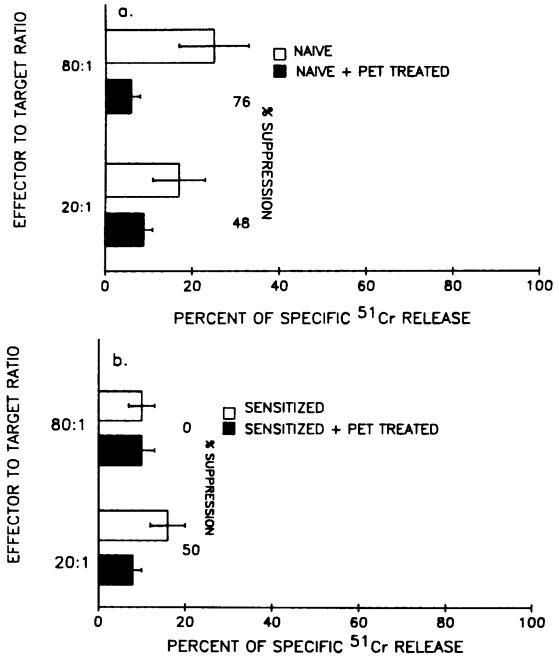

FIG. 4. Inhibition of autologous cytotoxic T-cell response. **a.** Naive BALB/c mice splenocytes or **b.** sensitized BALB/c mice splenocytes exposed to H-2^k, Mls^d alloantigen *in vitro* and tested for lysis of BALB/c (H-2^d, Mls^b) ^{51}Cr-labeled targets at increasing effector-to-target ratios and compared to lysis elicited when cells from PET-treated mice were admixed 1:1 to (*a*) naive BALB/c or (*b*) sensitized BALB/c mice splenocytes.

compared with positive controls; however, no suppression of the DTH response was demonstrated when PET-treated mice were primed and challenged with lymphocytes bearing the unrelated antigen H_2^b.

When BALB/c mice treated with photoinactivated naive cells were primed in the flank and seven days after the footpad challenge with H-2^k, Mls^d alloantigen-bearing lymphocytes, however, no suppression of DTH response (footpad swelling) was demonstrated as compared with controls (Fig. 6).

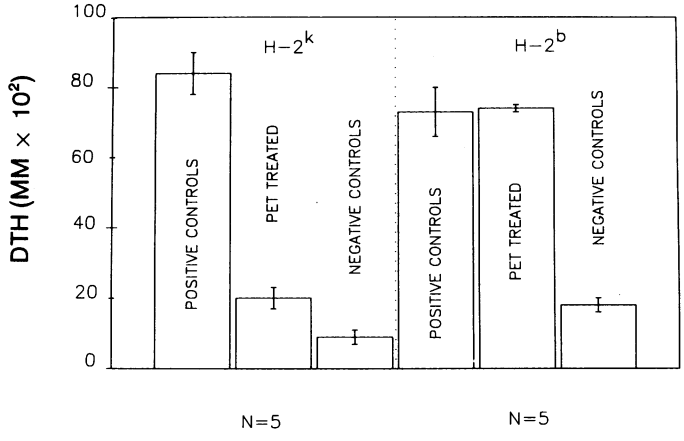

FIG. 5. Specific inhibition of delayed-type hypersensitivity response. PET-treated BALB/c mice primed in the flank and footpad challenged with either CBA/j (H-2^k, Mls^d) (*left*) or B10 (H-2^b, Mls^b) (*right*) splenocytes and the measurements of footpad swelling compared to the swelling elicited in positive and negative controls.

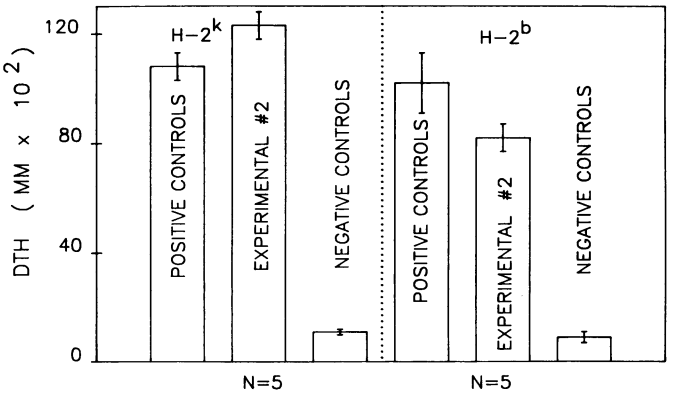

FIG. 6. Delayed-type hypersensitivity of experimental control group 2. BALB/c mice which had received intravenous infusions of 8-MOP/UVA photoinactivated syngeneic naive splenocytes were primed in the flank and footpad challenged with splenocytes from either CBA/j (H-2^k, Mls^d) (*left*) or B10(H-2^b, Mls^b) (*right*) mice and footpad swelling compared to swelling elicited in control mice.

