LOSS OF EPIDERMAL INTEGRITY BY T CELL-MEDIATED ATTACK INDUCES LONG-TERM LOCAL RESISTANCE TO SUBSEQUENT ATTACK

I. Induction of Resistance Correlates with Increases in Thy-1$^+$ Epidermal Cell Numbers

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Skin is known to be one of the worst affected target organs in graft-versus-host disease (GVHD)[1] (1). The cutaneous lesions are characterized by the epidermal cell death in association with a prominent T cell infiltrate (2). It is therefore thought that the cutaneous lesions occur as the result of a T cell-mediated immune attack on epidermal cells, either directly or via the release of various cytokines from the T cells, although the problem of whether the T cells toward the epidermal antigens cause these lesions has been still unclear (3). While much attention has been directed to effector T cells capable of mediating GVHD, almost nothing is known about the mechanism(s) by which the integrity of organ structures can be protected from such a T cell-mediated immune attack in GVHD. This is due, in part, to a difficulty in producing experimental models of GVHD to be able to investigate the protection mechanism(s).

We have recently demonstrated that cutaneous GVHD, resembling lichen planus and lupus erythematosus in humans, can be induced in normal mice by intradermal inoculation into the footpads of cloned, autoreactive CD4$^+$ T cells with cytolytic activity (4-6). The experimentally induced cutaneous GVHD is basically composed of two types of reactions: delayed-type hypersensitivity (DTH) reactions and epidermal cell damage defined histologically. DTH responses measurable by footpad swelling peaks at 24-48 h and then gradually recedes (5). Histologically, the DTH responses are characterized by the diffuse infiltration of mononuclear cells and prominent edema in the dermis. In contrast, epidermal lesions characterized by the severe infiltration of T cells associated with the consequent epidermal cell damage reach maximum

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[1] *Abbreviations used in this paper:* DTH, delayed-type hypersensitivity; GVHD, graft-vs.-host disease; PLN, popliteal lymph node; Thy-1$^+$ EC, Thy-1$^+$ epidermal cells.

severity at 4 and 5 d (5). The lesions, however, subside rapidly and the epidermis returns to normal by 14 d (6). Such rapid recovery from the experimentally induced cutaneous GVHD suggested to us that suppression mechanisms could play a role in recovering the epidermal integrity from the destruction and in protecting it from an additional attack by T cells. To investigate this possibility, the present study asks the following questions. (*a*) Are those mice that spontaneously recovered from the experimentally induced cutaneous GVHD resistant to subsequent attempts to induce the cutaneous GVHD? and (*b*) if so, which cells are responsible for the resistance to cutaneous GVHD?

Our results indicate that those mice recovered from the experimentally induced cutaneous GVHD acquire long-term resistance to the cutaneous GVHD. This resistance was not due to the generation of antiidiotypic T cells. Unexpectedly, this long-term, local resistance to cutaneous GVHD was accompanied by a nearly 30-fold increase in the number of Thy-1$^+$ epidermal cells (Thy-1$^+$ EC). Although Thy-1$^+$ EC bearing TCR-γ/δ have recently been the focus of intense research interest, evidence for the physiologic role in situ is still lacking (7, 8). In this paper we provide for the first time evidence indicating that Thy-1$^+$ EC play an important role in protecting the epidermal integrity from a T cell-mediated immune attack.

Materials and Methods

Mice. Female C57BL/6 (B6) mice were obtained from Charles River Japan, Inc., Atsugi, Kanagawa, and female B10.Thy-1.1 mice were bred in our laboratories. They were used predominantly at 8–12 wks old.

Cloned T Cell Lines. General characteristics of the cloned T cell lines used are shown in Table I. The derivation and maintenance of the T cell lines have been described in detail previously (4, 9–11). BB5, SK1, and J403 cells are capable of migrating into the epidermis upon their intradermal inoculation into the footpads of the syngeneic (in case of BB5) or appropriate allogeneic recipients with I-A^{k,b,f,r} (in case of SK1) or with H-2K^b (in case of J403) and cause the destruction of the epidermis, histologic changes identical to those seen in human cutaneous GVHD (4, 5). In contrast, other cloned T cells are completely incapable of migrating into the epidermis: they are unable to induce cutaneous GVHD even when injected into the footpads of appropriate recipients with relevant antigens (4, 5).

Assay for DTH Responses. As described previously (5), these cloned T cells were harvested from culture 7–10 d after antigenic stimulation. Before the T cells were used in experiments, dead cells in the preparation were removed on a lymphocyte separation media (Sigma Chemical Co., St. Louis, MO) density gradient. After being washed with HBSS three times, remaining cells with >97% viability were injected intradermally, in a volume of 25 μl, into hind footpads of recipient mice. The resultant swelling of the footpads was measured with

TABLE I

Description of T Cell Clones

Clone	Source	Strain of origin	Antigen specificity	Function	Ability to produce cutaneous GVHD
BB5	LN*	B6	I-A^b (self)	Cytotoxic/helper	+ + +
C10	Spleen	B6	I-A^b (self)	Cytotoxic/helper	–
82F12	LN*	B6	CGG + I-A^b	Helper	–
SK1	Spleen	A.TH	I-A^{k,b,f,r}	Cytotoxic/helper	+ +
J403‡	Spleen	B10.BR	H-2K^b	Cytotoxic	+ ~ + +

* LN, lymph node.
‡ Uncloned cell line.

a dial thickness gauge at several time points after injection of the T cells. The results are expressed as the arithmetic mean of measurements obtained from six mice.

