

PHENOTYPE, SPECIFICITY, AND FUNCTION OF T CELL SUBSETS AND T CELL INTERACTIONS INVOLVED IN SKIN ALLOGRAFT REJECTION

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The T cell populations involved in the rejection of skin allografts remain controversial (1-6). It is unclear whether skin allograft rejection is mediated by lymphokine secreting T helper (Th) cells, cytolytic T killer (Tk)¹ cells, dual function T cells (7, 8), or by interactions between functionally distinct T cell subsets. It has recently been appreciated (9-11) that phenotypically distinct populations of L3T4⁺ and Lyt-2⁺ T cells are each able to mediate skin allograft rejection. It is also known that phenotypically distinct populations of L3T4⁺ and Lyt-2⁺ T cells are functionally heterogeneous, each containing lymphokine-secreting Th cells and cytolytic Tk cells (12-15). Moreover, phenotypically distinct populations of L3T4⁺ and Lyt-2⁺ T cells express distinct recognition specificities (16), as do functionally distinct but phenotypically identical populations of Th and Tk cells (17-19).

Despite the complexities, it is important to understand the T cell mechanisms involved in skin allograft rejection. Recently, by correlating the phenotype, specificity, and function of H-2^b T cells responding in vitro to MHC class I K^b mutant alloantigens with the in vivo rejection by H-2^b mice of K^b mutant skin grafts, we identified class I-allospecific Lyt-2⁺ Th cells as the critical cell type determining the rejection rate of class I-disparate skin allografts (9). By using further in vitro insights to analyze in vivo allograft rejection, we have now compared the phenotype, specificity, and functional capabilities of defined T cell subpopulations with their ability to initiate and mediate the in vivo rejection of skin allografts expressing a variety of defined alloantigens. The results of this analysis are straightforward: without exception, skin allograft rejection occurred only in mice containing T cell populations that, by in vitro assessment, contained both Th and Tk cells specific for the alloantigens of the graft. However, for graft rejection to occur, the cellular elements of the skin allograft must be able to trigger the Th and Tk cells present in the host.

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¹ *Abbreviations used in this paper:* FCM, flow cytometry; Tk, T killer; TR, Texas Red.

TABLE I
MHC Alleles of the Mouse Strains Used in this Study

Strain	H-2 alleles				
	K	IA	IE	D	Qa1
C57BL/6 (B6)	b	b	—	b	b
C57BL/10 (B10)	b	b	—	b	
B6.PL-Thy-1 ^a (B6-Thy-1.1)	b	b	—	b	
B10.BR	k	k	k	k	
DBA/2	d	d	d	d	
B6.CH-2 ^{bm1} (bm1)	bm1	b	—	b	
B6.CH-2 ^{bm6} (bm6)	bm6	b	—	b	
B6.CH-2 ^{bm12} (bm12)	b	bm12	—	b	
B6.T1a ^a	b	b	—	b	a

Materials and Methods

Animals. B10 *nu/nu* female mice were obtained from the Small Animal Section, NIH, Bethesda, MD. C57BL/6 (B6), B10, B10.BR, and DBA/2 mice were obtained from The Jackson Laboratory, Bar Harbor, ME. B6.PL-Thy-1^a (B6-Thy-1.1) mice (20) were a generous gift of Dr. John Wunderlich from the Frederick Cancer Research Facility, Frederick, MD. B6.CH-2^{bm1} (bm1) (21), B6.CH-2^{bm12} (bm12) (22), B6.CH-2^{bm6} (bm6) (23), (bm6 × bm12)_{F1}, and B6.T1a^a mice (24) were bred in our own animal facility. B10 nude mice were 6–14 wk old at the time of engraftment and adoptive transfer. B6 mice were 13–16 wk old at the time of engraftment. The MHC haplotypes of the various strains are listed in Table I.

Monoclonal Antibodies. Anti-L3T4 mAb was either a culture supernatant of the hybridoma cell line GK1.5 (25), generously provided by Dr. Frank Fitch, University of Chicago, Chicago, IL, or an ascitic fluid of the hybridoma cell line RL-172/4 (26), generously provided by Dr. Ada Kruisbeek, NIH. Anti-Lyt-2.2 mAb was a culture supernatant of the hybridoma cell line 83-12-5, generously provided by Dr. Jeffrey Bluestone, NIH.

Isolation of T Cell Subpopulations. Depletion of L3T4⁺ T cells or Lyt-2⁺ T cells was accomplished by incubating spleen cells at a density of 10⁷ cells/ml with anti-L3T4 (1:2 dilution of GK1.5 or a 1:100 dilution of RL-172/4) or anti-Lyt-2.2 (1:5 dilution) mAbs for 30 min at 37°C. Cells were then pelleted, resuspended in C, and incubated for 50 min at 37°C. The cells treated with GK1.5 were resuspended at 10⁷/ml in rabbit complement (Cedar Lane Laboratories Ltd., Ontario, Canada) diluted 1:10. The cells treated with RL-172/4 and 83-12-5 were resuspended at 10⁸/ml in Guinea pig complement (Gibco Laboratories, Grand Island, NY) diluted 1:3. Treated cells were washed three times before injection into experimental animals. To avoid the confusion of referring to T cell populations in negative terms, anti-Lyt-2 plus C-treated spleen cells will be referred to as L3T4⁺ T cells and anti-L3T4 plus C-treated spleen cells will be referred to as Lyt-2⁺ T cells.

Skin Grafting of Normal B6 Mice. B6 mice were engrafted either on the left flank or on both flanks with tailskin grafts according to an adaptation of the method of Billingham and Medawar (27). Bandages were removed on day 7 and the grafts were scored daily until rejection (defined as loss of >80% of the grafted tissue) or the end point of the experiment.

Skin Grafting and Adoptive Transfer. Female B10 nude mice were engrafted on the thorax on day 0 with tailskin allografts from donor mice according to an adaptation of the method of Billingham and Medawar (27). On day 1, the mice were injected intravenously with 5–7 × 10⁷ spleen cells from unprimed B6 mice or B6 Thy-1.1 congenic mice that had been (a) untreated, (b) treated with anti-L3T4 plus C in vitro, or (c) treated with anti-Lyt-2.2 plus C in vitro according to the above procedures. Bandages were removed

on day 7 and the grafts were scored daily until rejection (defined as loss of >80% of the grafted tissue) or the end point of the experiment.

Reagents Used for Flow Cytometry (FCM). Anti-Fc receptor mAb (2.4G2) (28) and biotin-conjugated monoclonal anti-L3T4 (GK1.5) (25) were generous gifts from J. Titus (Immunology Branch, National Cancer Institute). FITC anti-Thy-1.2 (clone 30-H12), FITC-anti-Leu-1, biotin-conjugated anti-Lyt-2 (clone 53-6), and biotin-conjugated anti-Leu-1 were purchased from Becton Dickinson Immunocytometry Systems (Mountain View, CA). Texas Red (TR)-Streptavidin was purchased from Bethesda Research Laboratories, Bethesda, MD. Anti-Fc receptor antibody (2.4G2) was used to block Fc receptor-mediated antibody binding during the staining procedures.

