PRESENTATION OF THE H-Y ANTIGEN ON LANGERHANS' CELL-NEGATIVE CORNEAL GRAFTS DOWNREGULATES THE CYTOTOXIC T CELL RESPONSE AND CONVERTS RESPONDER STRAIN MICE INTO PHENOTYPIC NONRESPONDERS

By JOHN S. PEELER,* DAVID G. CALLANAN,‡ MARTHA W. LUCKENBACH,‡ AND JERRY Y. NIEDERKORN‡

*From the Departments of *Cell Biology and ‡Ophthalmology, The University of Texas Southwestern Medical Center, Dallas, Texas 75235*

The CTL response to the male-specific histocompatibility antigen (H-Y) in mice is controlled by gene products encoded at both H-2 and non-H-2 loci (1). The best characterized of these regulatory genes have been mapped based on the existence of "response-permissive" and "nonpermissive" alleles to loci within the murine MHC, H-2, and are expressed phenotypically as membrane proteins that function as restricting elements for T cell recognition of H-Y (2). In mice carrying all or part of the $H-2^b$ haplotype, the inductive phase of the CTL response is controlled by $I-A^b$, a dominant genetic locus mapping within the class II region of H-2 (2–4). T helper cell recognition of H-Y in association with $I-A^b$ molecules expressed on the surface of APC is required for the delivery of proliferation and differentiation signals to primed CTL precursors and the maturation of effector function (3–5).

Understanding the cellular events leading to the activation of T cells responding in vivo to cell surface alloantigens expressed on transplanted tissue, or during mixed lymphocyte responses in vitro, is complicated by the presence of two populations of APC. The relative contributions of graft-derived APC and host-derived APC during the initiation of immune responses to transplanted allogeneic tissue are not well understood.

Langerhans' cells (LC)[1] are bone marrow–derived dendritic cells found almost exclusively within stratified squamous epithelium of mammals and they form a reticulum of APC at the most peripheral outpost of the immune system (6). Recent studies suggest that epidermal LC represent immature precursors of the "interdigitating" dendritic cells found within T cell areas of the spleen and lymph nodes (7–8). Although cell surface expression of Ia molecules is induced on epidermal keratinocytes during delayed-type hypersensitivity (DTH) reactions in skin (9) and after grafting of syngeneic or allogeneic skin onto nude mice (10), results of studies carried out

[1] *Abbreviations used in this paper:* CM, conditioned medium; DTH, delayed-type hypersensitivity; LC, Langerhans' cells; PGE_2, prostaglandin E_2; RI, rejection index.

in vitro (11) and in vivo (12–14) indicate that constitutive expression of Ia molecules and the antigen-presenting capacity of epidermal cells are exclusive properties of the resident LC population. Thus conventional protein antigens or haptens introduced through epithelial tissues are believed to be processed by LC and "carried" to a regional lymphoid organ where further maturation of LC occurs and the antigen is presented to T cells.

The cornea of rodents and other mammalian species represents a naturally occurring hole in the antigen trap formed by LC because IA^+ CL are normally absent from its stratified squamous epithelium (15). In the present study we have used the murine cornea as a graft model to investigate the role of donor-derived LC in the regulation of anti-H-Y CTL and graft rejection in responder strain mice. Our results show that there are differential requirements for processing and presentation of alloantigens. In the absence of LC of graft origin, the H-Y antigen does not appear to be reprocessed in immunogenic form, but instead is presented in a manner that leads to downregulation of the anti-H-Y CTL response; however, H-2 alloantigens and some non-H-2 alloantigens are presented to host T cells either directly by nonspecialized graft-derived APC or after reprocessing by host APC.

Materials and Methods

Mice. Adult male and female C57BL/6 ($H-2^b$) (B6), C57BL/10 ($H-2^b$) (B10), B10.BR ($H-2^k$), B10.D2 ($H-2^d$), A.TL ($H-2^{t1}$), A.TH ($H-2^{t2}$), BALB/c ($H-2^d$), and DBA/2 ($H-2^d$) mice were purchased from The Jackson Laboratory, Bar Harbor, ME.

Culture Media. Cell cultures were performed in RPMI 1640 (KC Biological, Lenexa, KS) containing 10% heat-inactivated FCS, 2.0 mM L-glutamine, 5×10^{-5} M β2-ME, 10 mM Hepes buffer, 1.0 mM sodium pyruvate, penicillin (100 IU/ml), streptomycin (100 μg/ml), and Fungizone (0.25 μg/ml) (complete media). Full-thickness corneal tissue was incubated in McCarey-Kaufman media (MK) (16) containing 10% FCS, 20 mM Hepes, 1.0 mM sodium pyruvate, 2 mM L-glutamine, antibiotics, and other additives as indicated.

Antibodies and Reagents. Monoclonal hybridomas 25-9-3s (anti-IA^b, mouse IgM), MK-D6 (anti-IA^d, mouse IgG2a), 26-7-11s (anti-IA^k, mouse IgM), and GK1.5 (anti-L3T4, rat IgG2b) were obtained from the American Type Culture Collection (Rockville, MD). Hybridoma culture supernatants were used directly or after precipitation of the Ig fraction with $(NH_4)_2SO_4$ (3.9 M, 0°C). FITC-conjugated goat anti–mouse IgG (Becton Dickinson & Co., Mountain View, CA) and FITC-conjugated or peroxidase-conjugated goat anti–mouse IgM (Cappel Laboratories, Malvern, PA) were used as secondary reagents (1:40 dilution). Purified rIFN-γ (rat, 10^7 U/mg) was obtained from Amgen Biologicals, Thousand Oaks, CA. rIL-2 (mouse, 10^6 U/g) was from Genzyme, Boston, MA. Prostaglandin-E_2 was from Sigma Chemical Co., St. Louis, MO.

Cornea Grafting. Full-thickness corneal grafts were prepared and grafted to heterotopic subdermal beds on the thoraces of recipient mice as described (17). Two corneas were grafted onto each recipient mouse. LC^- central corneal grafts were cut to a diameter of ∼2.0 mm. Corneal limbus (LC^+) grafts were cut to a diameter of ∼3.2 mm to include a rim of LC-containing conjunctival tissue. Grafts were evaluated at weekly intervals and given a score from 0 to 4 based on corneal clarity. Clear corneas were scored as 0. Completely opaque or absent corneas were scored as 4 and considered rejected. This rejection index (RI) was shown previously to correlate well with histopathologic findings (17–18). Grafts lost before day 7 were scored as technical failures (<1%) and eliminated from the study.

Skin Grafting. Full-thickness abdominal skin was taken from donor animals and 3.0-mm diameter circular grafts similar in size to the murine cornea were cut with a surgical trephine. The skin was grafted to subdermal beds (two grafts/bed) as described (19). Skin grafts were also assigned a numerical score of 0 to 4 based on the percentage of necrotic tissue.

Mixed Leukocyte Cultures. Single cell suspensions were prepared from lymph nodes or spleens

harvested from normal and grafted mice. Splenic stimulator cells were prepared in a similar manner and irradiated (3,000 rad, ^{137}Cs source). Responder cells (5 × 10^{6}) were added to equal numbers of stimulator cells in 24-well trays and cocultured for 2–5 d at 37°C in a humidified 5% CO_2 atmosphere.

