

EFFECTOR CELLS IN ALLELIC H-2 CLASS I-INCOMPATIBLE SKIN GRAFT REJECTION

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The effector cell mechanisms involved in allograft or tumor rejection are still controversial (1). Sprent et al. (2) recently demonstrated that injection of Lyt-2⁺ cells (positively purified by the panning method) into adult, thymectomized, bone marrow-reconstituted (ATXBM)¹ B6 mice resulted in rejection of skin from the H-2K^b mutant strain of B6.C-H-2^{bm1} (bm1), but not the H-2IA^b mutant strain of B6.C-H-2^{bm12} (bm12), whereas injection of L3T4⁺ cells into these mice resulted in rejection of skin from bm12, but not bm1 mice. Similarly, Rosenberg et al. (3) reported that bm1 skin grafts were rejected by B10 nude mice reconstituted with B6 spleen cells depleted of L3T4⁺ cells, but not of Lyt-2⁺ cells by pretreatment with mAb and complement. These studies suggested that the rejections of skin grafts differing at H-2 class I and class II loci are mediated by Lyt-2⁺ and L3T4⁺ T cells, respectively. Although there have been several studies on B6 H-2^b mutant strains by investigating the immune responsiveness of B6 mice against them, there have been few on the cellular mechanisms of the immune responses against allelic MHC class I or II antigens.

Previously, we and others demonstrated that injections of anti-Lyt-2 and anti-L3T4 mAb into mice caused selective depletions of Lyt-2⁺ and L3T4⁺ cells, respectively (4-7). In this study we investigated the effect of *in vivo* administration of anti-Lyt-2.2 mAb, anti-L3T4 mAb, or both to recipient mice on skin graft survival and found that, besides Lyt-2⁺ effector cells, L3T4⁺ cells can also mediate skin graft rejection with allelic H-2 class I differences in two different ways, depending on the strain combination.

Materials and Methods

Mice. The strains of mice used and their respective genotypes are listed in Table I. C57BL/6 (B6), B10.BR, and B10.A mice were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan). B6.C-H-2^{bm1} (bm1) and B6.C-H-2^{bm12} (bm12) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B10.A(2R), B10.AKM, and B10.AQR mice were provided by Dr. K. Moriwaki, National Institute of Genetics (Mishima, Japan). These mice were bred in our colony.

¹ *Abbreviation used in this paper:* ATXBM, adult, thymectomized, bone marrow-reconstituted mice.

TABLE I
H-2 Haplotypes of Mouse Strains Used in This Study

Strain	H-2 haplo- type	H-2 regions				
		K	A	E	S	D
C57BL/6 (B6)	b	b	b	b	b	b
B6.C-H-2 ^{bm1} (bm1)	bm1	bm1	b	b	b	b
B6.C-H-2 ^{bm12} (bm12)	bm12	b	bm12	b	b	b
B10.BR	k	k	k	k	k	k
B10.A	a	k	k	k	d	d
B10.A(2R)	h2	k	k	k	d	b
B10.AKM	m	k	k	k	k	q
B10.AQR	y1	q	k	k	d	d

Monoclonal Antibodies. Anti-L3T4 mAb, a rat antibody of the IgG2b immunoglobulin class, produced by hybridoma GK1.5 (8, 9), was kindly provided by Dr. F. Fitch, University of Chicago (Chicago, IL). Anti-Lyt-2.2 mAb and other mAb used have been described previously (4). The titers of both anti-L3T4 and anti-Lyt-2 mAb determined by antibody-mediated complement-dependent cytotoxicity assay were 1:20,000. These antibodies were used in the form of ascites from hybridoma bearing mice. The concentrations of anti-L3T4 and anti-Lyt-2.2 mAb in pooled ascites were 2.8 and 7.1 mg/ml, respectively, as quantified by protein assay (Bio-Rad Laboratories, Richmond, CA), and by quantitative cellulose acetate electrophoresis.

Antibody Administration. Mice were anesthetized with ether and 0.2 ml of antibodies (ascites), diluted 1:8 with MEM, was injected through the retrobulbar venous plexus.

Skin Grafting. Full-thickness skin (0.8–1.2-cm-diam) from the back of a donor mouse was grafted onto the side of the thorax of a recipient mouse by suturing it in place with nylon string (Nichou Co., Tokyo, Japan). The graft was covered with gauze and a plaster cast for 6 d and then examined daily to score the percentage of complete rejection. Statistical analyses were done by Student's *t* test.