Assay for Cutaneous GVHD. Footpad skin biopsies taken from mice at several time points after the injection of the T cells were fixed in 3.7% buffered formaldehyde and were embedded in paraffin. Sections 2 μm thick were stained with hematoxylin and eosin. The cutaneous GVHD was evaluated both semiquantitatively by the intensity of epidermal invasion of the injected T cells and the severity of epidermal cell damage, as previously described (6). The semiquantitative evaluation of epidermal invasion by the injected T cells was performed by counting lymphoid cells within the epidermis, because our previous immunohistochemical studies showed that 72 h after the injection of the T cells intraepidermal lymphoid cells are mainly composed of the injected T cells, with small numbers of recruited T cells of host origin (4). At least 30 serial sections were examined for each individual footpad, and of these, six sections in which the epidermis was regarded to be most heavily infiltrated with lymphoid cells were enumerated. The results were expressed as the number of lymphoid cells per linear millimeter of epidermis that was calculated from that within the total epidermal length surveyed. The severity of the epidermal cell damage was scored according to the grading system described by Lerner et al. (12) for cutaneous GVHD. This evaluation was performed in a blind fashion by two investigators who were not informed of the treatment.

Antibody Reagents. The mAbs we used were anti-Thy-1.2 (rat IgG 2b; Becton Dickinson & Co., Mountain View, CA), anti-Thy-1.1 (mouse IgG 2b; Meiji Laboratories, Tokyo, Japan), anti-I-$A^{b,k}$ (mouse IgG 2a; Meiji), anti-CD3ε chain (145-2C11; hamster IgG), anti-CD4 (GK1.5; rat IgG 2b), anti-CD8 (anti-Lyt-2.2; mouse IgG 2a; Meiji) and anti-asialo GM_1 (rabbit IgG).

Immunofluorescence Staining. Epidermal sheets from the footpads were separated from the dermis by incubation in 0.02% EDTA for 2 h at 37°C as described (5). The sheets fixed in acetone for 10 min were extensively washed in PBS and incubated with appropriate dilutions of first-step antibodies for 16 h at 4°C. Then they were washed in PBS for 1 h and incubated for 1 h at 37°C with appropriate second-step antibodies labeled with FITC: FITC-$F(ab')_2$-goat anti-mouse IgG, FITC-$F(ab')_2$-goat anti-rat IgG, FITC-$F(ab')_2$-goat anti-rabbit IgG (Tago Inc., Burlingame, CA) and FITC-$F(ab')_2$-goat anti-hamster IgG (Cappel Laboratories, Cochranville, PA). For anti-I-A/anti-Thy-1.2 double labeling, the sheets were incubated with biotinylated anti-I-$A^{b,k}$ (Meiji Laboratories) at 1:20, followed by Texas Red-labeled avidin (Tago Inc.) at 1:400 and FITC-anti-Thy-1.2 (Becton Dickinson Inc.). The stained specimens were mounted in glycerin PBS and viewed through a fluorescence microscope.

Statistical Analysis. The statistical significance of the differences between different groups of mice was determined using Student's *t*-test; $p < 0.05$ (two-tailed test) was considered significant.

Results

Induction of Resistance to Cutaneous GVHD. To determine whether those mice that spontaneously recovered from the experimentally induced cutaneous GVHD would be resistant to subsequent attempts to induce the cutaneous GVHD, 2×10^6 autoreactive cloned T cells capable of producing cutaneous GVHD, termed BB5, were injected into the same footpad sites of syngeneic (B6) mice that had received 2×10^6 BB5 cells 2 wk before. While untreated mice were highly susceptible to the active induction of the cutaneous GVHD (Fig. 1 *A*), those mice that had spontaneously recovered from the cutaneous GVHD failed to develop the cutaneous GVHD defined by the epidermal invasion of the T cells and epidermal cell damage (Fig. 1 *B*): those mice had normal appearing epidermis with little or no intraepidermal lymphoid cells, although in the dermis there was a marked lymphoid infiltration indistinguishable from that observed in the untreated mice. Thus, the footpads of mice that had recovered from the cutaneous GVHD appeared to become resistant to the active reinduction of the cutaneous GVHD. However, pretreatment with 2 ×

