CELLS MEDIATING GRAFT REJECTION IN THE MOUSE

I. Lyt-1 Cells Mediate Skin Graft Rejection*

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It is commonly accepted that the in vitro lysis of allogeneic target cells by primed, cytotoxic T cells (Tc)[1] is an appropriate model for studying graft rejection. For example, Tc can be found in grafts undergoing rejection (1–3), in the spleen and other tissues of primed mice (1, 4, 5), and in sponge matrix grafts (6, 7). In addition, extensive in vitro studies have demonstrated the induction and function of Tc, the requirement of helper T cells (Th) for their induction, and the simultaneous induction of suppressor T cells (5, 8–14). In these studies, it has become abundantly clear that the Lyt-123/23-Tc[2] is different from Th cells (which are Lyt-1), suppressor T cells, and other T cells. In spite of the many experiments that support the concept that Tc produce graft rejection in vivo, there is little direct evidence demonstrating this, and most of the foregoing evidence can be regarded as "indirect." Indeed, it has not been clearly demonstrated that Tc have the only role, a major role, or any role in graft rejection in vivo. With this in mind, adult, thymectomized, lethally irradiated, bone marrow-restored (ATXBM) mice were repopulated with sensitized cells, depleted of Lyt-1, Lyt-2, or Lyt-123 subpopulations, and were then examined for the phenotype of the T cell causing skin graft rejection. With antigenic differences arising from the *H-2* and non-*H-2* loci, it was shown that graft rejection was entirely dependent on the presence of Lyt-1 cells and, furthermore, was independent of the presence of Tc. It appears that traditional concepts, linking graft rejection and delayed-type hypersensitivity (DTH), may well be true, in that both are mediated by Lyt-1 cells.

Materials and Methods

Mice. CBA/H ($H\text{-}2^k$), C57BL/6J ($H\text{-}2^b$), and BALB/cBy ($H\text{-}2^d$) strains were maintained inbred. Male mice were used in all experiments.

ATXBM Preparation. Adult thymectomy (16) of CBA/H mice was performed at 5–7 wk of age, followed 2 wk later by lethal whole body irradiation (800–900 rad) and immediate intravenous reconstitution with 3×10^6 Thy-1$^-$ syngeneic adult bone marrow cells (depletion by 1.0 ml anti-Thy-1.2 serum plus 1.0 ml rabbit complement [RC′] per 2×10^8 bone marrow

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[1] *Abbreviations used in this paper:* ATXBM, adult thymectomized, lethally irradiated and bone marrow restored; CTL-P, precursor cell of the cytotoxic T lymphocyte; DTH, delayed-type hypersensitivity; MLC, one-way mixed lymphocyte culture; MST, mean survival time; PFC, plaque-forming cells; RC′, rabbit complement; SAMG, sheep anti-mouse immunoglobulin; SRC, sheep erythrocytes; Tc, cytotoxic T cell defined by cell-mediated lymphocytotoxicity; Th, helper T cell; T_{DTH}, T lymphocyte mediating DTH.

[2] We have not been able to demonstrate directly the presence of an Lyt-1$^-$ cell in CBA mice although such cells appear to exist as functional entities and we would agree that the Tc in CBA mice is Lyt-123 ([15]; and this paper).

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cells, incubated as described below). These mice were used in experiments after an additional 2–4 wk.

Antisera. The production and testing of conventional antisera and monoclonal antibodies has been described elsewhere (13, 17, 18).[3] Antisera with high cytotoxic titers were used in this way: (*a*) anti-Thy-1.2 antiserum was made as (B6.PL(74NS) × RF)F_1 anti-C57BL/6 (thymus titer ~1/1,000); (*b*) Lyt-1.1 monoclonal antibody was made as 129/ReJ anti-B6-*Ly-1*^{a} (thymus cytotoxic titer of supernate ~1/2,000); and (*c*) Lyt-2.1 monoclonal antibody was made as 129/ReJ anti-B6.PL-Lyt-2.1,3.1 (thymus cytotoxic titer of supernate 1/256).

Sensitization and Preparation of Donor Cells for Adoptive Transfer. CBA/H mice received at least two C57BL/6J or BALB/cBy skin allografts and an intraperitoneal dose of 2×10^7 lymphocytes, the last being 2–3 wk before their use as donors for the adoptive transfer of sensitized cells. Cells were obtained from the spleen and the mesenteric and axillary lymph nodes of sensitized mice, and were teased, depleted of erythrocytes by lysis in warm 0.83% wt/vol ammonium chloride, and resuspended as single cell suspensions. For depletion with Ly antibodies, 2-ml aliquots of 10^8 cells were mixed with 0.4 ml anti-Lyt-1.1 antibody (monoclonal), 0.5 ml anti-Lyt-2.1 antibody (monoclonal), or 1.0 ml anti-Thy-1.2 antiserum. Cells were then incubated for 30 min on ice, and washed and incubated with selected non-toxic RC′ at an optimal concentration for 45 min at 37°C. The viable cells were collected on Ficoll-Isopaque (sp gr 1.09), and the antibody and RC′ treatments were repeated as before. Although the doses of antibody were in excess for maximum cytotoxicity, the second treatment was required for successful adoptive transfer and presumably removed residual antigen-bearing cells that survived the first treatment. Testing depleted cell populations by cytotoxicity demonstrated >98% depletion and this was exemplified by assays of cells taken after transfer (see Results). Anti-Thy-1.2 or anti-Lyt-1.1 treatment killed 50% of the spleen plus lymph node cell suspension, whereas anti-Lyt-2.1 treatment killed 25% of the cells. Aliquots of cells were adoptively transferred, intravenously, in volumes of 0.15 or 0.20 ml into recipient ATXBM-CBA mice.