Antibody-mediated Complement-dependent Cytotoxicity. The procedure used has been described (10). Preselected rabbit serum was used as complement.

Protein A Assay. The method has been described (11).

Results

Estimation of Lyt-2⁺ and L3T4⁺ T Cells in Lymph Nodes of Mice Treated with Anti-Lyt-2.2 or Anti-L3T4 mAb. Clearance of anti-Lyt-2.2 and anti-L3T4 mAb in mice was studied by the cytotoxic test with B6 thymocytes as target cells, using serum specimens obtained every other day after in vivo administration of mAb. The antibody titer gradually decreased, and disappeared by day 13–15 after treatment with anti-Lyt-2.2 mAb, and by day 7 after treatment with anti-L3T4 mAb. Because of its rather rapid clearance, anti-L3T4 mAb was injected on day 14 as well as on days 0 and 4, on which days anti-Lyt-2.2 mAb was injected. Changes in the number of Lyt-2⁺ and L3T4⁺ T cells in the lymph nodes with time were estimated. As shown in Fig. 1, suppression of Lyt-2⁺ cells were observed until about day 50 after injection of anti-Lyt-2.2 mAb, whereas suppression of L3T4⁺ cells was observed until about day 30 after injection of anti-L3T4 mAb. Results in strains B6, B10.A, B10.AKM, B10.BR, and B10.A(2R) were not significantly different.

Effect of In Vivo Administration of Anti-Lyt-2.2 mAb on Skin Graft Rejection with Allelic H-2 Class I Difference. The effect of in vivo administration of anti-Lyt-

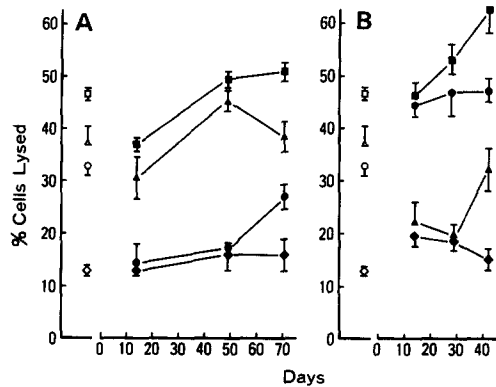


FIGURE 1. The phenotype of lymph node cells from B6 mice that were injected (*closed symbols*) or not injected (*open symbols*) with anti-Lyt-2.2 mAb (A) on days 0 and 4 or anti-L3T4 mAb (B) on days 0, 4, and 14 was determined kinetically with Thy-1.2 (□), L3T4 (Δ), or Lyt-2.2 (○) by antibody-mediated complement-dependent cytotoxicity assay. ◇; Background lysis. Tests were done in quadruplicate.

2.2 mAb on skin graft rejection was investigated with combinations of allelic H-2 class I difference using B10 H-2-congenetic strains. Donor skin was grafted onto the side of the thorax of recipient mice, and anti-Lyt-2.2 mAb was administered intravenously on days 0 and 4 after grafting. As shown in Fig. 2, the administration of anti-Lyt-2.2 mAb significantly prolonged graft survival in all three combinations when B10.A or B10.AKM was used as recipient, and in the combination B10.A(2R) → B10.BR. However, it did not prolong graft survival in two other combinations with B10.BR as recipient, or in all three combinations with B10.A(2R) as recipient, or in the reciprocal combinations of B10.AQR and B10.A. Injection of MEM as a control did not significantly affect graft survival with any combination tested. To determine whether the absence of effect of anti-Lyt-2.2 mAb in prolonging graft survival was due to injection of insufficient antibody and whether the effect of anti-Lyt-2.2 mAb could be enhanced by increasing the amount of antibody, we tested the effect on graft survival of three to five injections of anti-Lyt-2.2 mAb instead of two injections. However, these additional injections did not result in more or longer graft survival (Table II).