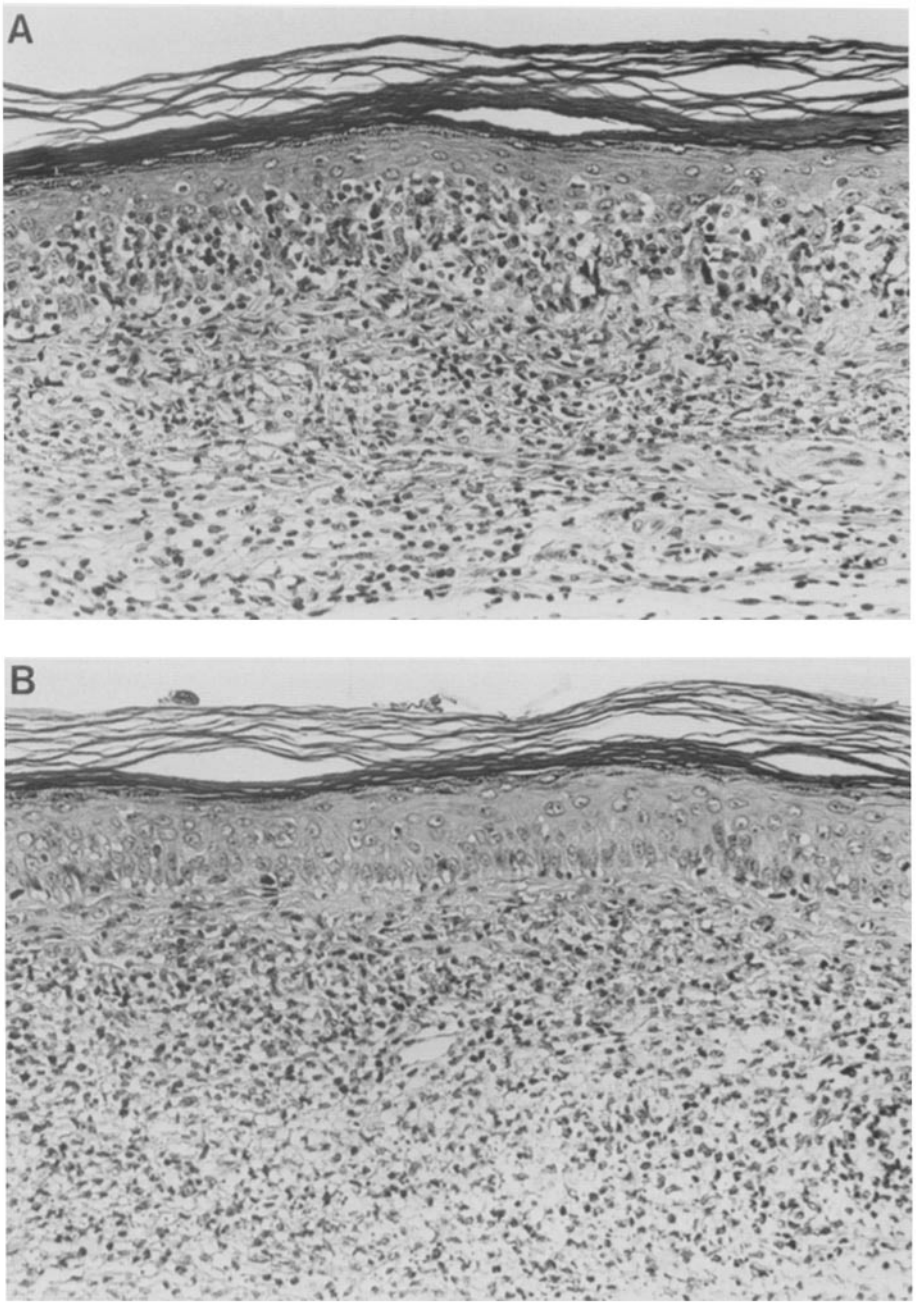

FIGURE 1. Cutaneous GVHD lesions in the footpads of B6 mice at 4 d after intradermal injection of 2 × 10^6 BB5 cells. The footpad skin of the control mouse (*A*) shows the destruction of epidermal structures associated with an extensive infiltrate of lymphocytes; in contrast, in the footpad skin of the mouse pretreated with 2 × 10^6 BB5 cells 2 wk before (*B*), no epidermal cell degeneration can be seen, although the infiltrative pattern in the dermis is similar to that shown in *A*. Hematoxylin and eosin, ×66.

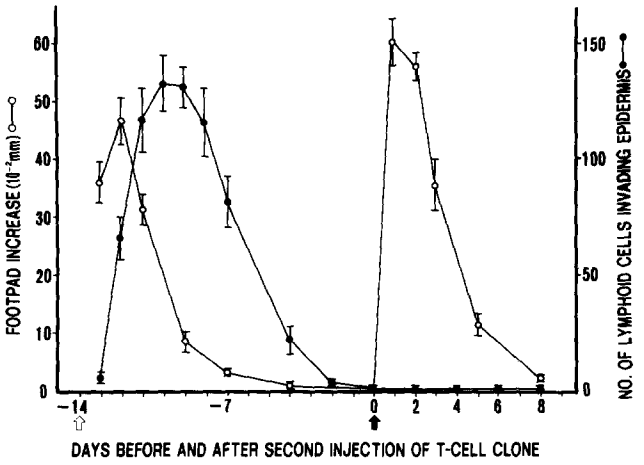

FIGURE 2. Kinetics of development of DTH responses measurable by footpad swelling (○) and cutaneous GVHD defined by the intensity of epidermal invasion of lymphocytes (●). An open arrow indicates first injection of 2 × 10^6 BB5 cells on day −14. A closed arrow indicates second injection of 2 × 10^6 BB5 cells on day 0. Each point represents the mean ± SE derived from 3–36 mice (DTH responses) or three mice (cutaneous GVHD).

10^6 BB5 cells did not affect the ability of the mice to mount DTH reactions by subsequent injection of 2 × 10^6 BB5 cells. Fig. 2 illustrates the typical time course of the development of the DTH reactions and cutaneous GVHD in those mice. In addition, with regard to the ability to exhibit the GVH reactions manifested by the enlargement of popliteal lymph nodes (PLN) after intradermal injection of BB5 cells, no significant differences were noted between untreated mice and those pretreated with BB5 cells 2 wk before (data not shown). As shown in Table II, mice whose right footpads had been injected with BB5 cells 2 wk before no longer developed the cutaneous GVHD, provided that BB5 cells were injected into the right footpads, while the untreated left footpads of the same mice remained susceptible to the active induction of the cutaneous GVHD by BB5 cells. These results suggest that resis-

TABLE II

Untreated Sites of Mice Injected with T Cell Clone Are Not Resistant to Subsequent Attempts to Induce Cutaneous GVHD

T cell clone injected on day −14 to cause GVHD resistance	Footpad site of injection of BB5 cells on day 0	No. of lymphoid cells invading the epidermis on day 4	No. of mice with cutaneous GVHD lesions on day 4 (grade)				
			0	+1	+2	+3	+4
BB5	Injected site*	14 ± 5‖	6	0	0	0	0
	Untreated site‡ (injected mouse)	96 ± 36	0	1	3	2	0
	Untreated site§ (control mouse)	111 ± 30	0	0	3	3	0

* On day 0, 2 × 10^6 BB5 cells were injected into the same footpad sites (right side) that had received 2 × 10^6 BB5 cells 2 wk before.

‡ On day 0, 2 × 10^6 BB5 cells were injected into the untreated footpad sites (left side) whose right footpads had received 2 × 10^6 BB5 cells 2 wk before.