Rosette Depletion. Sheep erythrocytes (SRC) coupled with sheep anti-mouse gammaglobulin (SAMG) and chromic chloride were used to form rosettes with Ig^+ cells (19). Nonrosetting cells were collected from the interface of Ficoll-Isopaque solution after centrifuging for 12 min at 1,000 *g*, and Ig^+ cells were recovered from the pellet. Less than 2% of interface cells were capable of forming Ig rosettes and >90% were sensitive to lysis by anti-Thy-1.2 antiserum plus RC′; pellets contained 90–95% rosetting lymphocytes. Hence the interface consisted of T cells, whereas B cells were isolated in the pellet. Rosettes formed with protein A-coupled SRC (20) were also used with monoclonal antibodies to detect spleen T cell subsets.

Skin Grafts. Skin for grafting was obtained from the dorsal or ventral surface of the donor mouse and grafts 0.5–1.0 cm^2 were applied to the thoracic wall (21). Graft rejection was read as total graft necrosis and expressed as the mean survival time (MST).

Plaque-forming Cell (PFC) Assay for Th Function. A splenic PFC to SRC was performed 5 d after intravenous injection of 0.2 ml of 2% vol/vol SRC (22). Both direct and indirect PFC (detected with rabbit anti-mouse Ig) were assayed, using guinea pig serum as a source of complement.

Cytotoxic T Lymphocyte Precursor Cell (CTL-P) Frequency Determinations. The frequency of CTL-P in lymphoid cell suspensions was determined as previously described (23). For each cell suspension in which CTL-P frequencies were determined, a minimum of 32 replicates for each of at least four responder cell doses were cultured in U-bottomed microtiter trays (Linbro Chemical Co., Flow Laboratories, Inc., Hamden, Conn.) with 3×10^5 C57BL/6-*nu/nu* or BALB/c-*nu/nu* spleen stimulator cells in modified Eagle's medium supplemented with 10% fetal calf serum. 7 d later, each microculture was assayed for cytotoxicity by replacing 100 μl of culture medium with 100 μl target cell suspension containing 10^4 ^{51}Cr-labeled EL4 or P815 tumor target cells. Wells were regarded as containing cytotoxic activity if they yielded specific ^{51}Cr release 3 SD above the mean isotope release from 10^4 cells cultured alone. A linear relationship existed between the dose of responder cells on a linear scale and the frequency of negative wells on a logarithmic scale. CTL-P frequencies were determined as the inverse of the responder cell dose required to generate 37% negative wells (24).

[3] Hogarth, P. M., J. Edwards, I. F. C. McKenzie, and F. Y. Liew. Monoclonal antibodies to the Lyt-2.1 murine cell surface antigens. Manuscript submitted for publication.

Conditioned Medium. CBA spleen cell suspensions were incubated for 2 h with 5 μg/ml concanavalin A, washed, and incubated overnight before collecting the supernatant fluid (conditioned medium), which was used as a source of helper activity.

DTH. A DTH response to allgeneic lymphocytes was elicited by sensitization with 3×10^7 spleen cells injected subcutaneously and followed 6 d later by footpad challenge with 5×10^6 lymphocytes in a volume of 0.05 ml. The 24-h footpad swelling was measured using Poco-Test calipers (Schnelltaster, Diatest U. K. Ltd., London, England) to ±0.05 mm. Results were expressed as the percent specific increase in footpad thickness.

Statistical Analysis. Data were calculated to give mean and standard errors, and statistical significance was determined by Student's *t* test.

Results

Dose Response Studies for Adoptive Cell Transfer and T Cell Dependence of Allograft Rejection. The protocol used to prepare ATXBM-CBA mice is outlined in Fig. 1. In periods of observation of 50–200 days ATXBM-CBA mice were incapable of rejecting skin allografts, as were animals repopulated with Thy-1.2-depleted sensitized lymphocytes (Table I). In contrast, mice receiving untreated sensitized cells rejected grafts in 10–14 d. It was found that 1×10^6 viable sensitized lymphocytes (spleen plus lymph node) restored the immune response in ATXBM-CBA mice to the maximum rate of allograft rejection where mean survival times in six different experiments varied