Flow Cytometry Analysis. Multicolor FCM was performed as previously described (29) using a modified FACS II (Becton Dickinson Immunocytometry Systems) equipped with (a) the manufacturer's filters and photomultiplier tubes, (b) argon ion laser for FITC excitation (488 nm), (c) pumped dye (rhodamine 6G) laser for TR excitation (590 nm), and it was interfaced to a Digital Equipment Corp. (Marlboro, MA) PDP 11/2423 (30). Fluorescence data were collected using logarithmic amplification on 50,000 viable cells as determined by forward light scatter intensity and propidium iodide exclusion. Logarithmic amplification was provided by a three-decade logarithmic amplifier constructed from an NIH-modified design of R. Hiebert, LASL (Los Alamos Scientific Laboratories, Los Alamos, NM). One-color fluorescence data were displayed as cell frequency histograms or immunofluorescence profiles in which log fluorescence intensity was plotted in 1,024 channels on the x-axis and cell number shown on the y-axis. Two-color immunofluorescence data were displayed as contour diagrams in which log intensities of green (FITC) fluorescence were plotted in 64 channels on the x-axis and the log intensities of red (TR) fluorescence were plotted on the y-axis. Constant values of the percentage of total cell number on the z-axis were selected to draw the rings or contours around peaks of cells correlating FITC and TR fluorescence.

Results

Experimental Model. To assess the T cell subpopulations involved in skin allograft rejection, we used an adoptive transfer model in which H-2^b nude mice were engrafted on day 0 with tailskin allografts and reconstituted on day 1 with 50–70 × 10⁶ syngeneic H-2^b spleen cell subpopulations. To determine if skin allograft rejection in this model depended upon reconstitution with immunocompetent T cells, B10 nude mice engrafted with B10.BR (H-2^k) skin were reconstituted with unfractionated spleen cells from either normal B10 or T cell-deficient nude B10 mice. As can be seen in Fig. 1, nude mice given normal B10 spleen cells rejected their skin allografts rapidly whereas nude mice given T cell deficient B10 spleen cells failed to do so. Unreconstituted nude mice also failed to reject the skin allografts (data not shown). These data indicated that the ability to reject skin allografts in this model was contingent upon the adoptive transfer of immunocompetent T cells.

Skin Allograft Rejection by Negatively Selected Populations of Phenotypically Distinct T Cells. We next tested the ability of phenotypically distinct T cell subpopulations to induce graft rejection. B10 nude mice engrafted with fully allogeneic DBA/2 skin grafts were reconstituted with H-2^b spleen cells that were (a) untreated, (b) treated with anti-L3T4 plus C, generating a T cell population that was overwhelmingly Lyt-2⁺ (<1% of the remaining T cells were L3T4⁺), or (c) treated with anti-Lyt-2 plus C, generating a T cell population that was overwhelmingly L3T4⁺ (<1% of the remaining T cells were Lyt-2⁺). To avoid the

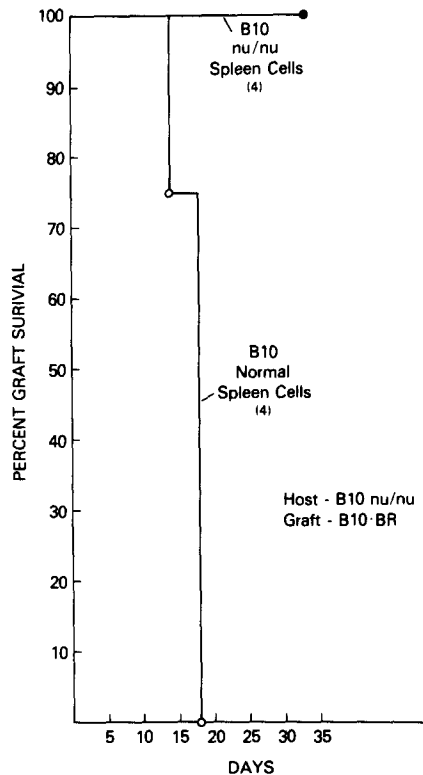


FIGURE 1. Rejection of skin allografts by nude mice reconstituted with normal but not T cell-deficient spleen cell populations. B10 nude mice were engrafted on day 0 with MHC-disparate B10.BR tail skin grafts, and were reconstituted on day 1 with 50×10^6 spleen cells from either B10 normal mice or T cell-deficient B10 nude mice. The number of mice in each group is indicated. The grafts were scored daily until rejection or death of the animal.

confusion of referring to T cell populations in negative terms, anti-Lyt-2 plus C-treated spleen cells are referred to as L3T4⁺ T cells and anti-L3T4 plus C-treated spleen cells are referred to as Lyt-2⁺ T cells. As can be seen in Fig. 2, all three H-2^b T cell populations effected the rapid rejection of DBA/2 skin allografts. In contrast to their rejection of allogeneic grafts, reconstituted nude mice failed to reject syngeneic B6 skin grafts (data not shown). These results indicated that negatively selected populations of L3T4⁺ and Lyt-2⁺ T cells were each able to mediate skin allograft rejection. However, it was possible that cells of the reciprocal phenotype derived from either the nude host or from residual cells in the donor inoculum contributed to the rejection of skin allografts in this adoptive transfer model.

To assess the possibility that cells derived from the nude host contributed to skin allograft rejection, we performed an experiment in which donor H-2^b spleen cells were from B6-Thy-1.1 congenic mice. Since mature T cells of B10 nude host origin would be Thy-1.2⁺, they would be discernible from T cells derived from the Thy-1.1 donor inoculum. Accordingly, B6-Thy-1.1 spleen cell populations were injected into B10 nude mice that had been engrafted with fully allogeneic DBA/2 skin grafts to provide maximal stimulation for the expansion of small numbers of host-derived T cells. Further, the mice were reconstituted with isolated T cell populations as well as unseparated T cell populations in the event that reconstitution with unfractionated T cell populations suppressed the

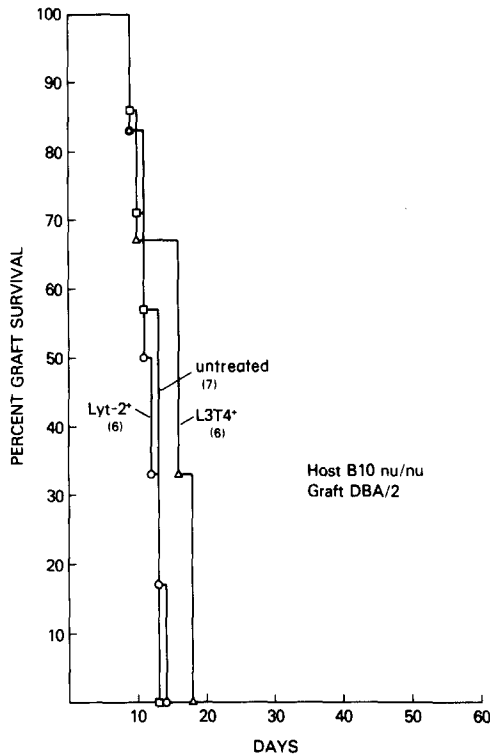


FIGURE 2. Isolated Lyt-2⁺ and L3T4⁺ T cell populations can reject fully allogeneic skin grafts. B10 nude mice were engrafted on day 0 with DBA/2 tail skin grafts, and were reconstituted on day 1 with 50×10^6 B6 spleen cells that were untreated, L3T4⁺ (negatively selected with anti-Lyt-2 plus C), or Lyt-2⁺ (negatively selected with anti-L3T4 plus C). The number of mice in each group is indicated. Grafts were scored daily until rejection. Control, reconstituted mice retained syngeneic B6 skin grafts indefinitely.