Cytotoxic T Cell Assay. Effector cells were harvested from mixed leukocyte cultures and Log_2 serial dilutions were dispensed in triplicate into 96-well round-bottomed plates. Blast target cells were prepared by culturing spleen cells for 5 d in media containing 15% supernatant derived from Con A–activated (5 µg/ml) rat spleen cells. Con A (2.0 µg/ml) or LPS (30 µg/ml) was added on the third day of culture. Blast cells were radiolabeled by incubation (60 min, 37°C) in HBSS (10[6] cells/ml) containing 200 µCi/ml $Na_2^{51}CrO_4$. E/T ratios usually ranged from 50:1 to 6:1. Supernatant was collected after a 4-h incubation and radioactivity was determined in a gamma counter. Spontaneous chromium release from blast target cells was usually <25%.

Elicitation of LC Migration into Corneal Epithelia. Introduction of inert phagocytic stimuli into the central corneal epithelium of mice was previously shown to induce migration of LC from the periphery (19). A superficial epithelial incision ~1 mm in length was cut into the central cornea of anesthetized mice using an ultra-thin razor blade. Polystyrene latex beads (1.0-µm diameter; Polysciences Inc., Warrington, PA) were sterilized by gamma irradiation (10,000 rad) and applied to the surface of the cornea. Migration of Ia^{+} dendritic cells (LC) into the central epithelium was shown by indirect immunofluorescence of corneal epithelial sheets removed from mice 2–7 d after implantation of latex beads. Latex beads were internalized by corneal epithelial cells and the corneas were free of inflammation and neovascularization 7 d after treatment. Latex bead–treated cornea (LC[+]) were excised from donor mice after 7 d and grafted onto recipient mice.

Conditioned Media (CM). Equal numbers (5 × 10^{6}) of lymph node cells from BALB/c and C57BL/6 mice were irradiated (3,000 rad) and cocultured in complete RPMI media for 48 h. Culture supernatant was concentrated 10-fold by vacuum dialysis and used as a source of Ia-inducing factors (i.e., IFN-γ). The activity of CM was assayed by its ability to induce Ia expression on an Ia[-] cell line (P388D1) (20) and was shown by indirect immunofluorescence to have maximal Ia-inducing activity when used at concentrations of 5% (vol/vol) (data not shown).

Induction of Ia Expression on Corneal Cells. LC[-] and LC[+] cornea were harvested and incubated for 2–3 d in MK culture media alone or in MK containing 5% conditioned media or 500 U/ml rIFN-γ (rat, 0.05 µg/ml) in the presence or absence of 10^{-6} M prostaglandin-E_2 (PGE_2).

Immunofluorescence Histology. Corneal epithelial sheets were separated from the underlying stroma after a 2-h incubation at 37°C in HBSS (without calcium and magnesium) containing 20 mM EDTA and 20 mM Hepes buffer (pH 7.3). Acetone-fixed epithelial sheets were incubated (12–16 h, 4°C) in anti-Ia hybridoma supernatant followed by 2-h incubation with FITC-conjugated goat anti-mouse Ig (1:40 dilution). LC were identified by dendritic morphology and cell surface Ia. In some experiments, corneas were removed from graft beds 3–10 d after grafting and observed for the presence of Ia[+] LC or the expression of Ia on corneal cells. Corneal tissue was fixed in acetone (45 min, 4°C) and incubated with anti-Ia antibodies (12 h, 4°C). Flattened whole mounts were observed for membrane expression of Ia on epithelial cells, endothelial cells, and stromal fibroblasts.

Results

Donor-derived LC Are Required for Activation of Anti-H-Y CTL in Grafted Mice. C57BL/6 female mice (three to four mice per panel) received either LC[-] central cornea grafts, LC[+] corneal limbus grafts, or LC[+] skin grafts from syngeneic male donors. Additional panels of mice received LC[+] central corneal grafts that were treated in situ with sterile latex beads 7–10 d before removing the cornea from the donor. LC were absent from the central corneal epithelium of >90% of untreated mice (Fig. 1 *a*) but were always abundant in the peripheral areas of the cornea (limbus) and within
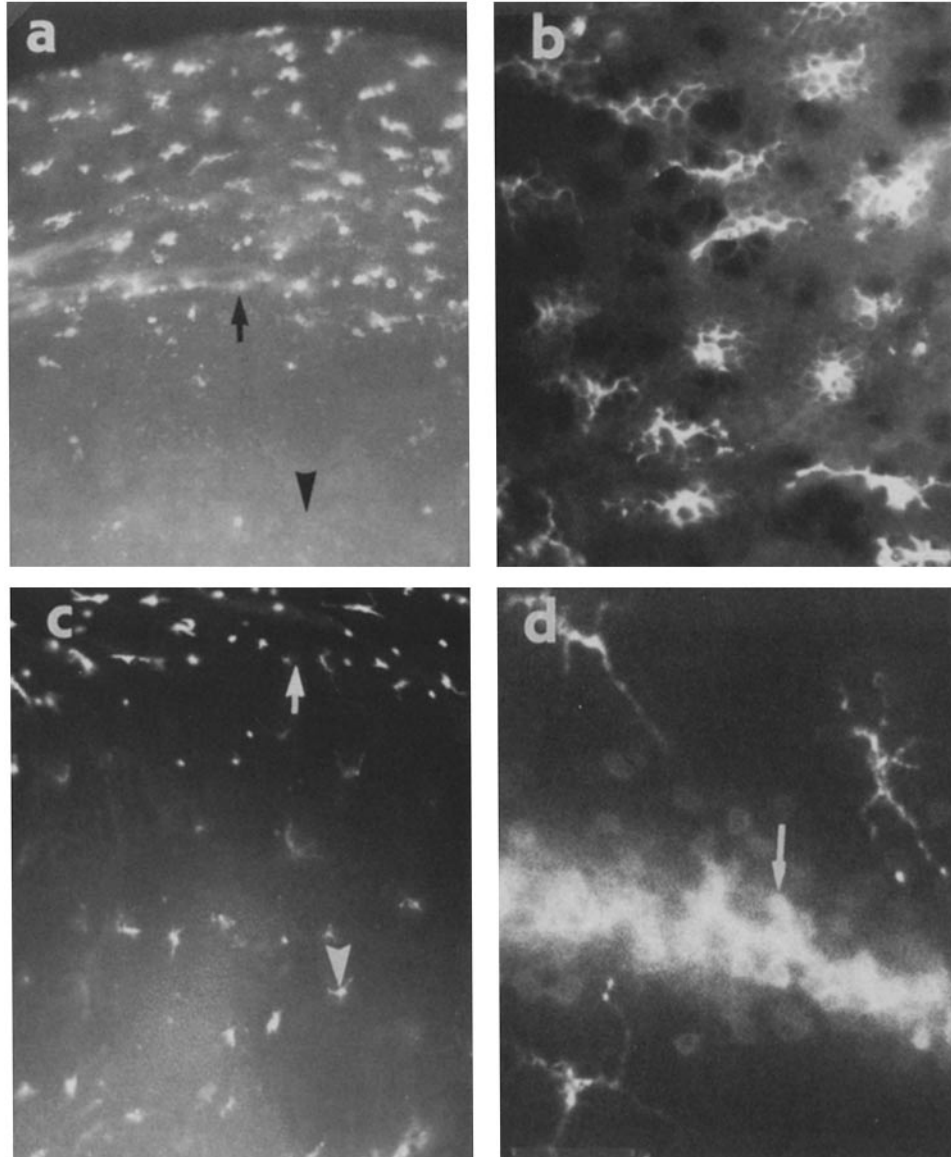
FIGURE 1.   Immunofluorescence detection of LC within corneal epithelium of C57BL/6 mice. Corneal epithelial sheets were removed from normal or latex bead–treated cornea and dendritic LC were detected by indirect immunofluorescence using anti-IA^b antibody (25-9-3s) followed by FITC-conjugated goat anti–mouse IgM. (a) IA$^+$ LC were absent from the central epithelium (arrowhead) but were present near the corneal-limbus junction (arrow) and (b) within the conjunctival epithelium adjacent to goblet cells. (c) Latex beads stimulated LC to migrate from the limbus (arrow) into the central corneal epithelium (arrowhead). (d) LC assumed a rounded morphology within the linear incision (arrow) in which the latex beads were deposited.