§ On day 0, 2 × 10^6 BB5 cells were injected into the untreated footpad sites of normal control mice.

‖ $p < 0.001$ compared with control mouse.

tance to cutaneous GVHD is restricted to the injection sites and that locally acting, downregulatory mechanisms could be involved in the resistance.

Specificity and Persistence of Resistance. We next examined whether the local resistance was specific for the T cell clone used for the induction of the resistance. Various T cell clones capable or incapable of producing cutaneous GVHD were tested for their ability to induce local resistance to the cutaneous GVHD in B6 mice. As shown in Table III, inoculation of cutaneous GVHD-producing T cell clones, such as SK1 and J403, also was able to induce local resistance to the subsequent induction of cutaneous GVHD by either SK1 or BB5. In contrast, resistance to the cutaneous GVHD was never induced by pretreatment with other cutaneous GVHD-nonproducing T cell clones, such as C10 and 82F12, even when used at supraoptimal doses (5×10^6–10^7 cells/footpad). These results indicate that the local resistance induced was not clonotypic.

To determine whether the destruction of epidermal structures by T cell clones is prerequisite for the induction of the resistance, suboptimal doses of either BB5 of SK1 cells that had been shown not to produce significant cutaneous GVHD lesions were injected and 2 wk later 2×10^6 BB5 cells were again injected into the same footpads. Table IV shows that the footpads of B6 mice pretreated with either BB5 or SK1 cells acquired resistance to the cutaneous GVHD in a dose-dependent manner: complete resistance was seen, when 2×10^6 BB5 cells were injected 2 wk before, while twofold more SK1 cells were required to achieve the same effect. The resistance induced by low doses of BB5 ($\leq 0.5 \times 10^6$) or SK1 cells ($\leq 1 \times 10^6$) was not so complete as that induced by 2×10^6 BB5 cells and was able to be partially overcome with the active induction by high doses of BB5 cells ($\geq 4 \times 10^6$). The ability of these T cells to induce the resistance appeared to be proportional to their ability to induce the destruction of epidermal structures.

Further experiments were conducted to determine the persistence of the local re-

TABLE III

Specificity of Resistance to Cutaneous GVHD Caused by Injection with T Cell Clone

T cell clone injected on day −14 to cause GVHD resistance*	Injection of T cell clone on day 0‡	Footpad increase on day 2 (10^{-2} mm ± SD)	No. of lymphoid cells invading the epidermis on day 4	No. of mice with cutaneous GVHD lesions on day 4 (grade)				
				0	+1	+2	+3	+4
None	BB5	53.7 ± 4.4	128 ± 20	0	0	2	4	0
BB5	BB5	57.1 ± 2.9	7 ± 6§	6	0	0	0	0
C10	BB5	55.2 ± 5.1	121 ± 23	0	0	3	3	0
82F12	BB5	56.3 ± 4.6	114 ± 32	0	1	2	3	0
SK1	BB5	58.5 ± 2.9	24 ± 22§	5	1	0	0	0
J403	BB5	57.0 ± 2.2	9 ± 3§	3	0	0	0	0
None	SK1	38.5 ± 7.0	98 ± 29	0	1	3	2	0
SK1	SK1	42.7 ± 4.2	21 ± 14§	5	1	0	0	0
C10	SK1	41.3 ± 5.6	106 ± 24	0	1	1	4	0
BB5	SK1	39.8 ± 6.6	14 ± 6§	6	0	0	0	0

* On day −14, 2×10^6 T cell clones indicated were injected into the footpads of B6 mice to induce the resistance to cutaneous GVHD.

‡ On day 0, 2×10^6 BB5 or SK1 cells were again injected into the same footpad sites.

§ $p < 0.0005$ compared with control mice without pretreatment.

TABLE IV

Dose Dependence of Induction of Resistance to Cutaneous GVHD Caused by Injection of T Cell Clone

T cell clone injected on day −14 to cause GVHD resistance	Injection of T cell clone on day 0	No. of lymphoid cells invading the epidermis on day 4	No. of mice with cutaneous GVHD lesions on day 4 (Grade)				
			0	+1	+2	+3	+4
None	2×10^6 BB5	138 ± 17	0	0	1	3	0
0.1×10^6 BB5	2×10^6 BB5	82 ± 27*	0	2	0	2	0
0.5×10^6 BB5	2×10^6 BB5	92 ± 32	0	1	2	1	0
1×10^6 BB5	2×10^6 BB5	37 ± 9‡	3	1	0	0	0
2×10^6 BB5	2×10^6 BB5	16 ± 9‡	4	0	0	0	0
0.5×10^6 SK1	2×10^6 BB5	95 ± 37	1	0	1	2	0
1×10^6 SK1	2×10^6 BB5	62 ± 42	2	1	0	1	0
2×10^6 SK1	2×10^6 BB5	44 ± 21*	2	2	0	0	0
4×10^6 SK1	2×10^6 BB5	17 ± 5*	4	0	0	0	0

* $p < 0.05$ compared with control mice without pretreatment.
‡ $p < 0.0001$ compared with control mice without pretreatment.

sistance. At various lengths of time after receiving 2×10^6 BB5 cells or C10 cells, B6 mice were injected intradermally with 2×10^6 BB5 cells. By up to 8 mo the epidermis of footpads pretreated with BB5 cells never again became susceptible to destruction by BB5 cells, provided that the BB5 cells were injected into the same footpads that had received BB5 cells on day 0 (Table V). In ongoing experiments, we found that 1 yr after the first injection with BB5 cells, the complete resistance was still observed. Thus, long-term local resistance to the cutaneous GVHD demonstrated here may represent locally acting suppression mechanisms, rather than a result of generation of antiidiotypic responses that can specifically suppress the function of effector T cells.