Effect of In Vivo Administration of Anti-L3T4 mAb Alone or With Anti-Lyt-2.2 mAb on Skin Graft Rejection with Allelic H-2 Class I Differences. To determine whether L3T4⁺ cells were involved in skin graft rejection with an allelic H-2 class I difference, we then tested the effect of administration of anti-L3T4 mAb alone or with anti-Lyt-2.2 mAb on graft rejection with several combinations selected randomly from among those in which anti-Lyt-2.2 mAb prolonged graft survival and in which it had no effect. Combinations of B6 and bm1 or bm12 mutant strains were also included. As shown in Fig. 3, the administration of anti-L3T4 mAb prolonged survival of skin grafts from bm12 but not bm1, and administration of anti-Lyt-2.2 mAb prolonged survival of skin grafts from bm1 but not bm12. These results are consistent with previous findings (2, 3) that bm1 skin was rejected by Lyt-2⁺ cells but not L3T4⁺ cells, and bm12 skin was rejected by L3T4⁺ cells but not Lyt-2⁺ cells that were adoptively transferred to ATXBM or athymic mice. With the allelic H-2 class I difference, the administration of anti-L3T4 mAb alone did not prolong graft survival of any combination tested. Whereas anti-L3T4 plus anti-Lyt-2.2 mAb markedly prolonged graft survival in combinations in which anti-Lyt-2.2 mAb did not prolong graft survival, and enhanced the effect of anti-Lyt-2.2 mAb in combinations in which anti-Lyt-2.2