appearance of T cells of nude host origin. All mice reconstituted with either unfractionated or isolated H-2^b T cell populations rejected their DBA/2 skin grafts (MST ranged between 11 and 16 d for each of the three groups). 35 d after reconstitution, spleen cells from the reconstituted mice were examined for mature T cells of nude host origin by staining with fluoresceinated anti-Thy-1.2 and a mixture of biotinylated anti-Lyt-2 and anti-L3T4. It can be seen in Fig. 3a and Table II that while rare cells expressing Thy-1.2 were present in unreconstituted nude mice, they were overwhelmingly L3T4⁻,Lyt-2⁻. This contrasted markedly with spleen cells from either B10 normal mice (Fig. 3b) or B10 nude mice reconstituted with Thy-1.2⁺ spleen cells (Fig. 3c). In these latter animals 18–20% of spleen cells were positive for both Thy-1.2 and L3T4/Lyt-2. Spleens of nude mice reconstituted with either unfractionated or isolated Thy-1.1 congenic spleen cells contained significant numbers of Thy-1.2⁻ cells expressing L3T4/Lyt2, but Thy-1.2⁺ cells expressing L3T4/Lyt2 were virtually undetectable (<0.5%) (Fig. 3, d–f; and Table II). We assessed the same parameters on days 42 and 76 and observed identical results (data not shown). Thus, at least over the time period necessary for most skin graft experiments, there was no evidence indicating the appearance of mature T cells of nude host origin in the spleens of the adoptively transferred mice.

To assess the alternative possibility, that significant in vivo expansion of contaminating T cells from the donor spleen cell inoculum occurs, B10 nude

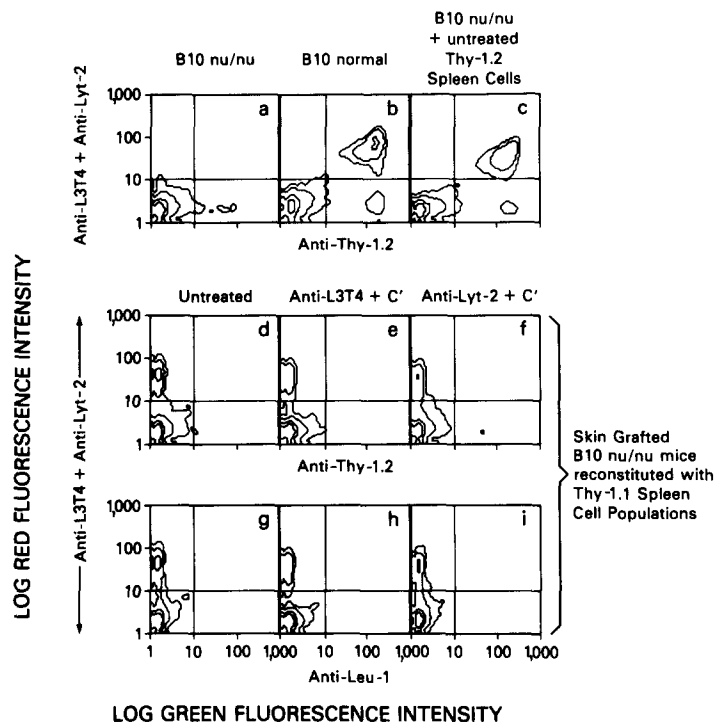


FIGURE 3. Failure to detect Lyt-2^+ or L3T4^+ T cells of nude host origin in adoptively transferred mice. B10 nude mice were engrafted on day 0 with a DBA/2 tail skin allograft, and were reconstituted on day 1 with 50×10^6 spleen cells from either syngeneic B6 or B6-Thy-1.1 congenic mice that were untreated, treated with anti-L3T4 plus C, or treated with anti-Lyt-2 plus C. On day 35, spleen cells from these experimental mice, as well as from unreconstituted B10 *nu/nu* and B10 normal mice, were analyzed by two-color FCM for cells expressing Thy-1.2 vs. L3T4/Lyt-2. Anti-Leu-1 is a negative control antibody. Quadrants selected for data integration are shown by solid lines on the contour profiles. These x and y levels were chosen as the intersection of profiles of positive and control negative antibodies reacted with spleen cells from B10 *nu/nu* animals that received untreated Thy-1.2 spleen cells. (Green: FITC-anti-Thy-1.2 vs. FITC-anti-Leu-1; red: biotin-anti-L3T4 plus biotin-anti-Lyt-2 vs. biotin-anti-Leu-1, data not shown). See Table II for percent of spleen cells stained.

mice engrafted with allogeneic skin grafts were reconstituted with either unfractionated or isolated spleen T cell populations. On days 30 and 60 following reconstitution, the phenotypes of their splenic T cells were assessed. As can be seen in Fig. 4, mice that had received untreated B6 spleen cells 30 d previously contained both Lyt-2^+ and L3T4^+ T cell populations. This contrasted markedly with mice receiving isolated T cell populations: mice receiving Lyt-2 plus C-treated cells had $<1.0\%$ Lyt-2^+ spleen cells 30 d after reconstitution, and mice receiving L3T4 plus C-treated cells had $<1.0\%$ L3T4^+ spleen cells. Similar results were obtained on day 60 after reconstitution (data not shown). From these studies it would appear that minimal expansion of contaminating T cells in the original donor inoculum occurred in vivo over the time course of the experimental assays.

Specificity of Skin Allograft Rejection by Phenotypically Distinct T cell Popula-

TABLE II
*Phenotypes of Spleen Cells Observed in Adoptively Transferred Mice
 Displayed in Fig. 3*

Panel*	Percent of Spleen Cells that Stained with:			
	Thy-1.2: L3T4/Lyt-2:	- +	+ -	+ +
a		1.3	1.8	0.14
b		1.0	3.7	20.20
c		1.6	2.0	18.11
d		16.0	1.1	0.37
e		6.7	0.6	0.03
f		12.4	1.2	0.04
	Leu-1: L3T4/Lyt-2:	- +	+ -	+ +
g		16.2	0.01	0.09
h		7.5	0.01	0.07
i		13.1	0.01	0.15

Numbers have not been corrected for background staining and thus represent the maximum possible frequency. Thy-1.2⁺, Leu-1⁺ background staining ranged between 0.07 and 0.15%.