the conjunctival epithelium adjacent to goblet cells (Fig. 1 *b*). Within 2–10 d after treatment of the cornea with latex beads, Ia$^+$ dendritic LC were seen within the central cornea in numbers approximately one-fourth to one-half the density of LC within the conjunctival epithelium ($\sim$800 LC/mm^2) (Fig. 1 *c*). LC migrated into the linear incision in which the latex beads were deposited and assumed a rounded morphology (Fig. 1 *d*). These rounded cells expressed membrane Ia and ATPase activity but surface Ig was absent. At higher magnification, short dendritic processes were observed but conspicuous vacuoles were absent. Based on these observations we believe these cells to be LC rather than B cells or macrophages. Cornea receiving only a superficial epithelial laceration without the addition of latex beads did not contain LC.

Cells were harvested from the draining lymph nodes of mice on various days after grafting and boosted in vitro for 5 d with irradiated spleen cells from syngeneic males before assaying on ^{51}Cr-labeled target cells. The results of representative experiments are shown in Table I. Secondary anti-H-Y CTL responses were not detected either 14, 21, 28, or 35 d after immunization of female mice with LC$^-$ male central corneal grafts. Spleen cells harvested from the same animals also failed to lyse syngeneic male target cells. By contrast, immunization of female mice with LC$^+$ skin, LC$^+$ corneal limbus, or LC$^+$ latex-treated corneal grafts from male donors elicited MHC-restricted anti-H-Y CTL responses. Less than 5% of the total number of animals immunized with LC$^-$ corneal grafts showed positive anti-H-Y CTL responses, while >90% of animals primed with LC$^+$ grafts responded.

Initiation of CTL Responses to H-2 or Multiple Minor-H Antigens Is Independent of Graft-derived LC. The requirement for graft-derived LC during the initiation of CTL

TABLE I

CTL Responses to the H-Y Antigen Presented on LC$^-$ or LC$^+$ Grafts

			Percent ^{51}Cr-release						
		Target cells:	B6♂				B10.BR♂	BALB/c♂	B6♀
Day	Graft type	E/T ratios:	50:1	25:1	12:1	6:1	50:1	50:1	50:1
14	None		6.8	5.1	<0	<0	<0	<0	<0
	Central		4.5	<0	<0	<0	<0	3.3	<0
	Skin		24.6	21.9	11.4	3.9	1.0	<0	<0
	Limbus		38.0	28.9	14.6	6.1	<0	0.4	<0
	Latex		49.8	33.5	24.8	10.9	3.4	1.2	0.6
21	None		2.8	0.9	<0	<0	<0	<0	<0
	Central		1.3	<0	<0	<0	<0	<0	<0
	Skin		29.0	27.5	10.2	4.8	0.6	<0	<0
	Limbus		47.5	45.8	27.1	18.9	3.2	2.1	3.6
	Latex		65.3	44.5	29.5	19.7	4.8	5.7	2.5
28	None		2.9	2.7	1.3	1.0	<0	ND	ND
	Central		3.6	2.9	1.8	1.8	7.9	ND	ND
	Skin		48.3	46.9	37.6	34.1	0.3	ND	ND
	Limbus		45.7	35.4	29.5	23.4	1.2	ND	ND
35	None		3.2	2.1	0.2	<0	9.8	ND	4.3
	Central		4.5	0.9	0.9	0.6	0.5	ND	2.2
	Skin		41.2	33.9	21.0	8.5	2.1	ND	9.0
	Limbus		46.1	39.9	24.0	10.5	<0	ND	2.8

responses to H-2 alloantigens and multiple minor-H antigens was also examined. Primary CTL responses to class I + class II differences were assayed directly on chromium-labeled Con A blast target cells. Detection of CTL responses to class II MHC alloantigens required restimulation in vitro for 2 d with alloantigen and the effector cells were assayed on LPS-activated (30 µg/ml) blast target cells (19). CTL responses to multiple minor-H antigens required restimulation for 5 d in vitro before assaying on Con A-activated target cells. The results (summarized in Table II) demonstrated that CTL responses to class I and class II alloantigens (H-2^d and H-2^k), class II alloantigens only (H-2I^k), or multiple minor-H alloantigens were initiated independently of graft-derived LC. Both LC$^-$ and LC$^+$ corneal allografts efficiently immunized recipient mice.

Rejection Responses to H-Y Require Initiation by Donor-derived LC. LC$^-$ male corneal grafts survived for prolonged duration (average RI = 1.4, day 35) on syngeneic female recipients when compared with LC$^+$ corneal limbus grafts (RI = 3.8, day 35) or LC$^+$ skin grafts (RI = 4.0, day 35) (Fig. 2). Preservation of tissue integrity was observed for both LC$^+$ female cornea (Fig. 3 a) and LC$^-$ male cornea (Fig. 3 b) 21 d after heterotopic grafting. These grafts contained few inflammatory cells and minimal vascularization. Descemet's membrane was intact but corneal endothelial cells were not found. By contrast, LC$^+$ cornea grafts (day 21) contained lymphocytes, neutrophils, and macrophages invading the corneal stroma and epithelium from the graft periphery and posterially through breaks in Descemet's membrane (Fig. 3 c).