TABLE V

Persistence of Resistance to Cutaneous GVHD Caused by Injection of T Cell Clone

First injection of T cell clone to cause GVHD resistance		Injection of T cell clone on day 0	No. of lymphoid cells invading the epidermis on day 4	No. of mice with cutaneous GVHD lesions on day 4 (grade)				
Weeks after injection*	Injected T cell clone			0	+1	+2	+3	+4
4	BB5	BB5	19 ± 5‡	5	0	0	0	0
	C10	BB5	140 ± 25	0	0	1	4	0
	None	BB5	131 ± 31	0	0	1	3	0
8	BB5	BB5	25 ± 11‡	4	1	0	0	0
	C10	BB5	125 ± 26	0	0	2	3	0
	None	BB5	125 ± 28	0	0	2	3	0
16	BB5	BB5	16 ± 6‡	4	0	0	0	0
	None	BB5	115 ± 22	0	0	2	2	0
32	BB5	BB5	20 ± 4‡	4	0	0	0	0
	None	BB5	136 ± 34	0	0	1	3	0

* At various lengths of time indicated after first injection of 2×10^6 BB5 or C10 cells, 2×10^6 BB5 cells were again injected into the same footpad sites.
‡ $p < 0.0001$ compared with control mice without pretreatment.

Lesional Epidermis Contains Large Numbers of Thy-1$^+$ EC. These results prompted us to investigate whether the resistance was due to a loss of some epidermal cell populations necessary for epidermal invasion of the T cells or if there was the appearance or the incresae in numbers of epidermal cell populations able to protect the integrity of epidermis from an additional attack by the T cells. The preparation of epidermal sheets obtained from footpads either before or after the injection of.2 × 10^6 BB5 cells were examined by the immunofluorescence technique. Our earlier studies indicated that Thy-1$^+$ EC present in footpad epidermis are different from those in other sites, such as ear and body wall skin, with respect to their distribution and morphology: the former Thy-1$^+$ EC occur predominantly at peripheral, hair-bearing areas, but rarely at central, non-hair-bearing areas, while the latter cells are evenly distributed; and the former cells, particularly those at central, non-hair-bearing areas, are angular and less dendritic, while the latter cells are predominantly dendritic. By contrast, there were no site variations in the densities of I-A$^+$ Langerhans cells.

As described above, the epidermis rapidly recovered from the destruction induced by the T cells and 14–21 d after the injection of the T cells no histologically detectable lesions were observed in the epidermis (Fig. 3): as far as hematoxylin eosin staining sections were concerned, the histologic findings of the epidermis were indistinguishable from that before injection of the T cells. The normal appearing epidermis, however, contained a large number of Thy-1$^+$ EC (Fig. 4 *A*): The number of Thy-1$^+$ EC/mm^2 was 20–30-fold higher in the epidermis of the footpads that had received 2 × 10^6 BB5 cells 2 wk before than that in untreated footpads (Fig. 4 *B*). In addition, the majority of the Thy-1$^+$ EC observed in the former

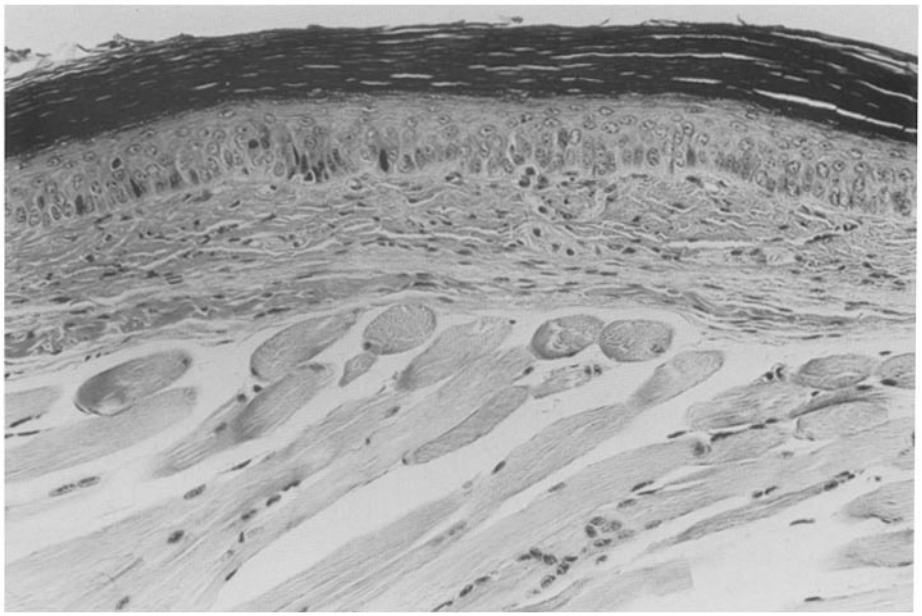

FIGURE 3. Histology of the footpad skin of B6 mice that received 2 × 10^6 BB5 cells 2 wk before and that recovered from the destruction. Hematoxylin and eosin, ×66.