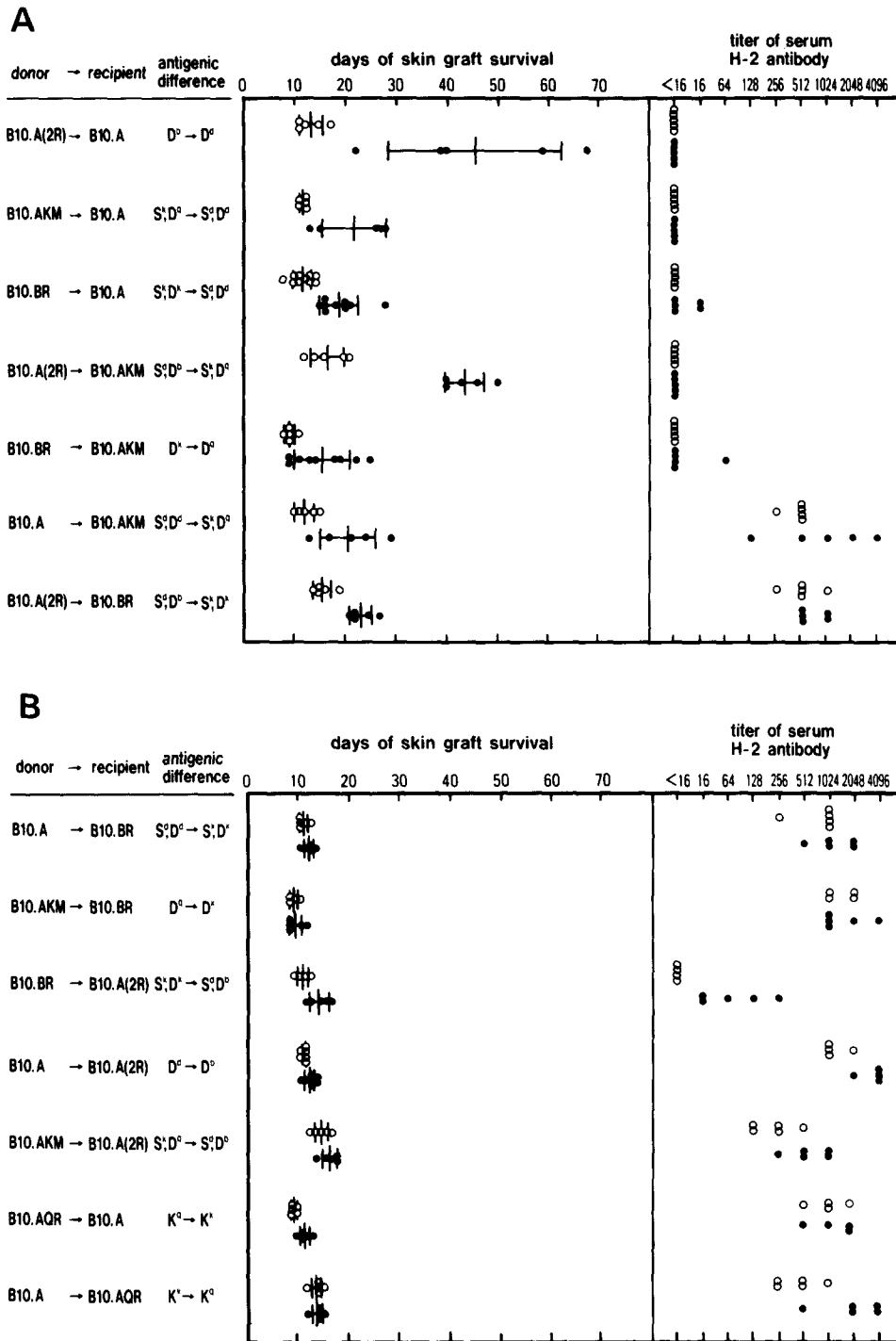


FIGURE 2. Effect of *in vivo* administration of anti-Lyt-2.2 mAb on skin graft survival and the production of H-2 antibody in recipient mice in combinations with H-2 class I differences. Each dot represents the value for an individual mouse. Anti-Lyt-2.2 mAb (closed symbols) or MEM (control) (open symbols) was injected into recipient mice on days 0 and 4 after grafting. A positive effect on graft survival was defined as >50% increase in the mean graft survival time over that in control mice. A, strain combinations in which anti-Lyt-2.2 mAb had a positive effect on graft survival; B, combinations in which anti-Lyt-2.2 mAb did not have a positive effect. The titer of H-2 antibody (at a dilution giving 50% rosette-forming cells) was determined by protein A assay on serum collected on day 21 after grafting. Spleen cells from donor mice that had been passed through nylon wool column were used as target cells. Cells from B6-Lyt-2.1,3.1 (H-2^b), CBA/N (H-2^k), or DBA/2 (H-2^d) were also used as targets, depending on the donor H-2 antigen to confirm that anti-Lyt-2.2 mAb has not been detected in mice treated with the antibody.

TABLE II
 Comparison of the Effect of Two and Three to Five Injections of Lyt-2.2 mAb
 in Prolonging Graft Survival

Donor	Recipient	Injection days	Graft survival (mean \pm SD)
B10.A	B10.A(2R)	None	10.7 \pm 0.5
		0, 4	11.9 \pm 1.1
		0, 4, 7	11.8 \pm 1.1
B10.A(2R)	B10.A	None	13.2 \pm 2.4
		0, 4	45.6 \pm 17.2
		0, 4, 14, 28, 42	42.4 \pm 11.5

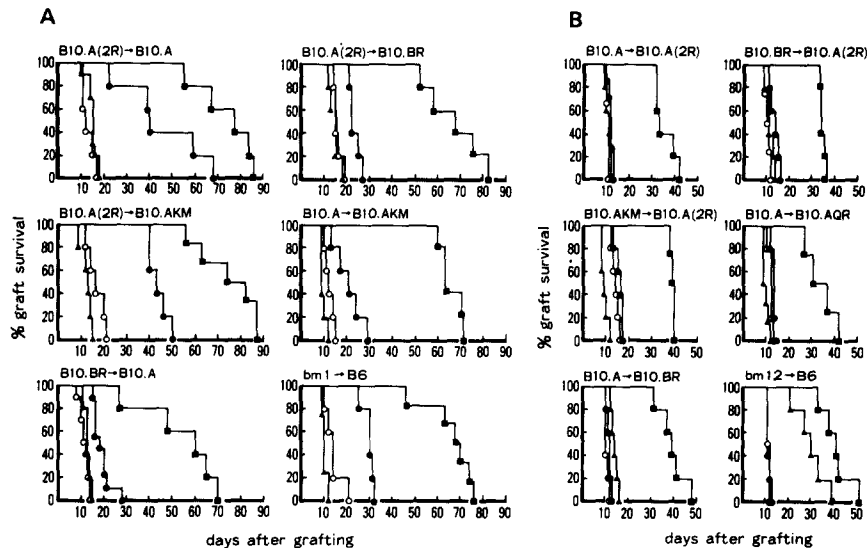


FIGURE 3. Effects of in vivo administration of anti-L3T4 mAb alone (\blacktriangle) or with anti-Lyt-2.2 mAb (\blacksquare) on skin graft survival in the combinations in which anti-Lyt-2.2 mAb (\bullet) alone prolonged (A), or did not prolong (B) survival. Anti-L3T4 mAb was injected into recipient mice on days 0, 4, 14, 21, and 28 in A, and on days 0, 4, and 14 in B. Anti-Lyt-2.2 mAb was injected on days 0, 4, 14, and 28 in A and on days 0 and 4 in B. \circ ; MEM was injected.

mAb did have an effect. The effect of anti-L3T4 plus anti-Lyt-2.2 mAb in prolonging survival was augmented by additional injections besides the routine injections of both mAbs (anti-Lyt-2.2 mAb on days 0 and 4; anti-L3T4 mAb on days 0, 4, and 14).