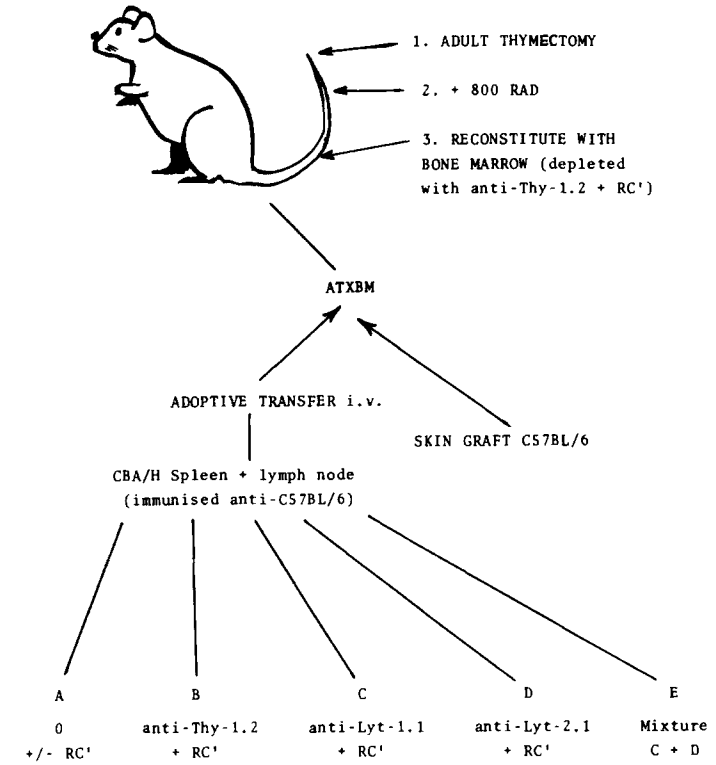

FIG. 1. Scheme of preparation and use of ATXBM-CBA/H mice. Adult thymectomized mice were lethally irradiated (800 rad) and reconstituted with 3×10^6 bone marrow cells depleted of Thy-1.2 cells (antiserum plus RC') within 24 h of irradiation. After 3–4 wk, ATXBM mice were given variously treated spleen cells plus lymph node cells and a full thickness skin graft and were then observed for a specific response against the skin graft.

TABLE I

T Cell Dependence of the Adoptively Transferred Allograft Response

Experiment	Cells transferred*	Number of recipients	Graft survival‡
1	None	4	>50
	7×10^6 untreated	5	14.2 ± 1.0
	8×10^6 Ig depleted	6	13.7 ± 0.7
	7×10^6 Thy-1.2 depleted	5	>44
2	None	5	>150
	10×10^6 untreated	8	10.1 ± 0.7
	8×10^6 Thy-1.2 depleted	6	>130
3	1.5×10^6 untreated	8	11.1 ± 1.4
	1×10^6 Thy-1.2 depleted	4	>60

* Viable spleen plus lymph node cells from sensitized donors; Ig depleted by separation of SAMG-rosettes on Ficoll-Isopaque gradients or depleted with anti-Thy-1.2 antiserum plus RC'.

‡ Mean ± SE or duration of observation.

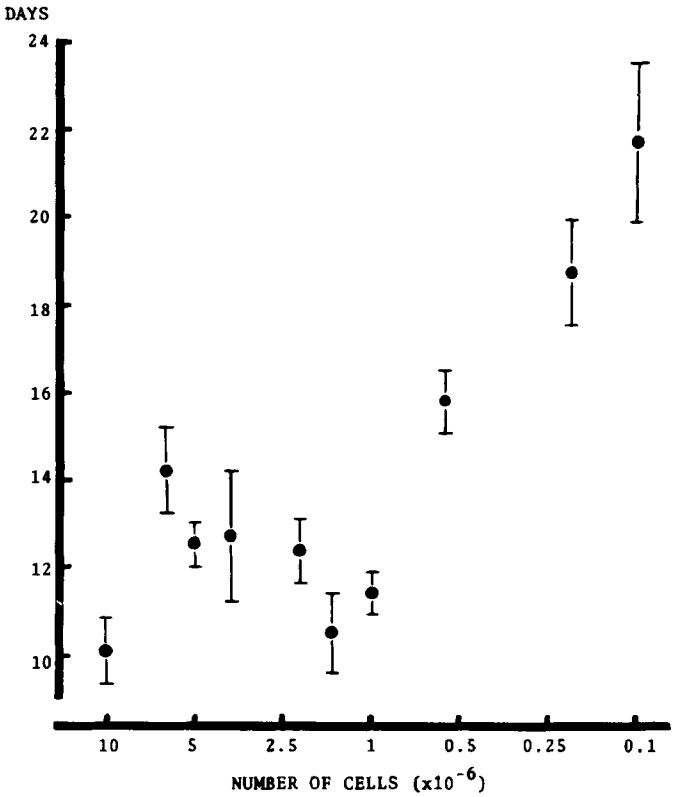

FIG. 2. Dose response of the adoptive transfer of sensitized lymphocytes into ATXBM-CBA mice and subsequent skin graft rejection. Groups of 5–10 mice received viable spleen cells plus lymph node cells 1 d before a C57BL/6 skin graft. Results are expressed as the MST ± SE.

between 9.7 and 14.2 d. The transfer of $<10^6$ viable lymphocytes resulted in a delayed rejection (>15 d) (Fig. 2), whereas up to 10^7 viable cells did not greatly affect the survival time over that produced with 10^6 cells. In most experiments, ~1–3 × 10^6 viable cells were adoptively transferred.