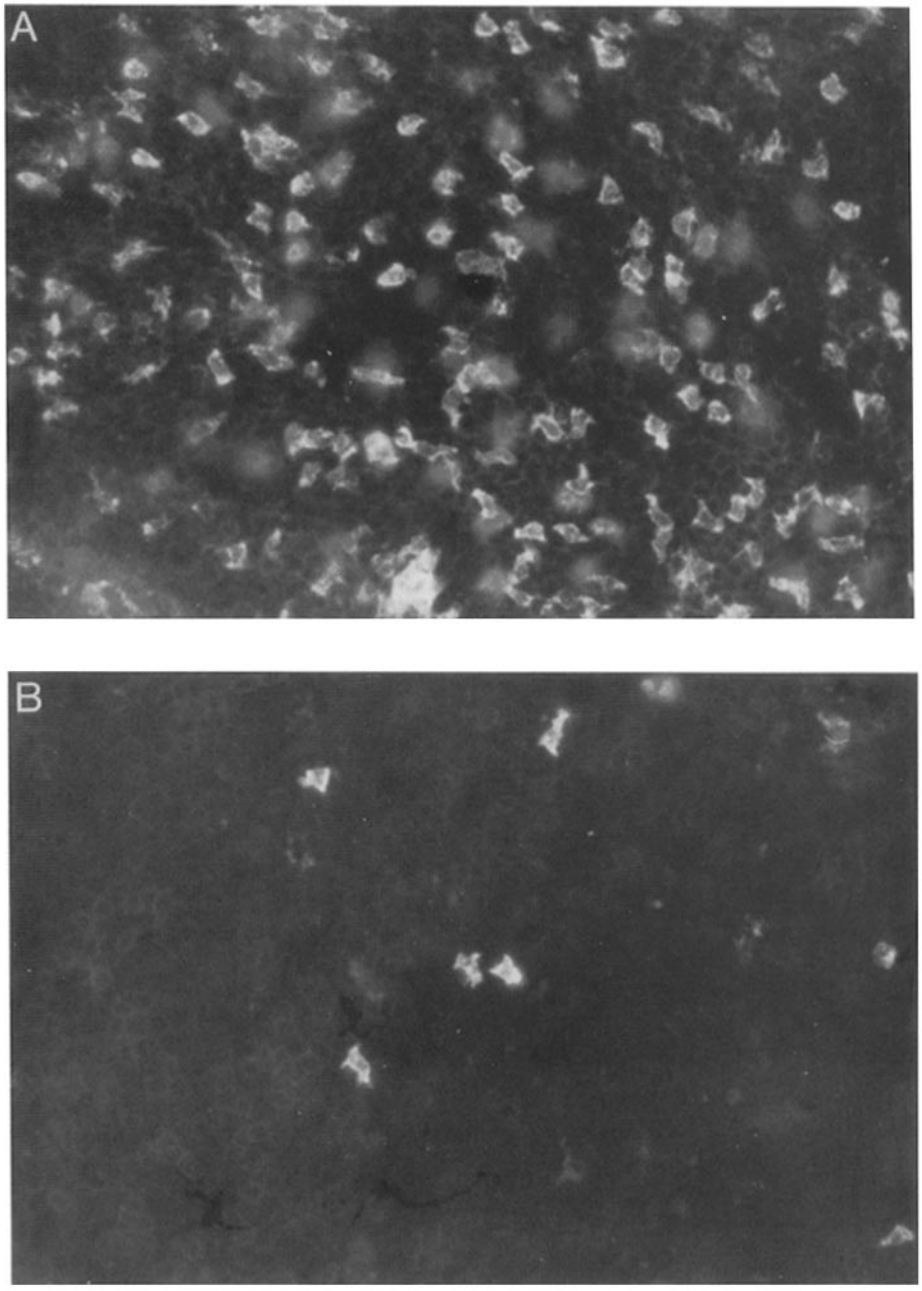

FIGURE 4. Immunofluorescence of Thy-1.2$^+$ EC in epidermal sheets from B6 murine footpad. (*A*) At 2 wk after injection of 2 × 10^6 BB5 cells, the footpad epidermis contains a large number of Thy-1$^+$ EC despite normal appearance of the histologic finding. (*B*) The footpad epidermis of normal mouse is relatively deficient in Thy-1$^+$ EC. ×80.

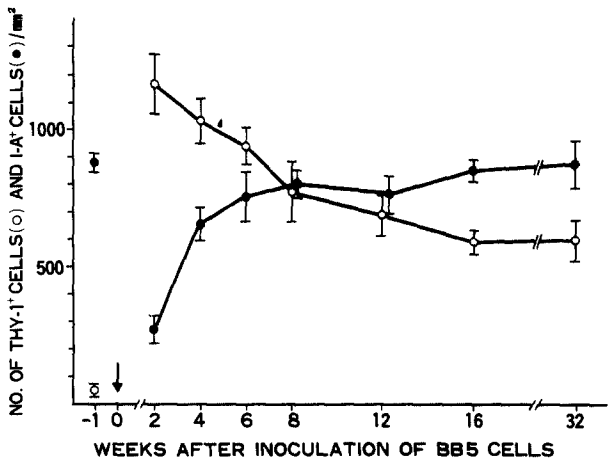

FIGURE 5. The densities of Thy-1⁺ EC (○) and I-A⁺ Langerhans cells (●) in the footpad epidermis of B6 mice following injection of 2 × 10^6 BB5 cells. A closed arrow indicates the injection of BB5 cells on day 0. Each point represents the mean ± SE derived from six to seven mice. During the development of the cutaneous GVHD, it was almost impossible to quantitatively assess the absolute numbers of positively stained cells due to the intense nonspecific staining as a result of the extensive destruction of epidermal structures. The recovery from the destruction 2 wk after injection of BB5 cells made it possible to determine the absolute numbers of positively stained cells in the epidermal sheet preparations.

epidermis, unlike their normal counterparts observed in ear or body wall skin, were primarily angular and only <5% of them appeared dendritic in morphology. In some areas, they were observed to form small clusters. Double-staining experiments indicated that >70% of the Thy-1⁺ EC were positive for CD3, although not so intensely as those located in hair-bearing areas. Like the normal counterparts, they lacked I-A, CD4, and CD8 expression. Anti-asialo GM_1 stained a portion of the Thy-1⁺ EC strongly. The increase in Thy-1⁺ EC number was never observed in the footpads that had received T cell clones incapable of producing cutaneous GVHD, as was the resistance. In the footpads of B10.Thy-1.1 mice that had received BB5 cells (Thy-1.2⁺) 21 d before, Thy-1.1⁺ cells were found in increased numbers, while Thy-1.2⁺ cells were never observed (data not shown). It was therefore obvious that the increased Thy-1⁺ EC are exclusively of recipient origin and the injected T cells are eradicated from the epidermis 21 d after the injection. Thus, it is unlikely that the injected T cells remaining in the epidermis are directly involved in the resistance to cutaneous GVHD.