* Correspond to panels in Fig. 3.

tions. It was clear from the experiment shown in Fig. 2 that each phenotypically distinct T cell population could effect the rejection of fully allogeneic skin grafts. To more precisely identify the antigen specificities triggering allograft rejection responses by each isolated T cell phenotype, we engrafted B10 nude mice with (a) MHC class I-disparate bm1 skin grafts, (b) MHC class II-disparate bm12 skin grafts, or (c) multiple minor H-disparate BALB.B skin grafts and we reconstituted them with unfractionated or isolated H-2^b T cell populations. As can be seen in Fig. 5 (*left*), unfractionated T cell populations containing both L3T4⁺ and Lyt-2⁺ T cell populations effected the rapid rejection of MHC class I, MHC class II, and multiple minor H disparate grafts. As can be seen in Fig. 5 (*middle*), the isolated L3T4⁺ T cell population effected the rapid rejection of MHC class II and minor H disparate grafts, but did not effect the rapid rejection of MHC class I disparate grafts. Indeed, B10 nude mice reconstituted with isolated L3T4⁺ T cells retained MHC class I-disparate bm1 skin allografts indefinitely (>300 d). In contrast, it can be seen in Fig. 5 (*right*) that the isolated Lyt-2⁺ T cell population did effect the rapid rejection of MHC class I-disparate grafts, but did not effect the rapid rejection of MHC class II- or minor H-disparate grafts. It might be noted that the reciprocal abilities of isolated L3T4⁺ and Lyt-2⁺ T cell populations to effect the rejection of MHC class II- and class I-disparate skin grafts, respectively, but not vice versa, further support the conclusion that, in this experimental model, T cells of the inappropriate phenotype from either the nude host or the donor inocula do not expand to sufficient numbers to significantly influence the experimental outcome. Thus, this experiment demonstrates that isolated populations of L3T4⁺ and Lyt-2⁺ T cells can

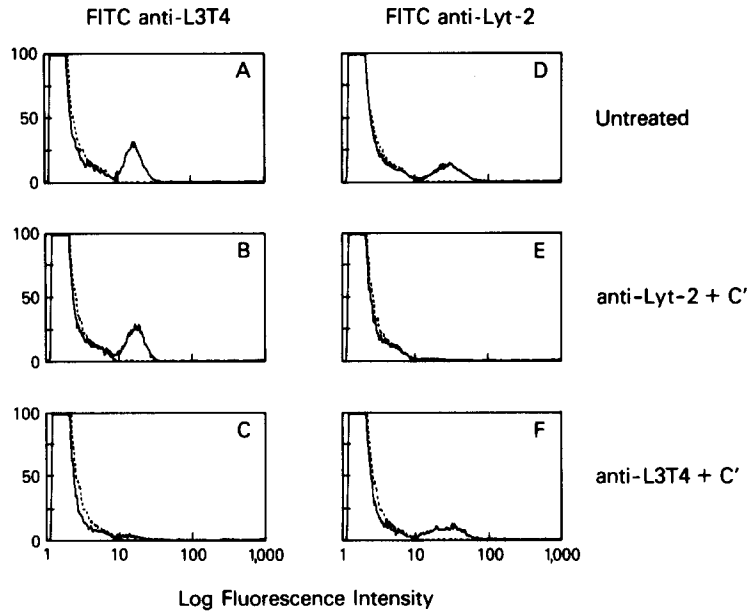


FIGURE 4. Failure to detect *in vivo* expansion of contaminating T cells after adoptive transfer. B10 nude mice were engrafted on day 0 with allogeneic skin grafts, and were reconstituted on day 1 with 50×10^6 B6 spleen cells that were untreated, treated with anti-Lyt-2 plus C, or treated with anti-L3T4 plus C. Before injection, these cell populations were analyzed by FCM, and it was found that the cells of the untreated donor inoculum were 18% L3T4⁺ and 11% Lyt-2⁺; the cells of the anti-Lyt-2 plus C-treated donor inoculum were 16.6% L3T4⁺ and <1% Lyt-2⁺; and the cells of the anti-L3T4 plus C-treated donor inoculum were <1% L3T4⁺ and 10% Lyt-2⁺. 30 d after reconstitution, the phenotypes of the spleen cells of mice from each group were assayed by FCM. Solid lines represent staining with either fluoresceinated anti-L3T4 or anti-Lyt-2 mAbs. Dotted lines represent control staining with fluoresceinated anti-Leu-1 mAb. Intensity levels for curve integration were chosen as the intersection of profiles for positive (—) and negative (---) antibodies reacted with spleen cells from animals that received untreated cells. Percentages of positive cells were determined as the difference above these levels between positive cells incubated with the experimental antibody and positive cells incubated with the control antibody.

each individually effect allograft rejection, but only in response to specific and different alloantigens.

This observation indicates that within each phenotypically distinct T cell subpopulation are cells with the appropriate specificity and the requisite functional abilities to mediate the rejection process. Interestingly, Lyt-2⁺ T cell populations, which rejected class I-disparate grafts, are known from *in vitro* studies to possess both lymphokine-secreting Th cells and lymphokine-responsive Tk cells specific for class I alloantigens (9, 15, 16, 19, 31), and L3T4⁺ T cell populations, which rejected MHC class II- and multiple minor H-disparate grafts, are similarly known to possess both lymphokine-secreting Th cells and lymphokine-responsive Tk cells specific for these alloantigens (13, 14, 17, 19, 31). Lyt-2⁺ T cell populations that failed to rapidly reject MHC class II- and minor H-disparate grafts contain Tk cells (13, 14, 19, 32-34) but lack Th cells (19, 31, and Mizuochi, T., A. S. Rosenberg, and A. Singer, manuscript in

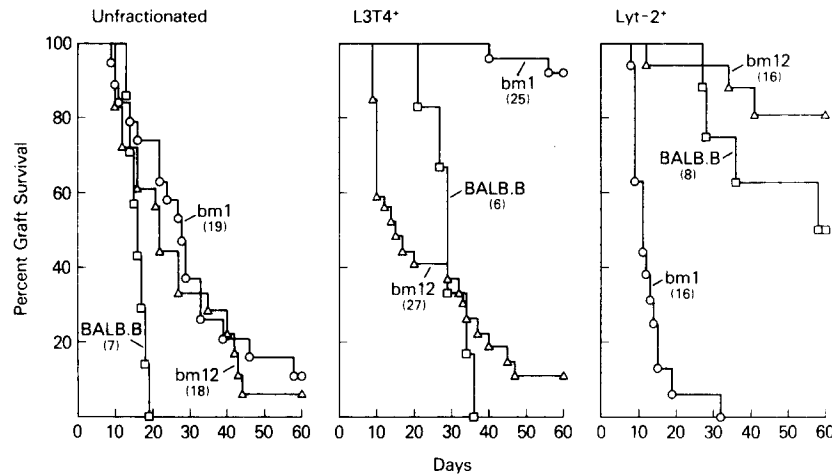


FIGURE 5. Reconstituting spleen cell populations. Specificity of skin allograft rejection by phenotypically distinct T cell populations. B10 nude mice were engrafted on day 0 with bm1, bm12, or BALB.B skin grafts, and were reconstituted on day 1 with 50×10^6 B10 spleen cells that were unfractionated (*left*), L3T4⁺ by negative selection with anti-Lyt-2 plus C (*center*), or Lyt-2⁺ by negative selection with anti-L3T4 plus C (*right*). Number of mice in each group is indicated. Grafts were scored daily until rejection or day 60. Control, reconstituted mice retained syngeneic B6 skin grafts indefinitely.