As reported previously (19) LC$^-$ or LC$^+$ corneas grafted onto H-2-disparate hosts are rejected within 14–18 d, while LC$^-$ cornea grafted onto class II–disparate hosts enjoy indefinite (15) or prolonged (19) survival. However, these cornea were rejected

TABLE II

CTL Responses to H-2 and Multiple Minor-H Alloantigens Presented on LC$^-$ or LC$^+$ Grafts

| Host	Donor	Graft type	Genetic disparity	E/T ratios:	Percent ^{51}Cr-release					
					50:1	25:1	12:1	6:1	50:1	50:1
					B10.D2				B10.BR	BALB/c
C57BL/10	None	None	H-2^d		6.8	5.9	4.8	3.6	2.0	<0
C57BL/10	B10.D2	Central	H-2^d		31.8	21.2	9.3	4.8	1.4	38.5
C57BL/10	B10.D2	Limbus	H-2^d		52.0	34.5	20.3	12.6	3.8	43.6
C57BL/10	B10.D2	Latex	H-2^d		41.9	29.5	13.6	8.9	4.1	52.4
C57BL/10	B10.BR	Central	H-2^k		6.7	–	–	–	37.5	5.3
					A.TL				B10.BR	BALB/c
A.TH	None	None	H-2I^k		5.3	8.0	2.7	3.1	8.4	4.3
A.TH	A.TL	Central	H-2I^k		29.4	19.2	11.1	7.1	32.7	4.6
A.TH	A.TL	Limbus	H-2I^k		23.4	18.9	13.8	8.9	36.7	9.5
A.TH	A.TL	Latex	H-2I^k		49.1	28.6	19.2	5.4	52.4	8.6
					DBA/2				B10.BR	B10.D2
BALB/c	None	None	Minor-H		9.6	4.4	0.9	<0	<0	<0
BALB/c	DBA/2	Central	Minor-H		49.9	40.6	29.4	21.5	<0	<0
BALB/c	DBA/2	Limbus	Minor-H		42.7	29.0	14.6	8.3	<0	<0
BALB/c	DBA/2	Latex	Minor-H		51.5	35.1	20.7	13.5	<0	<0

CTL responses were measured 14 d after grafting.
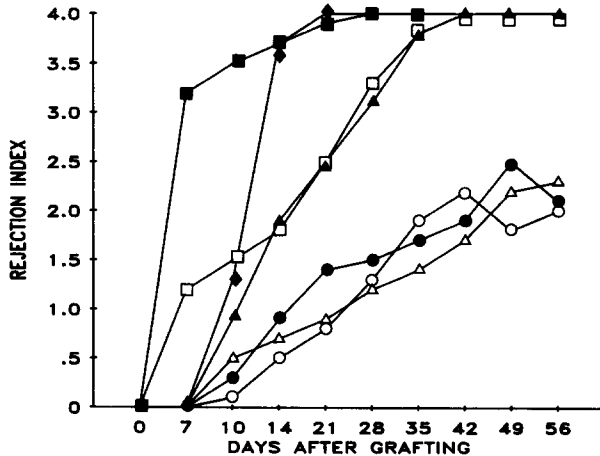
FIGURE 2. Rejection kinetics of LC⁻ and LC⁺ male cornea after heterotopic grafting to syngeneic female mice. Female LC⁻ cornea (O), female LC⁺ limbus cornea (●), male LC⁻ cornea (△), male LC⁺ cornea (▲), or male skin (◆) from C57BL/6 donor mice was grafted onto normal syngeneic female mice. Additional female mice were presensitized with male skin grafts 21 d before grafting them with male LC⁻ cornea (□) or male LC⁺ cornea (■). At least 20 individual corneal grafts on 10 individual recipient mice were scored based on graft clarity. For comparison with corneal grafts, skin grafts were evaluated (based on graft necrosis) using the same RI scale.

within 16–20 d by hosts immunized with skin grafts, indicating that class II antigens can be expressed on LC⁻ cornea (data not shown). LC⁻ cornea grafts differing from the host at multiple minor-H loci were rejected within 21–28 d (data not shown).

The triple correlation between the presence of LC within the cornea graft, the ability of the graft to immunize for anti-H-Y CTL responses, and prolonged graft survival might indicate that H-Y antigens are expressed only by LC and not by other cellular components of the central cornea. However, female mice that were sensitized with male skin grafts rejected LC⁻ male corneal grafts (RI = 3.8, day 35), although the rejection kinetics was retarded compared with LC⁺ cornea grafted onto presensitized recipients (Fig. 2). 21 d after grafting, these cornea were characterized by a pronounced proliferation of fibrovascular tissue and an absence of blood vessels, inflammatory cells, and stromal fibroblasts indicative of ischemic necrosis (Fig. 3 d).

Allogeneic Cross-Priming for Anti-H-Y CTL Responses Requires Graft-derived LC. Based on functional evidence, H-Y antigens were expressed by cells of LC⁻ cornea, yet did not appear to be processed by host APC in an immunogenic form. Experiments were carried out to determine if mice could be primed allogeneically (21) with H-Y antigens presented on LC⁻ H-2-incompatible corneal grafts. C57BL/10 female mice were grafted with LC⁺ or LC⁻ cornea from syngeneic male donors or from H-2-incompatible B10.BR or B10.D2 male donors. Allogeneic cross-priming for CTL responses did not occur unless donor-derived LC were present in the immunizing graft (Table III). B10 female recipients of B10.BR male LC⁻ central corneal grafts were not primed to respond to H-Y in the context of H-2ᵇ stimulators and target cells, while male B10.BR LC⁺ limbus grafts efficiently cross-primed B10 female mice. Similar results were obtained with cells from B10 female mice primed with B10.D2 male corneal grafts. Spleen cells from B10 female mice primed in vivo with H-Y antigen on B10.BR LC⁺ limbus grafts did not reprocess H-Y presented in vitro on B10.BR male stimulator cells since they failed to lyse syngeneic male target cells.

Corneal Cells Are Induced to Express Ia after Grafting of LC⁻ Cornea Independent of Rejection. Experiments were carried out to determine if host-derived LC migrated into corneal allografts after grafting. LC⁻ corneas from either male or female
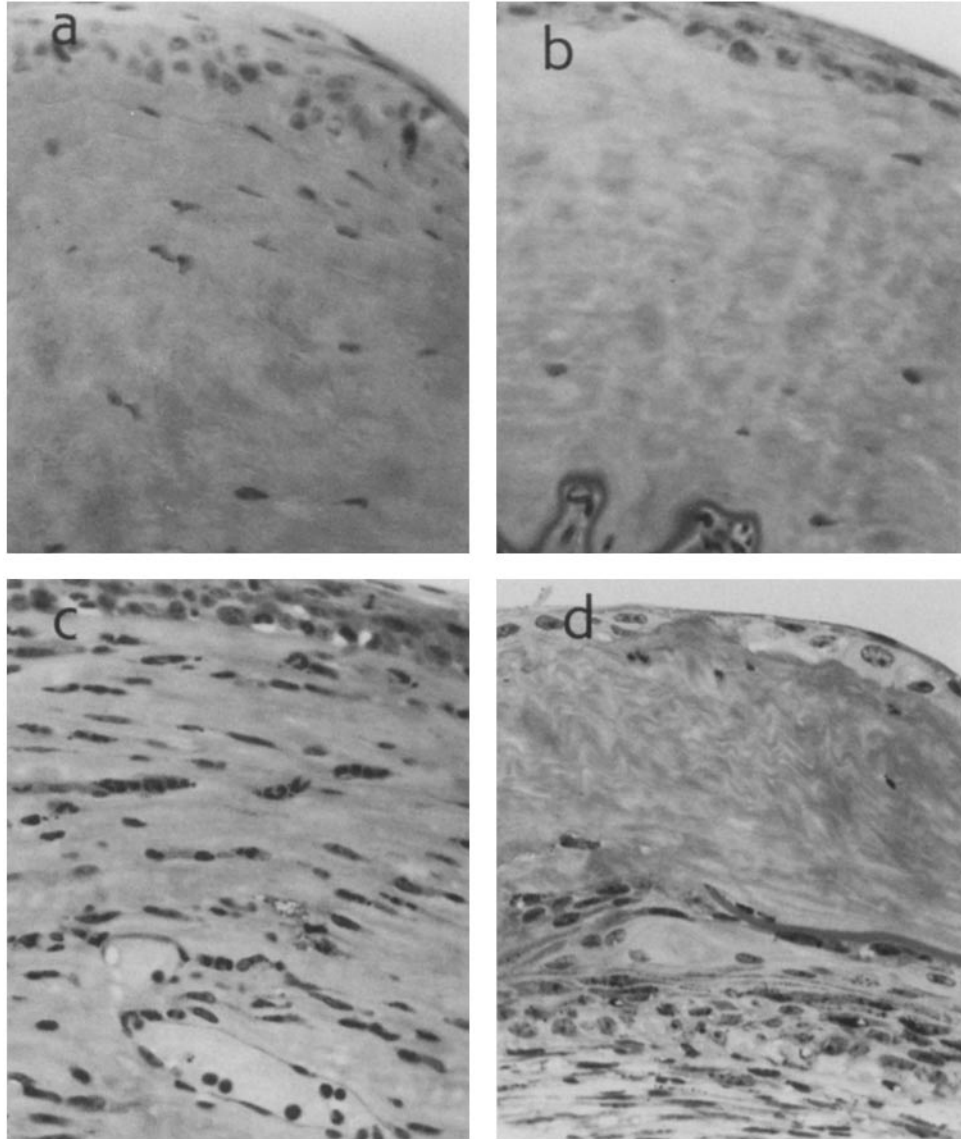
FIGURE 3.   Histology of LC⁻ and LC⁺ male and female cornea 21 d after heterotopic grafting onto syngeneic female mice. Heterotopic corneal grafts were removed from grafted animals and 3.0 μm sections were cut and stained with PAS. (a) Female LC⁺ cornea; (b) Male LC⁻ cornea; (c) Male LC⁺ cornea; (d) Male LC⁻ cornea grafted to presensitized female (fibrovascular scar tissue [arrow] is all that remains of this graft).