Allograft Retention

Finally, BALB/c mice which had been repeatedly infused with PET cells recognizing the H-2^k, Mls^d (CBA/j) alloantigen were challenged with a fresh CBA/j skin transplant, the survival of which was visually and histopathologically followed. Six PET-treated BALB/c female mice received CBA/j skin transplants, in parallel with naive BALB/c female mice which also received identical skin grafts. Treated mice continued to receive intravenous PET infusions on a weekly basis, following engraftment.

All control naive BALB/c mice demonstrated evidence of rejection by seven days after transplant. In contrast, none of the PET-treated mice demonstrated any visual evidence of rejection. Three PET-treated mice were sacrificed 18 days post-transplantation. Their skin grafts did not show visual evidence of rejection at that time, whereas 100 percent of the control mice transplants had already completely necrosed.

The surviving PET-treated mice were sacrificed at subsequent intervals for histological evaluation of the graft. Although these mice did not demonstrate clear visual evidence of rejection by the end of the third and seventh weeks, respectively, the histopathology evaluation performed at the third week post-transplantation revealed epidermotropism of mononuclear inflammatory cells within a viable epidermis. Confluent and focal vacuolar epidermal basal cell degeneration and focal areas of mononuclear cell infiltration of engrafted epidermis with a denser dermal mononuclear cell infiltrate were seen in the pathological specimens obtained from the fifth and seventh week post-engraftment. Four BALB/c mice, however, which had received similar amounts of photoinactivated splenocytes from syngeneic mice rejecting a CBA/j skin graft, then received a skin graft from B10 mice, which was rejected within seven days post-engraftment. The histologic evaluation of these skin grafts revealed separation of a necrotic epidermis from the dermis, which contained a very dense mononuclear cell infiltrate and thrombi formation within dermal vessels.

Therefore, pre-exposure of the mouse immune system to PET cells generated during skin transplant rejection rendered the secondary syngeneic recipient relatively, but not

necessarily absolutely, tolerant to the alloantigen carried by the transplanted donor used to generate the immunizing effector cell population. In spite of these *in vivo* lymphoid infiltrates suggesting a cellular response to the graft, substantial suppression of MLC, CTL, and DTH was demonstrated, and prolongation of specific allograft survival was observed.

DISCUSSION

Murine skin transplantation across H-2 barriers results in transplant rejection principally mediated by activated T lymphocytes of the recipient [5], which have been identified as Lyt1+2+ [15] and L3T4$^+$ positive [22].

In our study, CBA/j mice (H-2^k, Mls^d) were selected as skin transplant donors because of their disparity in the H-2 locus with BALB/c (H-2^d, Mls^b) and their stimulatory phenotype in the Mls locus, theoretically maximizing the expansion of effector cells. The Mls locus is of interest because it is the only known genetic locus other than the MHC in which strong proliferative response is observed between unprimed individuals displaying disparity on it [23]. The simplest view of the Mls system at present is that it is composed of a silent allele (Mls^b) and two independent stimulatory alleles (Mls-a and c) [24] and the co-expression of both stimulatory determinants (Mls^{a+c-d}) results in the Mls^d phenotype. By transplanting CBA/j skin onto BALB/c mice, the allografted mice should develop Tc effector cells which recognize Class I antigens and Tdth lymphocytes, possibly of the T_h1 [25] helper cells which recognized Class II antigens, and possibly, L3T4$^+$ cells responding to the Mls-modified products of the MHC [26]. We appear to have inhibited this response significantly with our PET immunization protocol.