preparation) specific for these alloantigens; and L3T4⁺ T cell populations that failed to rapidly reject MHC class I-disparate grafts contain Th cells (15, 19, 31, 35-37) but lack Tk cells (14, 17, 19) specific for MHC class I alloantigens. Thus, in Fig. 5, it appears that isolated Lyt-2⁺ and L3T4⁺ T cell populations rejected only those skin allografts for which they contained both Th and Tk cells. With regard to minor H-specific T cell populations, we have consistently failed to detect minor H-specific Lyt-2⁺ T cells that either secrete lymphokine or provide help for in vitro CTL responses, despite numerous and intense efforts involving multiply immunized and skin engrafted animals (Mizuochi, T., A. S. Rosenberg, and A. Singer, manuscript in preparation). However, Lyt-2⁺ proliferative cytolytic T cell clones specific for the minor locus H-1 have been reported (38), although it is unclear whether H-1 is unique among minor H loci. Nevertheless, it is conceivable that minor H-specific Lyt-2⁺ Th cells are present in frequencies that have escaped our detection but that are responsible for the slow rejection of minor H-disparate BALB.B skin allografts in some of the experimental mice (Fig. 5, *right*).

Skin Allograft Rejection Resulting from Interactions Between Phenotypically Distinct T Cell Subsets. To directly test the implication that both inducer and effector T cell functions are required to initiate and mediate skin allograft rejection, we turned to a system in which Th and Tk in vitro functions are mediated by phenotypically distinct T cell populations and so are readily separable (32, 33, 39, 40). The generation in vitro of cytolytic immune responses to the male-specific antigen H-Y required collaboration between L3T4⁺ Th cells and Lyt-2⁺ Tk cells (40). To determine the phenotype of the T cells required in vivo to reject H-Y skin allografts, we engrafted female H-2^b nude mice with B6 male

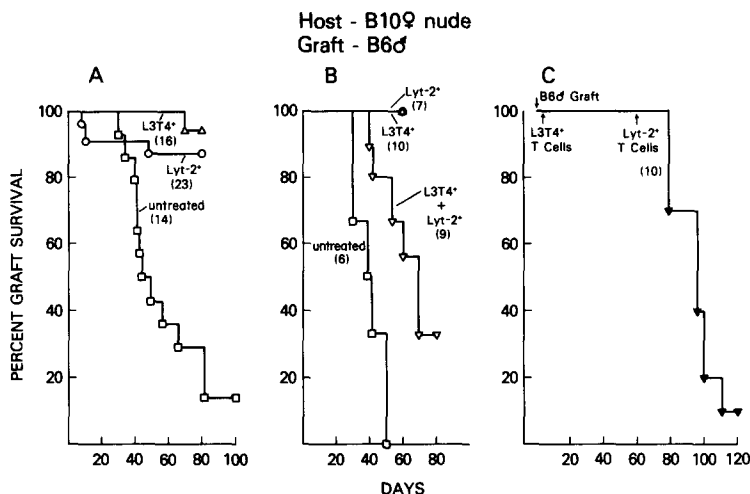


FIGURE 6. Interactions between phenotypically distinct T cell populations in the rejection of H-Y-disparate skin allografts. (A) Female B10 nude mice were engrafted with B6 male tail skin grafts on day 0 and the following day were reconstituted with 70×10^6 female B6 spleen cells that were untreated, L3T4⁺ by negative selection with anti-Lyt-2 plus C, or Lyt-2⁺ by negative selection with anti-L3T4 plus C. (B) Female B10 nude mice were engrafted with B6 male tail skin grafts on day 0 and the following day were reconstituted with 70×10^6 unfractionated or fractionated female B6 spleen cells. Mice reconstituted with both L3T4⁺ and Lyt-2⁺ spleen cell populations received 35×10^6 of each. (C) Female B10 nude mice were engrafted with B6 male tail skin grafts on day 0 and the following day were reconstituted with 50×10^6 female B6 spleen cells that were L3T4⁺ by negative selection with anti-Lyt-2 plus C. On day 60, the mice were injected with an additional 50×10^6 female B6 spleen cells that were Lyt-2⁺ by negative selection with anti-L3T4 plus C. Grafts were scored daily until rejection or the end of each experiment. Number of mice in each group is indicated. Control, reconstituted mice retained B6 female skin grafts indefinitely.

tailskin grafts and reconstituted them with H-2^b female spleen cell subpopulations. Fig. 6a reveals the results of our initial studies: neither isolated L3T4⁺ nor Lyt-2⁺ T cell populations effected the rejection of H-Y disparate skin grafts, whereas the untreated T cell population that contained both L3T4⁺ and Lyt-2⁺ T cells did effect the rejection of these grafts. To more formally test the need for T cells of both phenotypes to effect the rejection of H-Y disparate grafts, we engrafted H-2^b female nude mice with B6 male skin and reconstituted them with untreated, L3T4⁺,Lyt-2⁺, or a mixture of T cell populations from female H-2^b mice (Fig. 6b). Total cell number was kept constant among the groups. In Fig. 6b it is clear that only mice reconstituted with both L3T4⁺ and Lyt-2⁺ T cell subpopulations rejected H-Y-disparate skin grafts. In a final experiment, H-2^b female nude mice engrafted with B6 male grafts were initially reconstituted with isolated L3T4⁺ T cells (Fig. 6c). As before, such mice failed to reject their grafts. On day 60 these mice were additionally given isolated Lyt-2⁺ T cells, and shortly thereafter, began rejecting their H-Y-disparate skin allografts (Fig. 6c). In contrast, such mice not additionally given isolated Lyt-2⁺ T cells retained their H-Y-disparate skin grafts indefinitely (>300 d). Thus, unlike the rejection of MHC class I and class II disparate skin allografts, the rejection of H-Y-disparate skin allografts required interactions between L3T4⁺ and Lyt-2⁺ T cells. Since

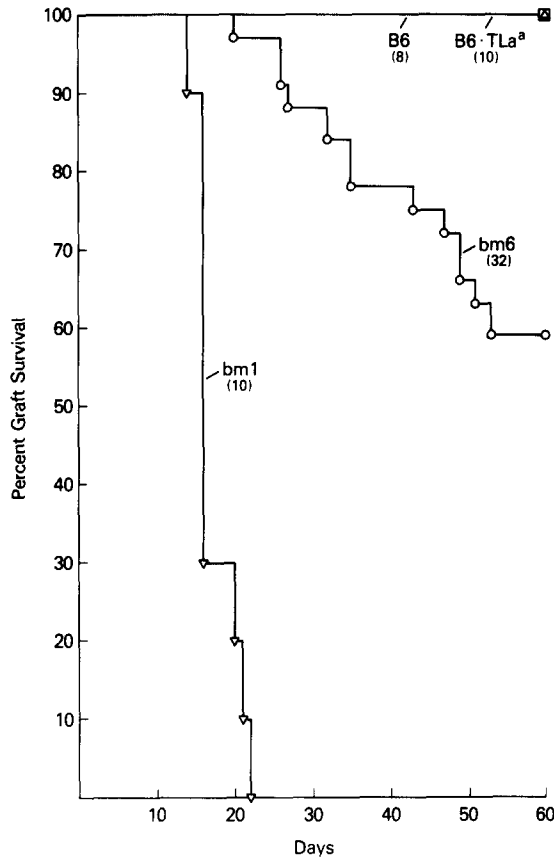


FIGURE 7. Rejection responses of B10 normal mice engrafted with B6.Tla^a or K^{bm6} skin allografts. B10 normal mice were engrafted with B6, bm1, bm6, or B6.Tla^a skin on day 0. Grafts were scored daily until rejection or day 60. Number of mice in each group is indicated.