TABLE II

Adoptive Transfer of Lyt-1-depleted Sensitized Cells to ATXBM-CBA Mice

Experiment	Adoptive cell dose*	Lyt-1 depleted	Number of recipients	Graft survival‡
1	33×10^5	−	7	12.7 ± 1.5
	65×10^5§	+	9	34.9 ± 4.1
2	10×10^5	+	7	>62
	8×10^5	+	4	>210
3	40×10^5	−	4	27.5 ± 4.9
	13×10^5	+	4	>68
4	50×10^5§	−	4	12.5 ± 0.5
	33×10^5	−	5	18.2 ± 1.6
	31×10^5	+	3	>100

* Viable cell dose of spleen plus lymph node cells, either treated (+) with anti-Lyt-1.1 antibody plus RC′, or not (−).

‡ Mean ± SE or duration of observation.

§ Second adoptive transfer of spleen plus lymph node cells 3–5 mo after the first skin graft (see text).

To exclude any role of B (Ig^+) cells in allograft rejection, Ig^+ cells were removed from a sensitized spleen plus lymph node cell suspension. Mice given untreated cells rejected their grafts in 14.2 d, whereas those given B-depleted cells (Ig^-) rejected grafts in 13.7 d, i.e., Ig^+ B cells were not essential for, and did not mediate the rapid rejection response to allografts (Table 1).

Lyt-1 Dependence of the Allograft Response. Depletion of the primed cell inoculum of cells bearing the Lyt-1 specificity prevented graft rejection. Four groups of mice receiving Lyt-1^--sensitized T cells were observed for at least 60 d, and some were observed until death at 210 d. In all groups, rejection was delayed: from 12.7 to 34.9 d (experiment 1, Table II) and from 62 to 200 d in the other experiments, i.e., in the latter experiments the grafts did not reject in mice lacking Lyt-1 cells (Table II). One group of mice that had rejected grafts in 34.9 ± 4.1 d (experiment 1) was used as donors for a second adoptive transfer assay. In Table II (experiment 3), it can be seen that alloreactivity was transferable from these mice (MST 27.5 ± 4.9 d), but that the rejection response was sensitive to anti-Lyt-1.1 antibody treatment because the grafts on treated mice did not reject during an observation period of >68 d. Presumably the shortened survival (35 d) in the first transfer was due to residual Lyt-1 cells that were eradicated in the second transfer. If the cells were taken from ATXBM-CBA mice repleted with untreated cells (experiment 4; MST 12.5 ± 0.5 d), a similar result was observed: untreated cells transferred alloreactivity (MST 18.2 ± 1.6 d), whereas Lyt-1-depleted cells were unable to transfer alloreactivity (no rejection at 100 d).

Lyt-2 Depletions and the Allograft Response. Cell depletion by monoclonal anti-Lyt-2.1 antibody plus RC′ did not significantly delay the graft rejection mediated by sensitized cells (Table III). In three experiments, mice that received 10^6 or more Lyt-2.1-depleted sensitized lymphocytes (i.e., Lyt-1^+2^- cells) rejected skin grafts in mean times ranging from 11.0 to 13.5 d. These results were similar to those found in mice that received untreated cells and rejected grafts in mean times of 11.6–12.7 d. Mixing Lyt-1-depleted cells with either Lyt-2-depleted cells or with a limiting dose of untreated cells was found to have no effect on graft rejection as mediated by either Lyt-2-depleted or untreated inocula (Table III, experiment 3). Injection of 2×10^6

TABLE III

Repletion of ATXBM-CBA Mice with Depleted Lymphocytes from C57BL/6-immunized CBA Donors

Experiment	Adoptive cell dose*			Number of recipients	Graft survival‡
	Untreated	Lyt-1 depleted	Lyt-2 depleted		
1	10×10^5	—	—	7	11.6 ± 0.7
	—	—	25×10^5	15	13.5 ± 0.4
2	10×10^5	—	—	6	12.7 ± 0.8
	6×10^5	—	—	5	15.8 ± 0.7
	—	—	30×10^5	5	11.0 ± 0.3
	—	—	10×10^5	8	12.0 ± 0.4
	—	—	5×10^5	7	15.4 ± 2.0
	—	—	—	5	>60
3	20×10^5	—	—	5	12.4 ± 0.7
	—	—	20×10^5	6	13.0 ± 1.5
	—	20×10^5	—	6	>40
	—	20×10^5	20×10^5	7	15.0 ± 1.1
	—	10×10^5	10×10^5	7	13.4 ± 0.6
	2×10^5	—	—	6	17.8 ± 0.8
	2×10^5	20×10^5	—	6	16.4 ± 0.5
	—	—	—	4	>80

* Viable spleen plus lymph node cells, either not treated or depleted as described in the text by anti-Lyt-1.1 monoclonal antibody or anti-Lyt-2.1 monoclonal antibody plus RC′.

‡ Mean ± SE or duration of observation.

Lyt-2-depleted cells resulted in mean graft survival of 13.0 ± 1.5 d, which was not affected by the addition of Lyt-1-depleted cells (MST 15.0 ± 1.1 d, $P < 0.15$). More important, a limiting dose of untreated cells (2×10^5; MST 17.8 ± 0.8 d) was not significantly affected when mixed with 2×10^6 Lyt-1-depleted cells (MST 16.4 ± 0.5 d, $P < 0.10$). Hence the allograft response in sensitized mice appeared to be mediated by Lyt-1^+2^- cells with no apparent synergistic effect of Lyt-1^-2^+ cells or Lyt-1^+2^+ cells.