C57BL/6 donor mice were grafted onto syngeneic female recipients. Grafts were removed after 7 d by excising full-thickness skin surrounding the graft bed, and epithelial sheets were stained with anti-IAᵇ antibody (clone 25-9-3s).

Host-derived IA⁺ LC were absent from male central corneal grafts 7 d after

TABLE III
Allogeneic Cross-Priming for Anti-H-Y CTL Responses Requires Graft-derived LC

					Percent ^{51}Cr-release					
		Graft	In vitro	Target cells:	B10σ				B10.BRσ	B10.D2σ
Host	Donor	type	boost	E/T ratios:	50:1	25:1	12:1	6:1	50:1	50:1
B10	None	None	B10		2.9	3.2	1.7	<0	4.6	3.1
			B10.BR		4.3	<0	<0	<0	35.5	<0
B10	B10	Central	B10		7.3	6.7	<0	<0	5.1	2.8
			B10.BR		2.1	<0	<0	<0	42.2	6.4
B10	B10	Limbus	B10		42.4	33.2	22.6	8.5	7.2	5.3
			B10.BR		6.4	3.5	<0	<0	48.6	3.9
B10	B10.BR	Central	B10		8.7	5.5	7.4	0.9	3.8	4.2
			B10.BR		6.4	5.7	4.3	<0	47.1	<0
B10	B10.BR	Limbus	B10		47.1	33.2	28.4	12.8	5.3	2.4
			B10.BR		7.9	6.4	<0	<0	55.2	5.1
B10	B10.D2	Central	B10		2.6	2.1	1.4	0.2	3.8	4.8
			B10.D2		4.1	<0	0.9	<0	5.4	43.6
B10	B10.D2	Limbus	B10		34.9	22.0	16.7	8.2	<0	5.3
			B10.D2		8.1	5.6	<0	0.5	7.3	51.6

grafting onto syngeneic females; however, corneal epithelial cells expressed membrane Ia determinants (Fig. 4 a). Examination of flattened whole mounts of cornea (epithelium removed) revealed that stromal fibroblasts from grafted cornea, but not normal control cornea, also expressed Ia (data not shown). Corneal endothelial cells were not found on grafted corneas. Induced expression of Ia molecules on corneal cells occurred despite the prolonged survival of LC⁻ male grafts. Moreover, expression of Ia was found on corneal epithelial cells after heterotopic grafting of female cornea onto syngeneic female recipients (data not shown). Thus LC⁻ corneal grafts expressed H-Y antigens (by functional criteria) as well as Ia antigens (immunofluorescence), yet did not induce CTL responses or graft rejection.

Induction of Ia Expression on LC⁻ Cornea Before Grafting Circumvents the Requirement for LC. LC⁻ and LC⁺ corneal grafts were prepared from C57BL/6 male donors and placed into MK media containing 5% MLR culture supernatant. This media contains factors (probably IFN-γ) capable of inducing Ia expression on P388D1, B16 melanoma, and a variety of other normal and transformed cell lines (data not shown). Corneas were cultured in conditioned media in the presence or absence of 10⁻⁶ M PGE₂ as an inhibitor of IFN-induced Ia expression (22). Corneas were incubated for 2-3 d at 37°C in an atmosphere of 5% CO₂. Epithelial sheets were removed and stained for surface Ia by indirect immunofluorescence. Corneal epithelial cells expressed Ia after incubation of full-thickness cornea in conditioned media (Fig. 4 d) but not when incubated in MK control media (Fig. 4 b) or in conditioned media containing PGE₂ (Fig. 4 c). Incubation of full-thickness corneal explants in conditioned media also resulted in expression of Ia by corneal endothelial cells and stromal fibroblasts (data not shown). Ia expression was also induced on corneal epithelial cells, endothelial cells, and stromal fibroblasts by purified rIFN-γ (rat) (500 U/ml), but was inhibited by coculturing with PGE₂ (data not shown).