L3T4⁺ T cell populations contain H-Y-specific Th cells and Lyt-2⁺ T cell populations contain H-Y-specific Tk cells, these results are consistent with a requirement for in vivo collaboration between H-Y-specific inducer and effector cells to reject H-Y-disparate skin grafts.

Skin Allograft Rejection Resulting from Interactions Between T Cells with Different Antigen Specificities. In the course of our studies on skin allograft rejection, we encountered two situations in which normal B6 mice failed to reject allogeneic skin grafts (9, 41). Thus, most B6 mice failed to reject either Qa1^a or K^{bm6} skin allografts (Fig. 7), indicating a deficiency in Qa1^a-specific and K^{bm6}-specific inducer T cell function, effector T cell function, or both. To examine whether an antigen-specific deficiency in inducer T cell function was the sole basis for the failure of normal B6 mice to reject these two types of skin allografts, we asked whether anti-Qa1^a or anti-K^{bm6} effector cells were present in B6 mice and whether they could be activated in vivo by inducer T cells with a different antigen specificity to effect the rejection of Qa1^a or K^{bm6} skin allografts. Consequently, in the following experiments normal B6 mice were engrafted simultaneously with two skin grafts: (a) on the left flank, an inducer graft bearing both the target antigen (either Qa1^a or K^{bm6}) as well as additional helper determinants,

TABLE III
Rejection of Qa1^a Skin Allografts Induced by H-Y Carrier Determinants

Group	Host	Left (inducer) graft					Right (indicator) graft				
		Strain	Sex	Antigenic disparity	MST	Fraction rejecting	Strain	Sex	Antigenic disparity	MST	Fraction rejecting
					<i>d</i>				<i>d</i>		
1	B6 F	B6.T1a ^a	F	Qa1 ^a	>83	0/4	B6.T1a ^a	F	Qa1 ^a	>83	0/4
2	B6 F	B6.T1a ^a	M	Qa1 ^a + H-Y	21	9/9	B6.T1a ^a	F	Qa1 ^a	23	9/9
3	B6 F	B6	M	H-Y	25	9/9	B6.T1a ^a	F	Qa1 ^a	>83	3/9
4	B6 M	B6.T1a ^a	M	Qa1 ^a	>83	0/3	B6.T1a ^a	F	Qa1 ^a	>83	0/3

and (b) on the right flank, an indicator graft bearing only the target antigen. In this way we asked if the T cell response to the inducer graft resulted in the activation of effector T cells capable of rejecting the indicator graft.

In the first experiment of this type, we assessed the ability of Qa1^a-specific effector cells to be activated by H-Y-specific Th cells to subsequently reject Qa1^a skin allografts (Table III). In the absence of an inducer graft expressing putative helper determinants, B6 female mice failed to reject Qa1^a disparate indicator grafts (group 1). However, in the presence of an inducer graft expressing both Qa1^a target antigens and H-Y helper determinants, B6 female mice rapidly rejected the indicator grafts expressing Qa1^a alloantigens alone, and also rapidly rejected the inducer grafts (group 2). This rejection of the Qa1^a indicator grafts reflected the activation of Qa1^a-specific effector cells and did not simply reflect crossreactive recognition of Qa1^a alloantigens by H-Y-specific effector cells since most mice engrafted with inducer grafts expressing H-Y-antigens alone failed to reject the Qa1^a indicator grafts (group 3). Further, the activation of Qa1^a-specific effector cells by Qa1^a plus H-Y-expressing inducer grafts required activation of H-Y-specific T cells since such inducer grafts, when placed on B6 male mice that cannot recognize H-Y allodeterminants, failed to activate effector cells capable of rejecting the Qa1^a indicator grafts (group 4). This experiment demonstrates that B6 mice do contain Qa1^a-specific effector cells and that such cells can interact with H-Y-specific T-inducer cells or their products to effect the rejection of Qa1^a-disparate skin grafts.

In the second experiment of this type, we asked if K^{bm6}-specific effector cells were present in B6 mice and whether they could collaborate with I^{bm12}-specific T-inducer cells to effect the rejection of K^{bm6} skin allografts (Table IV, Exp. 1). In the absence of an inducer graft expressing putative helper determinants, most B6 mice failed to reject the indicator K^{bm6} skin allografts (groups 1, 2). However, in the presence of an inducer graft expressing both the K^{bm6} target antigen and the I^{bm12} helper determinants, normal B6 mice rapidly rejected the indicator grafts expressing K^{bm6} alloantigenic determinants alone, as well as rapidly rejecting the inducer grafts (group 3). Even though it was extremely unlikely that the rejection of K^{bm6} indicator grafts resulted from crossreactive recognition by I^{bm12}-specific effector cells, we performed an experiment to document that L3T4⁺ I^{bm12}-specific T cells activated by the inducer grafts were not the cells directly responsible for rejecting the indicator grafts. B10 nude mice engrafted with both inducer skin grafts and K^{bm6} indicator skin grafts were reconstituted with either

TABLE IV
Rejection of bm6 Skin Allografts Induced by Allogeneic MHC Class II Helper Determinants

Exp.	Group	Host	Reconstituting H-2 ^b spleen cell population*	Left (inducer) graft				Right (indicator) graft			
				Strain	Antigenic disparity	MST	Fraction rejecting	Strain	Anti- genic disparity	MST	Fraction rejecting
						<i>d</i>			<i>d</i>		
1	1	B6	—	None	—	—	—	bm6	K ^{bm6}	>60	3/10
	2	B6	—	bm6	K ^{bm6}	>60	3/10	bm6	K ^{bm6}	>60	3/10
	3	B6	—	bm12 × bm6	I ^{bm12} + K ^{bm6}	14	12/12	bm6	K ^{bm6}	15.5	11/12
2	4	B10 Nude	L3T4 ⁺ + Lyt-2 ⁺	bm6	K ^{bm6}	>64	0/6	bm6	K ^{bm6}	>64	0/6
	5	B10 Nude	L3T4 ⁺ + Lyt-2 ⁺	bm12 × bm6	I ^{bm12} + K ^{bm6}	15	5/5	bm6	K ^{bm6}	18	5/5
	6	B10 Nude	L3T4 ⁺	bm12 × bm6	I ^{bm12} + K ^{bm6}	10	4/4	bm6	K ^{bm6}	>64	0/4

* Spleen cell populations from normal B6 mice that were either untreated and so contained both L3T4⁺ and Lyt-2⁺ T cells, or were L3T4⁺ by negative selection with anti-Lyt-2 plus C.

untreated or isolated L3T4⁺ T cell populations (Table III, Exp. 2). In the absence of an inducer graft expressing helper determinants, B10 nude mice reconstituted with untreated T cell populations failed to reject the K^{bm6} indicator grafts (group 4). However, in the presence of an inducer graft expressing both the K^{bm6} target antigen and the I^{bm12} helper determinants, such mice rapidly rejected their K^{bm6} indicator grafts, as well as their inducer grafts (group 5). This rejection of K^{bm6} indicator grafts was not mediated by I^{bm12}-specific effector cells, but rather was mediated by K^{bm6}-specific effector cells activated by I^{bm12}-specific T-inducer cells since the L3T4⁺ I^{bm12}-specific effector cells that rejected the inducer grafts were incapable of rejecting the K^{bm6} indicator grafts (group 6). Thus, the experiments in Table IV demonstrate that B6 mice do contain K^{bm6}-specific effector cells and that such cells can productively interact with K^{bm12}-specific inducer cells or their products to effect the rapid rejection of K^{bm6} skin allografts.