Lymphocyte Populations in the Spleens of Reconstituted ATXBM-CBA Mice. The cell surface phenotype was determined in the spleens of reconstituted mice to determine the efficacy of the depletion protocol. Protein A rosetting assays, in which the background was <10%, were used to determine the relative frequencies of Lyt-1, Lyt-2, and Thy-1 antigen-bearing cells 50–200 d after adoptive transfer. It was observed that ATXBM-CBA mice that had received sufficient cells (>1×10^6 sensitized lymphocytes) for graft rejection in minimum time (10–12 d) had normal total counts of splenic lymphocytes (7–10×10^7 cells; data not shown), but often had lower percentages of T cell subsets than normal mice (Fig. 3). For instance, results showed 18–21% Thy-1 and Lyt-1 cells compared with 30–35% in normal mice; and 13% Lyt-2 cells compared with 20% in normal mice. However, specific subset depletion was apparent in the spleens of mice that either had not been repleted (ATXBM) or had received anti-Thy-1.2-treated inocula (3% Thy, 2% Lyt-1, 2% Lyt-2), anti-Lyt-1-treated inocula (2% Lyt-1-, or anti-Lyt-2-treated inocula (2% Lyt-2) (Fig. 3). The anti-Lyt-1.1 monoclonal antibody reacted with slightly more cells than the anti-Thy-1.2 antiserum, presumably due to a higher affinity of binding. It was therefore concluded that antibody depletions were satisfactory both in vitro and for up to 200 d after adoptive transfer into ATXBM mice.

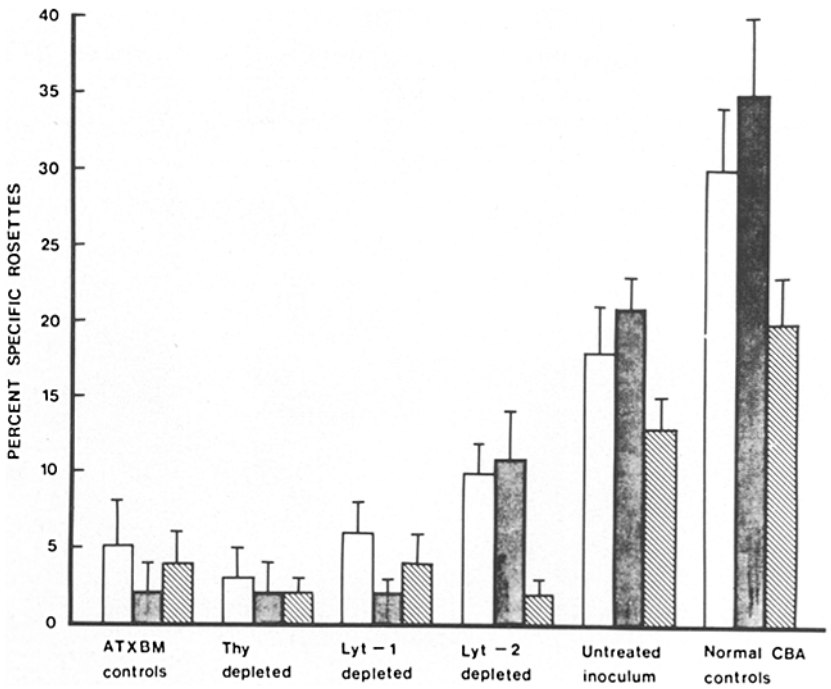

FIG. 3. Percentage of spleen T cell antigens in repleted ATXBM-CBA mice. All mice received a skin graft 1 d after the adoptive transfer of spleen cells plus lymph node cells and were killed 50–200 d later. No differences were found with time elapsed, so the data were pooled. Percent specific Protein A rosettes corrected for background reaction of 3–5%; □, Thy-1.2 cells; ■, Lyt-1.1 cells; and ▧, Lyt-2.1 cells.

TABLE IV

Helper Activity in Repleted ATXBM-CBA Mice as Indicated by a PFC Response

Experiment	Spleen donor*	PFC/10^6 IgM (direct)	Spleen cells IgM + IgG (indirect)‡
1	A. Repleted	16 ± 3	31 ± 8
	B. Lyt-1 depleted	<2	<3
	C. Lyt-2 depleted	49 ± 13	168 ± 28
	D. B + C	49 ± 10	72 ± 16
2	E. Repleted	44 ± 6	42 ± 8
	F. Lyt-1 depleted	10 ± 1	14 ± 2
	G. Lyt-2 depleted	35 ± 7	50 ± 12
	H. F + G	26 ± 8	33 ± 3
	I. Normal mouse control	267 ± 14	730 ± 48
3	J. Repleted	111 ± 7	162 ± 22
	K. Lyt-1 depleted	10 ± 2	3 ± 1

* ATXBM-CBA mice repleted with sensitized spleen plus lymph node cells as described in the text (50–100 d earlier) and killed 5 d after injection of SRC.

‡ Mean PFC ± SE for three donors in each assay.