LC⁻ or LC⁺ male corneas were incubated in conditioned media and grafted onto
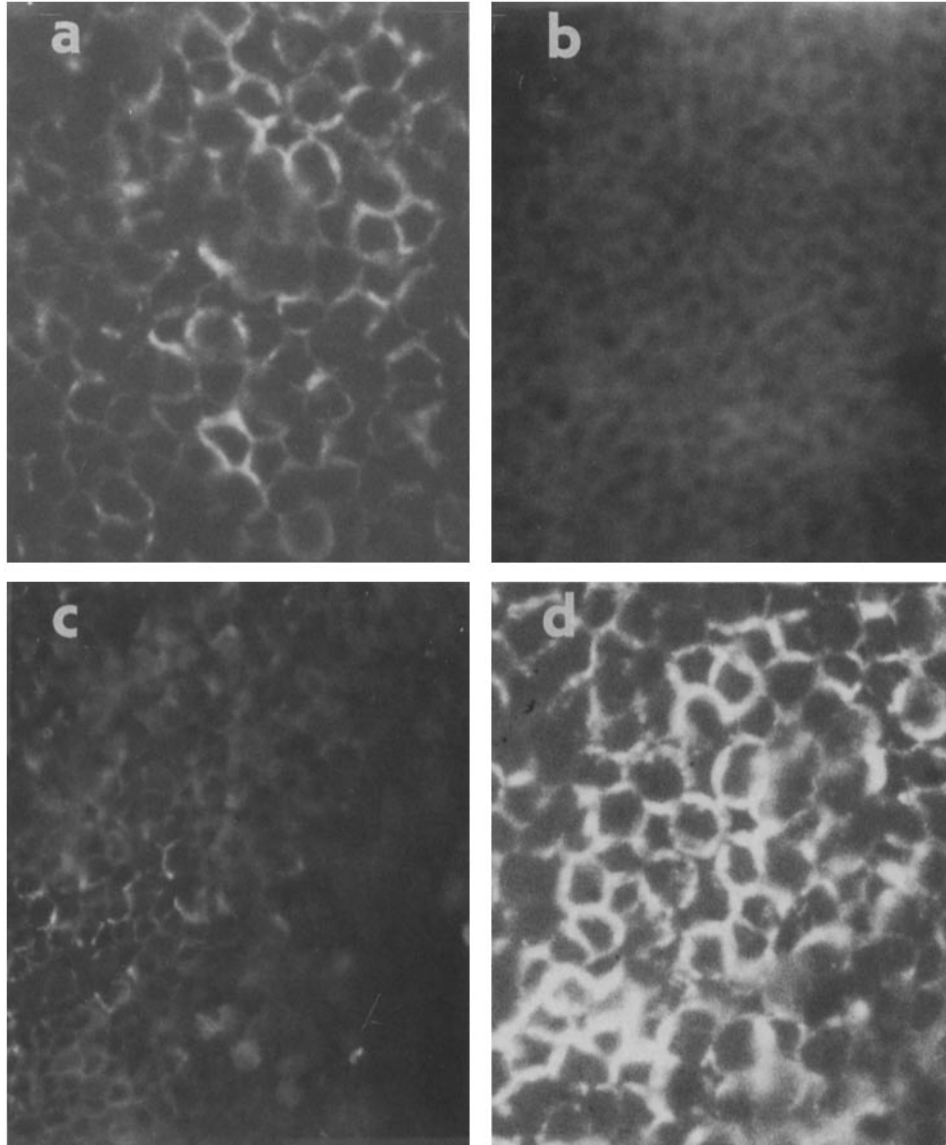
FIGURE 4.   Induced expression of Ia on corneal epithelial cells after heterotopic grafting or incubation of full-thickness cornea in CM. (a) C57BL/6 male LC⁻ corneas were excised from the graft beds 7 d after heterotopic grafting onto syngeneic female mice. Corneal epithelial sheets were stained by indirect immunofluorescence using anti-IA^b antibody 25-9-3s and FITC-conjugated goat anti–mouse IgM. Additional freshly prepared LC⁻ cornea grafts were incubated for 3 d in MK media (b) or in MK containing 5% CM in the presence (c) or absence (d) of 10^{-6} M PGE$_2$.

syngeneic C57BL/6 female recipients. Induction of Ia on LC⁻ corneas before grafting partially restored the ability of the graft to immunize the host for anti-H-Y CTL responsiveness (Table IV). The effect of conditioned media on CTL responses was reversed by co-incubation of LC⁻ corneas with PGE$_2$. The immunogenicity of

TABLE IV

Induced Ia Partially Restores the Anti-H-Y CTL Response to LC⁻ Cornea

| | | | Percent ^{51}Cr-release | | | | |
| | | | C57BL/6♂ | | | | B10.BR♂ |
Graft type	Graft treatment	Target cells: E/T ratios:	50:1	25:1	12:1	6:1	50:1
None	None		4.4	3.6	1.4	2.8	0.4
Central	MK media		5.1	0.9	0.6	1.6	<0
Limbus	MK media		52.5	53.6	39.8	31.5	1.9
Latex	MK media		35.6	27.4	18.9	13.8	<0
Limbus	MK + CM		46.3	39.5	22.4	16.6	2.3
Limbus	MK + CM + PGE₂		21.9	12.2	8.9	7.3	3.2
Central	MK + CM		18.7	13.5	9.9	6.5	<0
Central	MK + CM + PGE₂		4.3	3.5	4.8	2.1	0.7

Corneas taken from C57BL/6 male donor mice were incubated for 3 d (37°C, 5% CO_2) in MK media only or in MK containing 5% CM in the presence or absence of 10^{-6}M (PGE_2) before heterotopic grafting onto syngeneic female recipients. Cells were harvested from the draining lymph nodes of recipient mice 14 d later and secondary CTL responses were measured.

LC⁺ corneal limbus grafts after incubation in conditioned media was similar to that of untreated LC⁺ grafts but was partially reduced by the addition of PGE_2.

Donor LC are Required for the Activation of Anti-H-Y T Helper Cells During In Vivo Priming. The requirement for L3T4⁺ Th and IL-2 during restimulation with H-Y in vitro was investigated. IL-2-containing culture supernatant from Con A–activated rat spleen cells (IL-2 media) was added to secondary boost cultures as a source of nonspecific helper factors. Addition of 10% (vol/vol) IL-2 media did not restore the anti-H-Y CTL response to LC⁻ cornea (Table V). Addition of L3T4 antibody (clone GK-1.5) to the culture media (1:200 dilution) at the initiation of the boost cultures abolished the anti-H-Y CTL response to LC⁺ corneal limbus grafts but

TABLE V

T Helper Cell Requirements for Anti-H-Y CTL Activation

| | | | | Percent ^{51}Cr-release | | | | | | |
| Graft type | Additions in vitro | | Target cells: E/T ratios: | B6♂ | | | | B10.BR♂ | BALB/c♂ | B6♀ |
	Anti-L3T4	IL-2		50:1	25:1	12:1	6:1	50:1	50:1	50:1
None	−	−		3.4	4.2	3.6	1.1	6.8	8.2	3.4
None	−	+		6.3	5.4	4.8	<0	8.2	7.3	5.6
Central	−	−		0.6	1.6	1.2	2.4	0.5	ND	ND
Central	−	+		5.4	6.2	3.6	1.1	8.2	5.7	4.9
Limbus	−	−		44.4	38.3	21.2	14.2	8.2	7.4	3.6
Limbus	−	+		68.5	35.4	19.8	14.4	5.4	7.5	5.1
Limbus	+	−		6.3	5.8	4.9	<0	1.5	2.9	4.9
Limbus	+	+		48.3	36.8	25.6	10.6	4.8	6.5	3.9
Latex	−	−		44.2	33.4	25.2	19.5	6.8	7.5	4.3
Latex	−	+		67.8	40.8	33.2	20.1	5.5	4.7	6.2
Ia⁺LC⁻	−	−		26.4	12.6	8.2	5.4	5.6	7.4	5.1
Ia⁺LC⁻	−	+		53.5	33.6	19.7	9.6	9.2	14.1	6.2

IL-2-containing media (10%) and/or anti-L3T4 mAb (GK1.5, 1:200 dilution) was added at the initiation of the 5-d boost. Some panels of mice received central corneal grafts (Ia⁺ LC⁻) that had been incubated in conditioned media as described in Table IV.

the response was restored by the addition of 10% IL-2 media. Addition of purified rIL-2 (150 U/ml, 1.5 × 10-4 g/ml) also failed to restore the CTL response to LC⁻ cornea (data not shown).