Serum Antibody Response to Skin Grafts. Production of serum antibody after skin grafts was studied by antibody-mediated complement-dependent cytotoxicity and protein A assays. As shown in Fig. 2, no antibody was detected during an observation period of 8–11 wk in the serum of B10.A mice with grafted skin from B10.A(2R), B10.AKM, or B10.BR mice, in the serum of B10.AKM mice with grafted skin from B10.A(2R) or B10.BR mice, or in the serum of B10.A(2R) mice with grafted skin from B10.BR mice that were treated with medium.

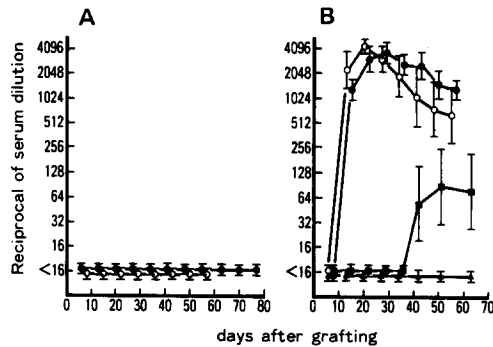


FIGURE 4. Geometric mean titer of H-2 antibodies in B10.A recipient mice (A) (five mice) with grafted skin from B10.A(2R), and in B10.A(2R) recipient mice (B) (four mice) with grafted skin from B10.A. Antibody production was determined by protein A assay. Recipient mice were treated with MEM (O), anti-Lyt-2.2 mAb (●), anti-L3T4 mAb (▲), or anti-Lyt-2.2 mAb plus anti-L3T4 mAb (■).

However, antibody was produced in recipient mice in other combinations tested, and its specificity was shown to be H-2 by testing several B10-congenetic mouse strains. The results of cytotoxicity and protein A assays were essentially the same. Typical kinetic profiles of antibody production, obtained with B10.A(2R) → B10.A and B10.A → B10.A(2R) combinations are shown in Fig. 4. B10.A(2R) recipient mice treated with both anti-Lyt-2.2 mAb and anti-L3T4 mAb started to produce antibody right after rejection (see Fig. 3B), indicating that L3T4⁺ cells with either helper or effector activity had recovered functionally as well as phenotypically (Fig. 1) by day 30–40.

Discussion

Injection of anti-Lyt-2.2 mAb into recipient mice prolonged skin graft survival in 7 of 12 combinations of H-2D-end difference, but not in 5 other combinations of D-end difference, or in 2 combinations of K-end difference. The finding that anti-Lyt-2.2 mAb prolonged survival of B10.A(2R) grafts on B10.A was consistent with previous reports by others (12). These findings might be explained by supposing that the positive and negative effects of anti-Lyt-2.2 mAb on graft survival are simply due to low and high antigenicity, respectively, of allelic H-2 class I antigens. This possibility, however, seems unlikely, because the mean graft survival times of control mice (treated with MEM) with these various combinations were not significantly different. On the other hand, the effect of anti-Lyt-2.2 mAb could be explained on the basis of differential stimulation of effector T cell populations in response to different H-2 class I antigens. Injection of anti-L3T4 mAb had no effect in prolonging graft survival of any combination of allelic class I differences tested, although it prolonged graft survival in a combination of bm12 → B6 used as a control. Injection of anti-L3T4 mAb together with anti-Lyt-2.2 mAb markedly prolonged graft survival in combinations in which anti-Lyt-2.2 mAb had no effect, and enhanced the effect of anti-Lyt-2.2 mAb in combinations in which anti-Lyt-2.2 mAb had an effect in prolonging graft survival. These results indicate that, in skin graft of combinations in which anti-Lyt-2.2 mAb had no effect, class I antigens stimulated L3T4⁺ effector cells when Lyt-2⁺ cells were blocked and Lyt-2⁺ effector cells when L3T4⁺ cells were blocked. This study did not show whether Lyt-2⁺ or L3T4⁺ cells were the primary effector cells in these combinations, but the selective stimulation of Lyt-2⁺ cells in in vitro mixed lymphocyte culture reactions in mouse strains with class