Helper Cell Activity in Reconstituted Mice. As an additional measure of the depletions and to examine the nature of the residual cell populations in reconstituted mice, the helper T cell activity for the antibody response to SRC was assayed 50–100 d after

TABLE V

Limiting Dilution Analysis of Cytotoxic Spleen Cells

Experiment	Treatment of spleen cells*	Target cell	CTL-P frequency‡	Percent depletion
Normal CBA responder				
1	None	P815	1/4,300	—
	A. Anti-Lyt-1.1	P815	<1/96,000	95.5
2	None	P815	1/31,500	—
	B. Anti-Lyt-2.1	P815	<1/315,000	>90
	A + B	P815	<1/285,000	>89
Sensitized CBA responder				
3	None	EL4	1/1,000	—
	A. Anti-Lyt-1.1	EL4	<1/120,000	>99
4	None	P815	1/2,200	—
	A. Anti-Lyt-1.1	P815	<1/3,000,000	>99.9
	C. Anti-Lyt-1.1 plus conditioned medium	P815	<1/3,000,000	>99.9
	D. Conditioned medium only	P815	1/1,900	—
5	None	P815	1/5,800	—
	B. Anti-Lyt-2.1	P815	<1/85,000	>93
	A + B	P815	<1/122,000	>95

* Responder spleen cells alone; A, treated with anti-Lyt-1.1 antibody and RC′; B, treated with anti-Lyt-2.1 antibody and RC′; C and D, adding conditioned medium.

‡ Limiting dilution analysis of cytotoxic precursors or the limit of detection of the culture system.

TABLE VI

Correlation of DTH Responses and Skin Graft Rejection

Status of responder	Skin graft rejection*	Number of recipients	Percent swelling‡	DTH response
ATXBM	No	4	3.2 ± 1.9	−
	Yes	6	17.6 ± 6.5	+
Lyt-1 depleted	No	13	3.6 ± 1.1	−
	Yes	4	20.1 ± 4.7	+
Lyt-2 depleted	No	0	—	
	Yes	32	35.7 ± 3.7	+
Thy-1.2 depleted	No	3	6.4 ± 4.6	−
	Yes	0	—	
Reconstituted	No	0	—	
(no depletion)	Yes	10	42.3 ± 5.9	+

* C57BL/6 skin graft 40–100 d after adoptive transfer of spleen plus lymph node cells.

‡ Percent increase in footpad thickness corrected for nonspecific swelling of the other footpad. Mean ± SE.

reconstitution and grafting. Lyt-1-depleted animals had barely detectable numbers of direct (IgM) PFC ($<10/10^6$ spleen cells), in comparison with mice that received untreated cells, Lyt-2-depleted cells, or a mixture of Lyt-1-depleted and Lyt-2-depleted lymphocytes (16–49 PFC/10^6 spleen cells) (Table IV). Fully repleted ATXBM mice (i.e., graft rejection within 10–15 d) had only 16–55 direct PFC per 10^6 spleen cells, compared with 267 ± 14 per 10^6 spleen cells in normal animals. In addition, mice that received untreated, Lyt-2-depleted, or the mixture of Lyt-1-depleted and Lyt-2-depleted cells, all had significantly more indirect (IgM plus IgG) PFC than those given Lyt-1-depleted cells. It was evident that Lyt-1-depleted mice lacked effective T helper function, whereas Lyt-2-depleted mice had normal or increased helper function, the increase presumably due to the removal of suppressor T cells.

In Vitro Determination of CTL-P Frequencies. Spleen cells from normal and reconstituted mice were used as responder cells in mixed lymphocyte cultures (MLC), which were assayed for cytotoxicity 7 d later. Using this culture method, the frequency of anti-H-2^d CTL-P in unprimed spleen cell suspensions was 1/5,000 and after specific sensitization this increased to 1/1,500 (Table V). Using responder cells depleted of Lyt-1 cells, the frequency of CTL-P was reduced by >95% and in two experiments by 99%. Although linear semilogarithmic plots of responder cell dose vs. proportion of negative cultures were consistently obtained, it was conceivable that the development of cytotoxicity was dependent upon the presence of helper cells provided by the responder cells, and that Lyt-1 depletion simply removed these accessory or helper cells. Therefore, the MLC was set up with Lyt-1-depleted responder cells plus *H-2* allogeneic nude spleen stimulator cells that were cultured in modified Eagle's medium supplemented with 10% conditioned medium obtained from concanavalin A-activated spleen cell suspensions. (This medium was known to contain helper activity necessary for the generation of CTL-P from limiting numbers of responder cells. Rh. Ceredig, unpublished observations.) Addition of conditioned medium had no effect on the responses of either Lyt-1-depleted or untreated cell suspensions (Table V), indicating that depletion of Lyt-1 cells did not merely remove T cell help, but in fact removed a Tc bearing the Lyt-1 specificity. Treatment of responder cells with the monoclonal anti-Lyt-2.1 antibody also reduced the CTL-P frequency by 89%, whether the responder cells were sensitized or not (Table V). Hence both Lyt-1 treatment and Lyt-2 treatment inhibited the generation of cytotoxicity, which indicates that the Tc was Lyt-123. However, this result could also be due to a synergistic interaction between Lyt-1 and Lyt-2 cells. Nevertheless, mixing Lyt-1^- and Lyt-2^- cell suspensions did not result in the generation of cytotoxicity in the MLC, because there was 89 and 95% depletion of CTL-P in two such experiments. Therefore, the CTL-P generated in MLC was Lyt-123 and the monoclonal antibodies could clearly deplete this population.