Interestingly, the great increase in the number of Thy-1⁺ EC following the destruction of epidermal structures in the cutaneous GVHD was accompanied by a profound decrease in Langerhans cell number. The number of Langerhans cells, however, recovered gradually and returned to the normal levels by 8 wk after the injection, as shown in Fig. 5. In contrast, the marked increase in number of Thy-1⁺ EC persisted for at least 8 mo, although its extent declined slowly over several months. These changes in the numbers of Thy-1⁺ EC and Langerhans cells were confined to the injected site: in other sites, such as another untreated footpad and ear, these changes were not observed, when examined 2 wk after the injection of BB5 cells (data not shown). Taken together, major differences between "resistant" and "susceptible" epidermis are found at the density of Thy-1⁺ EC of host origin.

Discussion

Recent studies with experimental models of organ-specific autoimmune disease (13–16) have suggested the existence of downregulatory mechanism(s) by which the

integrity of organ structures can be protected from a T cell–mediated autoimmune attack. Several hypothesis have been put forward to explain the protection mechanism(s). In some models, generation of antiidiotypic T cells that can inhibit the autoimmune effector T cells has been thought to be one of such mechanism(s) (13–15). An entirely different mechanism that has been offered is that organ-resident, nonlymphoid cells may suppress proliferation of autoimmune T cells, thereby representing an important component of a locally acting suppression mechanism against autoimmune T cells (16). The concept of the locally acting suppression mechanisms explains the ability of organ-resident cells to participate in protecting the integrity of organ structures without involving antiidiotypic suppressor T cells.

In this study we have demonstrated that long-term local resistance to cutaneous GVHD can be induced in mice by the intradermal injection of the cloned T cells capable of producing cutaneous GVHD but not of those incapable of it. The acquisition of resistance to cutaneous GVHD demonstrated here was confined to the epidermal structures, because the intradermal injection of the T cells did not affect the susceptibility of mice to subsequent active induction of DTH responses and GVH reactions manifested by the enlargement of the PLN. Thus far, resistance to GVH reactions has been extensively studied by the use of the PLN weight assay (17, 18), both because the enlargement of the lymph nodes that drain the site of injection was believed to represent a local GVH reaction and because of its ready availability. The PLN weight assay, however, may not necessarily be suitable for the detection of resistance to GVHD, because it has been suggested that in some experimental systems the state of tolerance could be manifested by the enlargement of lymphoid organs due to the extensive activation of all lymphocyte subsets (19). Resistance to DTH reactions has also been studied in some experimental systems: Perez et al. have demonstrated that mice that had received alloreactive T cells photoinactivated with the chemical crosslinker 8-methoxypsoralen (8-MOP) and UV A light failed to mount a DTH reaction to the alloantigen used for the vaccination, but not to other alloantigens (20).

Because DTH responses are thought to reflect the proliferation of lymphocytes, release of various cytokines, and recruitment of host cells, this method also can not be free from the drawbacks of the PLN weight assay. Although skin is one of the most severely affected organs in GVHD and the histopathological effects of cutaneous GVHD have been evaluated by the severity of epidermal cell damage, little attention has thus far been given to the epidermal lesions in experiments showing resistance to GVH reaction. Thus, one must realize that most of the information available on the resistance to GVH reactions has been derived from experiments using the PLN weight assay and DTH responses. In this study, the cutaneous GVHD was evaluated by the microscopic observations of the epidermal lesions in addition to the two conventional methods, PLN weight assay and DTH reactions. In fact, the resistance to cutaneous GVHD in mice pretreated with the T cells capable of producing cutaneous GVHD was demonstrated only by the microscopic observations of the epidermal lesions, but not by the two conventional methods. Our data did not provide any evidence indicating the generation of antiidiotypic suppressor T cells in resistant mice, which have been demonstrated as one of the main mechanisms of resistance in other experimental models (13–15), but instead demonstrated the existence of an epidermis-resident protection mechanism, which has never been

described. The choice of assay systems may have caused other investigators to miss such an epidermis-resident protection mechanism not involving suppressor T cells as demonstrated here.

The reason is not clear yet why only epidermal structures, but not dermal structures, can be protected from a T cell–mediated immune attack in the resistant mice, while in naive mice epidermal structures are relatively susceptible to destruction by the T cells as compared with dermal structures. Which subpopulation(s) in epidermis can serve to protect the integrity of epidermis from a subsequent attack by the T cells? The present results show that the footpads of mice that became resistant to subsequent induction of the cutaneous GVHD contained unexpectedly large numbers of Thy-1$^+$ EC in the epidermis. The numbers of the Thy-1$^+$ EC in the resistant mice were 20–30-fold higher than those in normal controls. Such a great increase in the Thy-1$^+$ EC number persisted for at least 8 mo, during which time complete resistance was seen. In addition, the increase was observed only in the injected sites, but not in the untreated sites of the same mice, which remained totally susceptible to the active induction of the cutaneous GVHD (Table II). Thus, resistance to the cutaneous GVHD as measured by the protection of epidermal structures from a subsequent T cell attack appears to closely correlate with the great increase in Thy-1$^+$ EC numbers.