The experiments summarized in Tables III and IV make two points. First, they demonstrate in normal mice that skin allograft rejection can be mediated by interactions between distinct subsets of T-inducer and T-effector cells, and that the interacting T cells need not recognize the same antigen specificities. And second, these experiments indicate that an inability to reject specific allografts can reflect a selective deficiency in antigen-specific Th/T-inducer cells.

Discussion

In the present study we used an adoptive transfer model to assess the phenotype, specificity, and functional capability of the T cells involved in initiating and mediating skin allograft rejection. The results of this study demonstrate that skin allograft rejection is neither unique to a specialized subset of T cells with a given Lyt phenotype, nor unique to a specialized subset of helper-independent effector T cells with so-called dual function capability. Rather, this study indicates that skin allograft rejection can be mediated by *in vivo* collaborations between T-inducer cells and T-effector cells, and that the two interacting T cell subsets can express different Lyt phenotypes as well as different antigen specificities. The present study also demonstrates that a selective deficiency in antigen-specific T-

inducer cells results in a failure to reject specific skin allografts despite the presence of antigen-specific T-effector cells. The Lyt phenotype and antigen specificity of T cells that function as T-inducer and T-effector cells for in vivo allograft rejection responses parallel closely those of T cells that function as lymphokine-secreting Th and lymphokine-responsive Tk cells for in vitro cytolytic T cell responses. Indeed, in vivo skin allograft rejection was observed only in those mice whose T cell populations contained, by in vitro assessment, both Th and Tk cells reactive against the alloantigens of the graft.

The experimental model system that we used involved the reconstitution of skin-engrafted B10 nude mice with either whole spleen cell populations or negatively selected spleen cell subpopulations from naive H-2^b mice. In theory, it was possible that reconstituted nude mice contained T cells derived either from the nude host animal (42) or from contaminants in the reconstituting T cell population. However, flow cytometry failed to detect significant numbers of mature (i.e., L3T4⁺ or Lyt-2⁺) T cells derived from any source other than that with which the experimental mice were reconstituted. Upon adoptive transfer, it was found that nude mice reconstituted with negatively selected populations of Lyt-2⁺ T cells rapidly rejected MHC class I-disparate skin allografts, whereas nude mice reconstituted with negatively selected populations of L3T4⁺ T cells rapidly rejected MHC class II- and minor H-disparate skin allografts. The simplest interpretation of these observations was that the rejection specificities expressed by adoptively transferred T cell populations merely reflected their antigen specificities (43). However, such a perspective did not explain why collaboration between phenotypically distinct populations of L3T4⁺ and Lyt-2⁺ T cells was required for the rejection of H-Y-disparate skin allografts. Similarly, even though multiple minor H-disparate skin allografts were rejected by isolated L3T4⁺ T cell populations (Fig. 5, *center*), their rejection by T cell populations containing both L3T4⁺ and Lyt-2⁺ cells was significantly more rapid (Fig. 5, *left*), indicating that cell interactions between phenotypically distinct T cell populations also enhanced the rejection of multiple minor H-disparate skin allografts.

Consequently, we thought that a better understanding of the roles performed by T cells in skin allograft rejection would result from the simultaneous consideration of their Lyt phenotype, recognition specificity, and functional capabilities (Table V). Because cytolytic T cell responses have been considered to be the in vitro counterpart of in vivo graft rejection (44, 45), the T cell functions we focused on were those involved in CTL responses, namely the stimulation of lymphokine-mediated T cell help and the activation of lymphokine-responsive Tk cells (15, 37). The usefulness of such an analysis is evident in Table V; without exception, skin allograft rejection occurred only in mice reconstituted with T cell populations that, as shown by in vitro assessment, contained both Th and Tk cells specific for the alloantigens of the graft. Because T-inducer cells for skin allograft rejection in vivo and Th cells for CTL responses in vitro are identical in their Lyt phenotypes, antigen specificities, and ability to activate effector cells, it is likely T-inducer and Th cells are one and the same. While we have not shown that T-inducer cells mediate their helper function in vivo by

TABLE V
Correlation of In Vitro T Cell Functions with Observed In Vivo Rejection Responses*

Antigenic disparity relative to B10	Lyt phenotypes of responding H-2 ^b T cells	In vitro T cell function		Skin graft rejection
		Th	Tk	
Fully allogeneic (DBA/2)	Lyt-2 ⁺ + L3T4 ⁺	+	+	+
	Lyt-2 ⁺	+	+	+
	L3T4 ⁺	+	+	+
MHC class I (bm1)	Lyt-2 ⁺ + L3T4 ⁺	+	+	+
	Lyt-2 ⁺	+	+	+
	L3T4 ⁺	+	-	-
MHC class II (bm12)	Lyt-2 ⁺ + L3T4 ⁺	+	+	+
	Lyt-2 ⁺	-	+	-
	L3T4 ⁺	+	+	+
H-Y	Lyt-2 ⁺ + L3T4 ⁺	+	+	+
	Lyt-2 ⁺	-	+	-
	L3T4 ⁺	+	-	-
Multiple minors (BALB.B)	Lyt-2 ⁺ + L3T4 ⁺	+	+	+
	Lyt-2 ⁺	-	+	-
	L3T4 ⁺	+	+	+
Qa-1 ^a (B6.T1a ²)	Lyt-2 ⁺ + L3T4 ⁺	-	+	-
K ^{bm6} (bm6)	Lyt-2 ⁺ + L3T4 ⁺	+	+	-

* References are cited in the text.

secreting lymphokines, we think it is highly likely that they do so, since given the recognition specificities of T-inducer and T-effector cells, they are unable to recognize each other directly. However, Dr. Kevin Lafferty (University of Colorado, Barbara Davis Center, Denver, CO, personal communication) has suggested the alternate possibility that activated macrophages, rather than soluble lymphokines, perform the intermediary role in vivo between Th/T-inducer cells and Tk/T-effector cells, such that Tk/T-effector cells are triggered only by interacting with macrophages that had previously interacted with Th/T-inducer cells. Since T-effector cells for skin allograft rejection in vivo and Tk cells for CTL responses in vitro are identical in their Lyt phenotypes, antigen specificities, and helper requirements, it is tempting to conclude that T-effector and Tk cells are also one and the same. Indeed, it has been previously shown (46-49) that Tk cells are among the cellular elements that infiltrate rejecting allografts. Nevertheless, it is not yet clear that the mechanism by which T-effector cells reject skin allografts involves cell mediated cytotoxicity.