DTH Responses Correlate with Graft Rejection. All groups of reconstituted mice were tested for the ability to elicit a DTH response generated ~50 d after adoptive transfer. Mice that were unable to reject a skin graft had no significant footpad swelling, whereas those which had rejected a skin graft, even in a delayed manner, were found to exhibit specific swelling of 18–44% (Table VI). This swelling was maximal at 24 h and rapidly reduced thereafter (data not shown). The ability to elicit the DTH response required an Lyt-1 cell, because ATXBM mice reconstituted with either Lyt-1-depleted or Thy-1-depleted cells, or no cells at all did not exhibit footpad swelling. In contrast, Lyt-2 depletion was without effect on the expression of either the DTH response or on allograft rejection. In all 72 mice examined, there was complete correlation between skin graft rejection and the DTH response (Table VI).

Discussion

There is evidence that Tc are generated during graft rejection in vivo (see Introduction), and it is established that Ly cell surface phenotypes distinguish the Tc subset from other T cell subsets. Strong evidence suggests that Tc are the prime mediators of rejection. However, our data show that this is not the case and that skin graft rejection in the mouse is mediated by Lyt-1 cells that do not have killer function and are distinct from the Lyt-123 (or Lyt-23) Tc. Furthermore, our studies suggest

that graft rejection and DTH responses are similar, if not identical, phenomena. These findings throw considerable doubt on the validity of using in vitro studies of Tc as the most appropriate model to examine graft rejection. Because of the implications of these studies our model needs to be discussed in detail and compared to other relevant studies. As indicated, the evidence of a role of Tc in graft rejection has been extensively reviewed elsewhere (5, 12, 13) and will not be reviewed here. However, it should be emphasized that most of the evidence accumulated is indirect, showing that Tc are generated during graft rejection, can be recovered during and after rejection, and are not induced in vivo apart from the ability to reject grafts. These findings do not prove that Tc do mediate graft rejection.

The model that we used appeared to be suitable for the study of the separate and joint roles of Lyt-1, Lyt-2, and Lyt-123 cells in graft rejection. As expected, the T cell-deficient ATXBM mice were unable to reject grafts. Direct typing of spleen (Table IV) and lymph nodes and examination for thymic remnants confirmed T cell deficiency (although several "ATXBM" mice did eventually reject their grafts and were found to have small numbers of T cells and residual thymic tissue; these were excluded from the study). The careful dose response studies (Fig. 2) in repopulated mice demonstrated a limiting cell effect of sensitized cells to mediate graft rejection and Thy-1 depletion showed the T cell dependence (Thy-1^+ Ig^- cell; Table I). Thus the model appeared to conform to the usual criteria applied to T cell-mediated reactions. However, the most crucial questions concern the cell depletions. Were the depletions adequate? Would the results be explained by a more thorough depletion with the Lyt-1.1 rather than the Lyt-2.1 antibodies? do the monoclonal antibodies really bind the defined Lyt-1 and Lyt-2 determinants? These antibodies do recognize the defined determinants based on their strain and tissue distributions, their reaction with *Lyt-1* and *Lyt-2* congenic strains, and a 67,000-mol wt molecule is precipitated by the anti-Lyt-1.1 antibody (17).[3] Furthermore, both reagents have been demonstrated to react with Tc (Table V; 17, 18), which, in CBA mice, is an Lyt-123 cell (15). In addition, the T helper cell was identified in these studies, as expected, as an Lyt-1^+2^- cell (Table IV). Thus, by the usual genetic and functional criteria, the two monoclonal antibodies appear to react with the Lyt-1 and Lyt-2 determinants. We also consider that the depletions were satisfactory. After exposing the cells twice to excess amounts of the monoclonal antibodies and to complement, we were unable to detect the appropriate cell and, at various times after adoptive transfer, these cells were not detected in mice in significant numbers by rosetting methods (Fig. 3). Finally, by repeating the depletion on Lyt-1.1-depleted mice (following a long-delayed skin graft rejection), the key role of the Lyt-1 cell in graft rejection was again demonstrated (Table II). In addition, the in vitro treatments were successful in the depletion of Tc with both antibodies (Table V) and with anti-Lyt-1.1 for the helper T cell. Although it may be considered that depletions are never 100% successful and that small numbers of cells could be transferred, and could multiply and produce effects, we were given no reason to interpret data in this way and were unable to detect such a clonal expansion of Lyt-1 or Lyt-2 cells in mice receiving cells depleted of these populations (Fig. 3). It could be suggested that the studies are deficient in that a "negative selection" has been used, and studies are in progress to transfer pure populations of selected cells. However, we would note that negative selection by the

removal of Lyt-1 cells is in fact a positive selection for Lyt-2 cells, and vice versa with Lyt-2 depletion (Table VII).