Presentation of H-Y Antigen on LC⁻ Grafts Downregulates the Anti-H-Y CTL Response. The possibility that presentation of the H-Y antigen on APC-deficient grafts resulted in active suppression (23) was investigated in cell mixing experiments. The results summarized in Table VI show the secondary CTL responses of individual mice immunized with H-Y presented on LC⁻ or LC⁺ corneal grafts. Positive anti-H-Y CTL responses were obtained from four mice immunized with LC⁺ limbus corneal grafts (Nos. 3-6). Only one animal (No. 9) responded to immunization with LC⁻ cornea. Coculture of spleen cells from limbus-immunized animals with equal numbers of cells from unsensitized (naive) animals had no effect on anti-H-Y CTL responses after a 5-d boost with male stimulator cells. However, coculture of cells from limbus-immunized animals with cells from mice immunized with LC⁻ central cornea reduced CTL responses to background levels in three out of four animals tested. Spleen cells from one animal (No. 9) that responded to immunization with LC⁻ grafts failed to suppress the response of limbus-primed cells.

Discussion

The activation of CTL responding to alloantigens in primary MLC requires participation of a specialized population of Ia⁺ accessory cells. Elimination of alloreactivity is achieved only by removing Ia⁺ APC from both responder and stimulator

TABLE VI

Presentation of the H-Y Antigen on LC⁻ Cornea Grafts Downregulates the Anti-H-Y CTL Response

Culture no.	Graft type	Target cells: E/T ratios:	Percent ^{51}Cr-release				
			C57BL/10				B10.BR
			25:1	12:1	6:1	3:1	25:1
1	None		<0	<0	<0	<0	<0
2	None		<0	<0 <0	<0	<0	<0
3	Limbus		26.1	25.4	15.5	8.9	2.8
4	Limbus		32.8	34.2	29.9	23.5	<0
5	Limbus		39.0	26.0	21.9	12.8	<0
6	Limbus		36.1	39.1	32.8	25.9	27.2
7	Central		<0	<0	<0	<0	<0
8	Central		<0	<0	<0	<0	<0
9	Central		24.9	15.7	9.3	0.6	<0
10	Central		2.2	<0	<0	<0	<0
Admixture of cultures							
1 + 3	Naive + limbus		27.8	10.6	4.0	2.2	<0
1 + 4	Naive + limbus		33.1	26.3	26.8	10.8	<0
1 + 5	Naive + limbus		41.8	30.3	17.2	7.7	<0
1 + 6	Naive + limbus		41.0	33.1	19.9	12.0	23.2
7 + 3	Central + limbus		<0	<0	<0	<0	<0
8 + 4	Central + limbus		5.3	0.8	<0	<0	<0
9 + 5	Central + limbus		28.5	22.4	11.2	2.8	<0
10 + 6	Central + limbus		<0	<0	<0	<0	19.8

The results indicated are from individual animals.

cell populations (24), which indicates that cell surface alloantigens can be presented by APC from the stimulator cell population (direct primed response) or, after reprocessing, by APC from the responder population (cross-primed response). Understanding the mechanisms of direct priming versus cross-priming are essential for transplantation immunology since current attempts to improve allograft survival both experimentally and clinically often include the putative removal of APC from the graft (23, 25). We have used the murine cornea as an allograft model to investigate the role of graft-derived LC during the induction of host CTL responses to the H-Y antigen.

Natural exclusion of LC from the immunizing corneal graft negatively modulated CTL and rejection responses to H-Y, while inclusion of LC resulted in the activation of anti-H-Y CTL and rapid rejection. Early studies by Gordon et al. (35) showed that grafts of male tail skin were only weakly immunogenic for anti-H-Y CTL responses by syngeneic females. The tail skin of rodents contains very low numbers of Ia^+ LC compared with body wall skin (13), and grafts of male tail skin survive on syngeneic female rats much longer than trunk skin (23). Moreover, the introduction of haptens through tail skin fails to initiate contact hypersensitivity in mice, but instead induces a state of active tolerance (13). Ray-Keil and Chandler (25) showed that 64% of male C57BL/6 corneas survived for 21 d when grafted to heterotopic beds on syngeneic female mice. In the present study, the incidence of graft prolongation was higher (90–95% on day 21, data not shown) than that reported by Ray-Keil and Chandler. This discrepancy might be explained by the fact that the LC^- corneal grafts used in our study were ~2 mm in diameter, while those reported in the study by Ray-Keil and Chandler were 3-mm diameter grafts. Since 3-mm diameter grafts would be cut very close to the corneal limbus junction, it is possible that a greater percentage of the grafts used in their study contained LC. Because of corneal desiccation and rapid epidermal invasion it was technically difficult to evaluate these delicate grafts long term, thus we could not determine if LC^- male grafts survived permanently. However, within the time-frame allowable for accurate graft evaluation (35–42 d), our observations indicate that the survival of LC^- grafts was significantly prolonged compared with LC^+ grafts.

Studies using F_1 mice derived from H-2 nonidentical parental strains have shown that immunization with multiple minor-H alloantigens expressed on cells bearing the H-2 haplotype of a single parent produces a population of primed T cells containing clones restricted by the H-2 haplotype of either parent. Further, genetic cross-priming occurs during in vivo sensitization rather than during restimulation in vitro (26–28). The best interpretation of this is that minor-H alloantigens expressed on the immunizing cells are reprocessed by host APC before being presented to host T cells. Studies with H-Y have shown no evidence for cross-priming for CTL in F_1 female mice immunized with parental strain male cells (21). However, parental strain female mice primed in vivo with H-Y on H-2-incompatible male skin grafts respond to H-Y presented in vitro on H-2-compatible male cells. Thus it appears that under certain conditions of priming, H-Y can be processed by host APC and presented in a self-restricted fashion. In the present study, mice were cross-primed allogeneically by immunization with H-2-incompatible LC^+, but not LC^- corneal grafts, indicating that the H-Y antigens expressed on male corneal grafts are reprocessed in vivo by host APC only when initially presented on graft-derived LC. The absence

of cross-priming may be an idiosyncracy of the H-Y antigen, rather than the absence of LC; however, it is likely that the migratory properties of LC facilitate delivery of H-Y antigens to the regional lymph nodes or spleen where reprocessing by host APC occurs. Experiments are in progress to further address this issue.

The results of the present study also suggest a differential ability of alloantigens to cross-prime the host. In the absence of donor-derived APC, the H-Y antigen does not appear to be reprocessed and direct-priming either does not occur or is suppressed (29); however, H-2 alloantigens and some multiple minor-H antigens are recognized on LC⁻ grafts either directly or after reprocessing by host APC. Some minor-H antigens may be recognized by host T cells directly on allogeneic cells without a requirement for host reprocessing (29).