I difference (13) suggest that Lyt-2⁺ cells are the primary effectors. Prolongation of skin graft survival by the injection of both anti-L3T4 mAb and anti-Lyt-2.2 mAb, but not by injection of either alone was also observed with a combination of H-2 and non-H-2 antigen difference (7). On the other hand, in combinations in which anti-Lyt-2.2 mAb prolonged graft survival, these antigens preferentially stimulate Lyt-2⁺ but not L3T4⁺ effector cells initially, although delayed activation of L3T4⁺ effector cells occurred when Lyt-2⁺ cells were blocked. This was also the case in the combination bm1 → B6.

In this study, we observed a significant correlation between the effect of anti-Lyt-2.2 mAb in prolonging graft survival and the failure of recipient mice to produce H-2 antibody. The finding of no antibody response against several H-2D antigens was consistent with previous results (14). The fact that injection of anti-L3T4 mAb abrogated antibody production (15, 16, and this study) indicates that antibody production was a consequence of activation of L3T4⁺ helper cells. Therefore, the absence of H-2 antibody production in some combinations of D difference could have resulted from the absence of stimulation of the L3T4⁺ cell population. This is consistent with the preferential stimulation of Lyt-2⁺ effector cells in the initial phase of skin graft rejection in these combinations. However, exceptions to this rule were observed. In the two combinations B10.A → B10.AKM and B10.A(2R) → B10.BR, injection of anti-Lyt-2 mAb prolonged skin graft survival, but H-2 antibody was produced during rejection. On the other hand, in the combination B10.BR → B10.A(2R), anti-Lyt-2.2 mAb had no effect, but no H-2 antibody was produced. We have no explanation for these discrepancies at present. It is possible that there are two distinct subsets of effector and helper cells among L3T4⁺ cells. If so, B10.A and B10.A(2R) skin may stimulate predominantly L3T4 helper cells but not effector cells in B10.AKM and B10.BR recipients, respectively, while B10.BR skin may stimulate L3T4 effector cells but not helper cells in B10.A(2R) recipients. The fact that injection of anti-L3T4 mAb together with anti-Lyt-2.2 mAb markedly prolonged survival of grafts in this third combination suggests the presence of L3T4 effector cells.

Summary

The cellular mechanisms of skin graft rejection with allelic H-2 class I differences were studied by examining the effect on graft survival of *in vivo* administration of anti-Lyt-2.2 mAb, anti-L3T4 mAb, or both to recipient mice. The injections of anti-Lyt-2.2 mAb and anti-L3T4 mAb caused selective depletions of Lyt-2⁺ cells and L3T4⁺ cells, respectively. Injection of anti-Lyt-2.2 mAb significantly prolonged graft survival in 7 of 12 combinations of H-2D-end difference, but did not prolong graft survival in 5 other combinations of H-2D-end difference, or in 2 combinations of H-2K-end difference. Injection of anti-L3T4 mAb did not prolong graft survival in any combinations with class I difference tested. Injection of anti-L3T4 mAb plus anti-Lyt-2.2 mAb markedly prolonged graft survival in the combinations with class I difference in which anti-Lyt-2.2 mAb had no effect and overcame the effect of anti-Lyt-2.2 mAb in those in which anti-Lyt-2.2 mAb had an effect in prolonging graft survival. These results indicated that in combinations in which anti-Lyt-2.2 mAb did not prolong

graft survival, class I antigen stimulated L3T4⁺ effector cells when Lyt-2⁺ cells were blocked and Lyt-2⁺ effector cells when L3T4⁺ cells were blocked. On the other hand, in the combinations in which anti-Lyt-2.2 mAb prolong graft survival, these antigens initially caused preferential stimulation of Lyt-2⁺ but not L3T4⁺ effector cells, although delayed activation of L3T4⁺ effector cells occurred when Lyt-2⁺ cells were blocked. Furthermore, a significant correlation was found between the effect of anti-Lyt-2.2 mAb in prolonging graft survival and the failure of recipient mice to produce H-2 antibody. These results can be taken as evidence that L3T4⁺ effector cells are not involved in the initial phase of graft rejection in these combinations.

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