On the basis of the foregoing points, the results can be summarized here and in Table VII as: (*a*) depletion of cells bearing the Lyt-1 antigen (Lyt-1 and Lyt-123) removed Tc, Th, and graft rejection capability; (*b*) depletion of cells bearing the Lyt-2 antigen (Lyt-2 and Lyt-123) removed Tc only, with Th and graft rejection ability remaining; (*c*) mixing of cells obtained in (*a*) and (*b*) demonstrated Tc to be absent, whereas Th and graft rejection were retained.

That the cell that mediates graft rejection has no killer function was clearly shown when Lyt-2 depletion removed the CTL-P but did not prevent skin graft rejection. Further, when in vitro helper activity for the generation of cytotoxicity was provided "in excess" by the addition of conditioned medium or by the mixing of Lyt-1-depleted and Lyt-2-depleted populations, cytotoxicity remained at background levels. Hence. the results show: graft rejection for T cell, Lyt-1; for Th, Lyt-1; for Tc, Lyt-123; i.e., there is a clear distinction between cells producing graft rejection and the Tc, and in addition, our studies using sensitized cells have not demonstrated any synergy in graft rejection between the three T cell subsets examined.

How do Lyt-1 cells produce graft rejection, and is there a relationship between this cell and other Lyt-1 cells (i.e., T lymphocytes mediating DTH (T_{DTH}) and Th)? At present we cannot distinguish these three possible Lyt-1 cell types. It seems that Th and T_{DTH} are different cells, e.g., help and DTH are different phenomena. However, there are compelling reasons to consider that T_{DTH} and T (graft rejection) are the same cell, and that DTH and skin graft rejection are different manifestations of the same response (Table VI). Traditionally, and before the discovery of T cell subsets, these phenomena were linked (25). However, in recent studies of DTH to the H-Y antigen, there is a close correlation between the DTH reaction and graft rejection (26). Furthermore, it should be noted that in the H-Y model, graft rejection and the detection of Tc are independent phenomena. These findings, taken together with ours, indicated that graft rejection and the detection of Tc are mediated by different cells in what may be a general dissociation of the two phenomena. Conversely, there appears to be a close association between graft rejection and the DTH phenomena for *H-2* differences (Table VI), *H-Y* differences (26), and for other antigenic differences (I. F. C. McKenzie, unpublished observations). The linking of graft rejection and DTH should have important implications for mechanistic and therapeutic models to study graft rejection in man and we note that one of the best predictive tests for graft survival in man is the DTH status of the recipient awaiting transplantation (27). Therefore, the studies reported here are important in both the analysis of the mechanism and the treatment of graft rejection, for if they are applicable to all types

TABLE VII

Summary of Findings of Functional T Cell Populations

Antibody	Cells removed	Cells present	Tc	Th	Graft rejection	DTH
A. None	None	Lyt-1, Lyt-123, Lyt-23	+	+	+	+
B. Anti-Lyt-1.1	Lyt-1, Lyt-123	Lyt-23	−	−	−	−
C. Anti-Lyt-2.1	Lyt-123, Lyt-23	Lyt-1	−	+	+	+
D. B + C	Lyt-123	Lyt-1, Lyt-23	−	+	+	+

of grafts (see below), then we should be considering ways to neutralize lymphokines and other secondary mediators involved in DTH responses (28), rather than considering the removal of the Tc.

What then is the role of the Tc if not for graft rejection? It is clearly the mediator of a sophisticated, potent system of specific in vitro cell lysis. At present, we are unable to answer this question but would suggest that cell lysis on a single cell contact basis may not be an efficient mechanism for the removal of a whole graft, where tissue repair mechanisms may maintain the status quo and the amplification mechanisms available via lymphokines, as in the DTH phenomenon, may be a better explanation of the rapid graft rejection. It may be that Tc are primarily involved in the lysis of virus-infected target cells and may also be the true mediators of several forms of autoimmune disease rather than of graft rejection. However, we should conclude on a note of reservation. Our studies have examined the response to the whole *H-2* complex (plus non-*H-2* differences) and have exclusively examined skin graft rejection. Preliminary data suggests that our results will also apply to lymphoid tumor grafts; however, they may not be applicable to vascularized grafts or to individuals receiving immunosuppressive agents. Current examination of these variables should have an important bearing on the understanding of the mechanisms and treatment in man of graft rejection.

Summary

The Ly phenotype of cells mediating skin graft rejection was determined using monoclonal anti-Lyt-1.1 and Lyt-2.1 antibodies in CBA mice that received CBA lymphoid cells from mice sensitized to C57BL/6; i.e., alloantigenic differences arising from the *H-2* and non-*H-2* loci. It was clear that graft rejection was due wholly to the presence of Lyt-1 cells in the inoculum and that Lyt-123 or Lyt-23 cells had no effect. Furthermore, no synergism was noted between Lyt-1 and Lyt-2 cells. In this model, both the cytotoxic T cell and cytotoxic lymphocyte precursors were shown to be Lyt-123 and these could be depleted from sensitized Lyt-1 populations that mediated graft rejection. Thus cytotoxic T cells are not responsible for skin graft rejection, but rather, this is mediated by an Lyt-1 cell. Whether this T cell is distinct from other Lyt-1 cells (T helper, T cells mediating delayed hypersensitivity) is not clear at present, but other evidence, and traditional concepts, link graft rejection and delayed type hypersensitivity as being different manifestations of the same mechanism.

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