Attempts to correlate graft rejection with T cell function have met with conflicting results and the actual mechanisms of allograft rejection remain controversial. Rejection of male skin grafts by syngeneic female mice correlates with DTH rather than CTL-activation when the responses are mapped using H-2-congenic mice on various genetic backgrounds (30–31). However, recent studies using B6.C-H-2^{bm12} mutants suggest that rejection of male skin by female mice of this genetic background correlates best with anti-H-Y CTL responses (32). Further, a requirement for Ia$^+$ bone marrow-derived cells of graft origin depends upon host-graft disparity (14) and the nature of the tissue transplanted (33). Previous work from our own laboratory (18, 19, 34) has shown that LC⁻ corneas grafted onto H-2 allogeneic hosts are rejected from heterotopic sites in the absence of Lyt-1$^+$,2$^-$ T cells and DTH responsiveness, while primary CTL responses are detected as early as 7 d after grafting. By contrast, LC⁻ corneas are not rejected by class II-disparate hosts (15) although allo–class II CTL are activated (19). H-Y-disparate skin grafts also survive on mice of some H-2 haplotypes despite concomitant induction of anti-H-Y CTL (31). The ability of a graft to activate a specific T cell effector mechanism may not necessarily be a predictor of the T cell effector involved in graft rejection. In the present study, grafts displayed distinct patterns of CTL activation and rejection responses depending on genetic disparity. H-2-disparate (class I+II) LC⁻ corneas stimulated strong primary CTL responses and graft rejection. Class II-disparate LC⁻ corneas stimulated secondary CTL responses (but not primary) and no primary rejection response. Multiple minor-H alloantigens stimulated secondary CTL and primary rejection responses, while the single minor antigen H-Y presented on LC⁻ grafts failed to stimulate secondary CTL responses or primary rejection. Thus the T cell subsets activated and their requirement for graft-derived Ia$^+$ APC depend on the genetic background of the host, graft–host genetic disparity, and graft type. We propose that LC⁻ corneal grafts and LC$^+$ skin grafts may be rejected from subdermal graft beds by different mechanisms.

Failure to respond to H-Y presented on LC⁻ corneal grafts was not due to the absence of H-Y antigen expression on corneal cells, since these grafts were rejected by animals presensitized with male skin. Thus H-Y antigens are expressed by cells of the central cornea and can act as targets for graft destruction. Although class II molecules are normally not expressed by corneal cells, unresponsiveness to H-Y could also not be attributed, at least qualitatively, to an absence of IA determinants on LC⁻ grafts, since corneal epithelial cells and stromal fibroblasts were induced to express IA^b determinants after grafting. We cannot rule out the possibility that

quantitative, temporal, or transitory aspects of induced Ia expression after grafting resulted in the inability of LC$^-$ cornea to present the H-Y antigen.

Failure to generate CTL responses after secondary stimulation with the H-Y antigen in vitro was also not due to the absence of nonspecific helper factors. Addition of IL-2-containing media at the initiation of the boost cultures did not restore responsiveness to LC$^-$ grafts. Addition of anti-L3T4 antibody to boost cultures abolished the CTL response to LC$^+$ grafts but was restored by the addition of IL-2. We interpret this as indicating that H-Y-bearing LC were required for the activation of H-Y-specific T helper cells in vivo.

Presentation of the H-Y antigen on LC$^-$ corneal grafts resulted in active downregulation of the anti-H-Y CTL response in responder strain C57BL/10 mice. The generation of CTL responses by lymphocytes from animals immunized with LC$^+$ cornea was suppressed when mixed in boost cultures with equal numbers of lymphocytes from mice immunized with LC$^-$ cornea. The secondary CTL response to LC$^+$ grafts was abolished by blocking T helper cell function in vitro with anti-L3T4 antibodies, but was restored by the addition of IL-2, while addition of IL-2 did not restore the response to LC$^-$ grafts. Thus the data suggest that presentation of H-Y on LC$^-$ grafts downregulates the T helper cell response during in vivo priming.

Maximum immunogenicity of male corneal grafts occurred when both constitutive Ia expression (by LC) and induced Ia expression (by other corneal cellular elements) were present. Artificial induction of Ia on corneal cells after incubation of LC$^-$ cornea in conditioned media partially restored graft immunogenicity. The amount of surface Ia expressed on corneal cells after incubation in conditioned media was greater than that expressed by LC-alone in an untreated cornea (Fig. 4), yet the CTL response to these grafts was weak and only partially restored (Table IV). This suggests that antigen-presenting potential is not entirely a function of Ia expression. It is possible that this result was due to "activation" of a few Ia$^-$ LC precursors present in the LC$^-$ cornea (7); however, most of the corneal epithelial cells expressed Ia after incubation in conditioned media so we were not able to detect Ia$^+$ dendritic cells against this background staining.

PGE$_2$ inhibited the CM-inducible expression of IA by epithelial cells but did not affect IA expression by LC and only partially reduced the immunogenicity of LC$^+$ limbus grafts. Thus induced expression of IA, whether on epithelial cells or LC precursors, appears to serve as an amplifying signal, but only after the response has been initiated by IA$^+$ LC. Moreover, it appears that the IA$^+$ initiation signal must be delivered to the host's immune system within a window of time beyond which the induced-IA$^+$ signal can neither initiate nor amplify the response. Both constitutive expression and inducible expression of IA were sufficient signals for responsiveness to H-Y when either was expressed at the time of grafting, whereas the natural induction of IA on LC$^-$ cornea after grafting did not result in CTL responsiveness or graft rejection. Taken together, these results are consistent with the proposal that presentation of H-Y in the absence of an IA$^+$ stimulatory signal results in active suppression.

Silvers et al. (23) have provided evidence that tolerance is induced after the presentation of histocompatibility antigens on grafts deficient in APC. Interestingly, the ability of such grafts to induce tolerance depends on the antigen involved as well

as the size of the graft, with single minor-H antigens presented on small APC-deficient grafts being most tolerogenic.

In summary, the results presented here indicate that: (a) the activation of CTL responses to H-Y antigens expressed on cornea is dependent upon presentation of H-Y by graft-derived LC; (b) the expression of H-Y and Ia molecules by cells of the graft is not sufficient for T cell activation; (c) in the absence of graft-derived LC, the H-Y antigen is not reprocessed by host APC in immunogenic form but instead, is presented in a manner that results in active downregulation of the anti-H-Y CTL response. LC appear to possess specialized characteristics enabling them to function efficiently as APC during the inductive phase of the allograft response. Moreover, inappropriate bypass of this requirement for a specialized APC results in active suppression. We suggest that the specialized qualities of LC are related both to their constitutive expression of large amounts of cell surface Ia molecules and to the migratory properties of these cells.

Summary

We have used the murine cornea is an allograft model to investigate the relative roles of graft-derived IA$^+$ APC (Langerhans' cells) and host-derived APC during the induction of CTL responses to H-Y. The natural exclusion of LC from the immunizing corneal graft led to a specific state of unresponsiveness to H-Y in responder strain mice, while inclusion of LC resulted in responsiveness. Failure to respond to H-Y could not be attributed to the absence of H-Y or IA antigen expression on the surface of LC-deficient grafts but instead, appeared to be due to active suppression of the T helper cell response during in vivo priming. Reprocessing of the H-Y antigen by host APC did not occur after immunization with H-Y presented on H-2-incompatible grafts unless presented initially by graft-derived LC. H-2 as well as some non-H-2 alloantigens were presented to the host without a requirement for donor-derived LC. Thus there appear to be differential requirements for the processing and presentation of alloantigens.

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