Consistent with the relationship between in vitro T cell responses and in vivo allograft rejection, the present in vivo demonstration of a selective Th cell deficiency in the failure of H-2^b mice to reject Qa1 disparate skin allografts parallels in vitro observations of a Th cell deficiency in Qa1-specific CTL responses (50, 51). Moreover, the ability of H-Y to function as a helper determinant for the in vivo activation of anti-Qa1 T-effector cells to reject Qa1-disparate skin allografts (Table III) also parallels the reported ability of H-Y to function as a helper determinant in vitro to activate anti-Qa1 Tk cells (50, 51).

While this study indicates that skin allograft rejection occurs only in those mice

containing both lymphokine-secreting Th and lymphokine-responsive Tk cells reactive against the alloantigens of the graft, it should be emphasized that the presence of both Th and Tk cells is not sufficient to predict that rejection will occur. For example, the majority of B6 mice fail to reject bm6 skin allografts despite the *in vitro* assessment that they contain both anti-K^{bm6} Th and Tk cells (Table V; reference 18), and despite their ability to generate anti-K^{bm6} CTL responses *in vitro* (18). Indeed, the present demonstration that a selective Th cell deficiency is responsible for the failure of H-2^b mice to reject bm6 skin allografts is consistent with our previous observations that H-2^b mice contain anti-K^{bm6} L3T4⁺ Th cells but are deficient in anti-K^{bm6} Lyt-2⁺ Th cells (18), and that it is the Lyt-2⁺ Th cell population that initiates the rejection of MHC class I-disparate skin allografts (9). Why do K^{bm6}-reactive L3T4⁺ Th cells function *in vitro* to initiate anti-K^{bm6} CTL responses (18) yet apparently fail to function *in vivo* to trigger rejection of K^{bm6} skin allografts? Failing to function *in vivo* to initiate skin allograft rejection is probably not unique to anti-K^{bm6} L3T4⁺ Th cells since we have never observed enhancement of any MHC class I rejection response by L3T4⁺ Th cells (Fig. 5, compare *left* and *right* panels). We think at least part of the explanation lies in the specificity of class I-allospecific L3T4⁺ Th cells for an antigenic complex composed of foreign class I plus self class II MHC determinants (19, 31, 37). We have recently shown that the Ia⁺ cells in the skin are markedly deficient in their expression of class I MHC determinants (52), so that the antigenic complex of class I plus class II MHC determinants may not be formed in sufficient quantity in skin allografts to activate class I-allospecific L3T4⁺ Th cells. Indeed, unlike class I-allospecific L3T4⁺ Th cells, class I-allospecific Lyt-2⁺ Th cells recognize MHC class I allodeterminants directly so that, in principle, Lyt-2⁺ Th cells can efficiently respond to allogeneic skin cells by recognizing class I allodeterminants that are either present in large amounts on the surface of Ia⁻ keratinocytes or are present in small amounts on the surface of Ia⁺ Langerhans' cells. Since Ia⁻ keratinocytes express on their surface 30 times as much MHC class I as do Ia⁺ Langerhans' cells (52), it is somewhat surprising that preliminary experiments indicate that class I-allospecific Lyt-2⁺ Th cells respond to skin cells because they respond to the small amount of MHC class I allodeterminants present on the surface of Ia⁺ Langerhans' cells (Mizuochi, T., S. Shimada, A. S. Rosenberg, S. W. Caughman, S. I. Katz, and A. Singer, unpublished results). Thus, we think that despite the presence of both Th and Tk cells, H-2^b mice fail to reject bm6 skin allografts *in vivo* because the K^{bm6}-specific Th cells they contain are exclusively L3T4⁺ whose recognition requirements are not fulfilled by the cellular elements constituting the bm6 skin allograft.

Much of the controversy surrounding the characterization of T cells involved in allograft rejection have resulted from attempts to deduce function from phenotype, even though T cell function does not strictly segregate with T cell phenotype (16, 19). The present results strongly support previous conclusions (1, 5, 7, 48) that both help and cytolytic T cell functions, whether provided by a single dual function T cell or by interactions between two functionally distinct T cells are necessary to initiate and mediate skin allograft rejection. However, the present observation that Lyt-2⁺,L3T4⁻ T cell populations are fully capable

of rejecting skin allografts expressing MHC class I disparities appears to conflict with the observation of Cobbold and Waldmann (53) that in vivo administration of anti-L3T4 antibody significantly inhibited the rejection of fully allogeneic skin allografts. Studies investigating this apparent discrepancy are in progress.

In conclusion, the present study indicates that skin allograft rejection can be reasonably viewed as the in vivo endpoint of an antigen-specific immune process initiated by lymphokine-secreting Th cells and mediated by lymphokine responsive Tk cells. Thus, a rejection response depends upon: (a) the presence of Th and Tk cells each specific for alloantigens expressed by the graft, and (b) the ability of cellular elements within the skin allograft to fulfill the recognition and activation requirements of the allospecific T cell populations present in the host.

Summary

In the present study we used an adoptive transfer model with athymic nude mice to characterize the T cells involved in initiating and mediating skin allograft rejection. It was found that skin allograft rejection in nude mice required the transfer of immunocompetent T cells and that such reconstitution did not itself stimulate the appearance of T cells derived from the nude host. Reconstitution with isolated populations of Lyt-2⁺/L3T4⁻ T cells resulted in the rapid rejection of MHC class I-disparate skin allografts, whereas reconstitution with isolated populations of L3T4⁺/Lyt-2⁻ T cells resulted in the rapid rejection of MHC class II-disparate and minor H-disparate skin allografts. By correlating these rejection responses with the functional capabilities of antigen-specific T cells contained within the reconstituting Lyt-2⁺ and L3T4⁺ T cell populations, it was noted that skin allografts were only rejected by mice that, as shown by in vitro assessment, contained both lymphokine-secreting Th cells and lymphokine-responsive Tk cells specific for the alloantigens of the graft. The ability of two such functionally distinct T cell subsets to interact in vivo to reject skin allografts was directly demonstrated in H-Y-specific rejection responses by taking advantage of the fact that H-Y-specific Th cells are L3T4⁺ while H-Y specific Tk cells are Lyt-2⁺. Finally, the importance of in vivo interactions between functionally distinct Th/T-inducer cells and T killer (Tk)/T-effector cells in skin allograft rejection was demonstrated by the observation that normal B6 mice retain Qa1^a and K^{bm6} skin allografts because of a selective deficiency in antigen-specific Th cells, even though they contain T-effector cells that, when activated, are able to reject such allografts.

Thus, the ability to reject skin allografts is neither unique to a specialized subset of T cells with a given Lyt phenotype, nor unique to a specialized subset of helper-independent effector T cells with so-called dual function capability. Rather, skin allograft rejection can be mediated by in vivo collaborations between T-inducer cells and T-effector cells, and the two interacting T cell subsets can express different Lyt phenotypes as well as different antigen specificities.

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