Is there any possibility that epidermal cell populations other than Thy-1$^+$ EC are involved in the induction of the resistance? We have previously reported that epidermal invasion of the GVHD-producing T cells was accompanied by Ia expression on keratinocytes (4, 5). Therefore, in view of previous suggestions by Gaspari et al. that Ia$^+$ keratinocytes may induce antigen-specific unresponsiveness in T cells (21), it is possible that Ia$^+$ keratinocytes could provide a defense mechanism against a T cell attack by inactivating and helping to eliminate the aggressor T cells. However, there are some strong arguments against a causal relationship between the presence of Ia$^+$ keratinocytes and the induction of the resistance: Ia expression on keratinocytes induced by the T cells was transient and no longer evident 2 wk after injection of the T cells, at which time the epidermal structures were protected from the T cell attack; although T cells incapable of producing cutaneous GVHD, e.g., C10 cells, can also induce Ia expression by keratinocytes upon the intradermal injection, they were unable to induce the resistance, as demonstrated in Table III. Thus, although Ia$^+$ keratinocytes may be playing some role in the spontaneous resolution of the cutaneous GVHD lesions induced by the T cells, it is unlikely that clonal anergy of the T cells by Ia$^+$ keratinocytes represents a major mechanism for the resistance demonstrated here.

In line with previous observations that Thy-1$^+$ EC bearing TCR-γ/δ are the main lymphocyte population residing in epidermis (22) and that these TCR-γ/δ cells have non–MHC-restricted cytotoxic function (23), the notion has been suggested, but not yet proved, that Thy-1$^+$ EC are able to recognize a novel antigen expressed by infected, transformed, or damaged keratinocytes and express cytotoxic activity, thereby protecting the integrity of epidermis (24). Our results provide experimental support for the proposed role of Thy-1$^+$ EC as a protection mechanism for the epidermal integrity. The present observations that the resistance to the cutaneous GVHD was not clonotypic and that there is a good correlation between the intensity of the destruction of epidermal structures induced by the T cells for the induction of resis-

tance and the degree of the resistance induced are in accordance with the hypothesis that Thy-1$^+$ EC may recognize a novel, common antigen expressed by damaged keratinocytes, but not conventional class I and II MHC molecules (24).

However, it should also be pointed out that the mere presence of high numbers of Thy-1$^+$ EC in epidermis is not sufficient to render the epidermis resistant to a T cell-mediated attack, since ear and body wall epidermis in naive mice contains high numbers of Thy-1$^+$ EC comparable to those in footpad epidermis 12 wk after injection of the T cells, but it is susceptible to destruction by the T cells (data not shown). There are at least two possible explanations for this discrepancy: one possible explanation is that extensive activation by damaged keratinocytes during the development of cutaneous GVHD would be required for Thy-1$^+$ EC residing in the epidermis to become functional. Consistent with this notion is our findings that the local resistance induced by the T cells was not clonotypic and that the ability of the T cells to induce the resistance paralleled the ability to induce the destruction of epidermal structures upon intradermal injection. Another possibility is that the Thy-1$^+$ EC that are found in increased numbers in the footpad epidermis of mice recovering from the cutaneous GVHD may represent a separate T lymphocyte lineage from Thy-1$^+$ EC originally residing in normal murine epidermis. Indeed, our preliminary staining analysis with the use of anti-TCR-γ/δ (3A10) and TCR-α/β (H57-597) mAbs indicates that a large number of the Thy-1$^+$ EC found in "resistant" epidermis seem to be TCR-γ/δ^+, while a small portion of the Thy-1$^+$ EC reacted with anti-TCR-α/β (Shiohara, T., N. Moriya, C. Gotoh, and M. Nagashima, unpublished data). In addition, our preliminary data using another set of anti-CD8 mAbs show that many if not all of the Thy-1$^+$ EC expressed very low levels of CD8. Given CD8 expression in the Thy-1$^+$ EC population, it is reasonable to speculate that the Thy-1$^+$ EC presumably involved in the resistance resemble intestinal intraepithelial lymphocytes (IEL) (25), a subset of γ/δ T cells resident in the gut, rather than Thy-1$^+$ EC originally residing in the epidermis. Because TCR gene usage is shown to be different in both T cell populations (26, 27), further investigations are certainly needed to identify TCR-γ/δ gene usage of the Thy-1$^+$ EC. Thus, studies to determine whether the Thy-1$^+$ EC could exhibit a limited diversity of TCR-γ/δ gene usage as do other T cells will provide important information about the heterogeneity of γ/δ TCR.

Understanding the cellular and molecular basis of the resistance to cutaneous GVHD will provide further insight into the protection mechanisms for epidermal integrity and provide alternative therapeutic approaches to cutaneous GVHD and other T cell-mediated skin diseases.

Summary

The cutaneous graft-versus-host disease (GVHD) lesions induced by intradermal injection of cloned autoreactive T cells have been shown to subside rapidly and the epidermis returns to normal 2 wk after injection. Those mice that had spontaneously recovered from the cutaneous GVHD became resistant to subsequent attempts to induce the cutaneous GVHD by the T cells while maintaining their activity to mount delayed-type hypersensitivity (DTH) responses and to induce the enlargement of the popliteal lymph nodes (PLN). The resistance appeared to be restricted to the epidermal structures of the injection sites, suggesting the involvement of lo-

cally acting suppression mechanisms. This local resistance was not specific for the clonotype used for the induction of the resistance. A loss of the epidermal integrity by an attack of T cells capable of producing cutaneous GVHD was a prerequisite for the induction of the resistance. By up to at least 8 mo after injection of the T cells, no mice became susceptible to the cutaneous GVHD again, provided that the T cells were injected into the same footpad sites that had initially received the T cells. This resistance correlated well with the great increase (20–30-fold) in Thy-1$^+$ EC number. The great increase in the number of Thy-1$^+$ EC following destruction of epidermal structures may be important in protecting the epidermal integrity from an additional attack by T cells.

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