Our data demonstrate that this novel immunization protocol leads to the development of significantly diminished responsiveness to alloantigen and that this inhibition reflects the presence of cells capable of inhibiting the specific response to H-2^k, Mls^d alloantigen in MLC, CTL, and DTH reactions. Moreover, a substantial increase in the length of skin graft survival was obtained. Our findings extend those reported in other systems by various investigators [27–29]. Rohowsky and Suciu-Foca [27] have shown that idiotype-like receptors for alloantigens on autologous T lymphoblasts stimulate the autologous MLC (AMLC) response in resting lymphocytes. In our studies, a substantial increase in the AMLC response has been observed in the splenocytes of PET-treated animals (Table 1). Dorsch and Roser [28] demonstrated a rapidly recirculating suppressor T-cell population in animals rendered tolerant to alloantigen by transfusion of allogeneic bone marrow cells during the neonatal period of life. These cells are capable of adoptively transferring tolerance, in irradiated syngeneic recipients, to skin transplants bearing the alloantigens to which the original tolerance was induced, without interfering with the rejection of an unrelated skin graft. In our system, we have demonstrated the presence of a cell population capable of specifically ameliorating the response in MLC, CTL, and DTH to the antigen used to generate the effector cell population, while sparing the response to an irrelevant antigen. Bellgrau et al. [29] induced tolerance to parental lymphocytes, in F_1 rats, by immunizing them with lymphocytes bearing the MHC receptors for specific parental strains of lymphocytes. In an analogous fashion, we induced hyporesponsiveness to alloantigen by immunizing syngeneic mice with splenocytes containing cells bearing the MHC receptor for a specific histoincompatible antigen carried by the skin graft.

In this report, we describe a method of inducing tolerance that may be practical even in a clinical setting. In our studies, the host selects the appropriate immunoreactive cell

type from a mixed splenocyte pool which includes an expanded population of effector BALB/c anti CBA/j lymphocytes. These anti H-2^k, Mls^d lymphocytes would be the cells most likely to induce a response in the secondary recipient naive BALB/c mice, on a mere numerical basis. Moreover, Ben-Nun and Cohen [30] have demonstrated that the induction of a suppressive clonotypic response is more efficiently attained if the EAE-inducive T-cell clones used for vaccination have been activated *in vitro* with the antigen and attenuated either with X-irradiation or mytomicin C prior to reinfusion. If, however, viable EAE-inducing T-cell clones are intravenously infused, acute onset paralysis characteristic of EAE develops in the recipient animals. In our protocol, we have used *in vivo* activation by skin grafting to expand effector cells and use these cells obtained at the peak of rejection for immunization. The anti-H-2^k, Mls^d T-cell-specific contribution to these results may be better ascertained after we have cloned, photoinactivated, and infused them (either alone or in varying ratios with bystander splenocytes). Under these conditions, it will also be of interest to determine whether nonspecific bystander splenocytes exert either an adjuvant or interference effect. These studies are in progress.

The identity of these cells remains to be established. It will be necessary to determine whether they are Ly2+ suppressor T cells [31] or Ly2+ anti-receptor anti-MHC cytotoxic T cells described by Kosmatopoulos et al. [32] or the CD8+ cells which specifically respond to determinants on the disease-mediating T-cell clones [33].

In our system, photoinactivation with 8-MOP might have been so effective in maximizing the development of a specific inhibitory response to alloantigen because it reflects the ability to control two variables (drug dose and UVA energy), which limit the chemical reaction to microseconds [34], and thus makes possible meticulous control of the dose-response curve of cellular inactivation to a level not previously attainable. Significantly, 8-MOP-UVA pre-treatment of cloned anti-basic myelin protein T cells [35] was more effective than X-irradiation of the same cells for vaccinating rats against the induction of EAE. Second, our studies on the suppression of DTH response to sheep red blood cells [36] have demonstrated that 8-MOP-UVA treatment of naive population of splenocytes is ineffective in inducing suppression, indicating that the presence of expanded population(s) of specific effector T cells are necessary. Third, Laskin et al. [37] have identified specific, saturable, high-affinity binding sites for 8-MOP on mice and human cell lines which might be a cell surface binding site that becomes covalently modified by exposure to UVA-modifying cellular antigenicity. It must be emphasized, however, that 8-MOP-UVA pre-treatment of effector cells may be merely one means of inducing the observed results.

In summary, we conclude that it is possible to induce a state of hyporeactivity against a specific set of MHC antigens. Syngeneic effector cells with specificity for these antigens can be photoinactivated and used as immunogens to induce this hyporesponsive state. Since a method for photoinactivating effector T cells in analogous fashion with that used here has been successful in the treatment of CTCL patients [4] and is currently being tested in other T-cell-mediated disorders, our protocol may have potential application in the field of transplantation in humans.

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