allogeneic class II MHC molecules induces neither tissue rejection nor clonal inactivation of alloreactive T cells. J Immunol 1990; 144: 334.
19. Johnson EW, Meunier SF, Roy CJ, Parenteau NL. The serial cultivation of normal human keratinocytes: a defined system for studying the regulation of growth and differentiation. In Vitro (in press).
20. Hall BM, Bishop A, Duggin GG, Horvath JS, Phillips J, Tiller DJ. Increased expression of HLA-Dr antigens on renal tubular cells in renal transplants: relevance to rejection response. Lancet 1984; Aug 4: 247.
21. Milton AD, Fabre JW. Massive induction of donor type class I and class II major histocompatibility antigens in rejection of cardiac allografts in the rat. J Exp Med 1983; 157: 1339.
22. Altman DM, Hogg N, Trowsdale J, Wilinkson D. Cotransfection of ICAM-1 and HLA-DR reconstitutes human antigen presenting cell function in mouse L cells. Nature 1989; 338: 512.
23. Umetsu DT, Katzen D, Jabara HH, Geha RS. Antigen presentation by human dermal fibroblasts: activation of resting T lymphocytes. J Immunol 1986; 136: 440.
24. Gaspari AA, Jenkins MK, Katz SI. Class II MHC bearing keratinocytes induce antigen specific unresponsiveness in hapten-specific TH_1 clones. J Immunol 1988; 141: 2216.
25. Demidem A, Faure M, Dezutter-Dambuyant C, Thivolet J. Loss of allogeneic T-cell activating ability and Langerhans cell markers in human epidermal cell cultures. Clin Immunol Immunopathol 1986; 38: 319.
26. Nickoloff BJ, Basham TY, Merigan TC, Torseth JW, Morhenn VB. Human keratinocyte-lymphocyte reactions in vitro. J Invest Dermatol 1986; 87: 11.
27. LaRosa RG, Talmage DW. MHC expression on parenchymal cells of thyroid allografts is not by itself sufficient to induce rejection. Transplantation 1990; 49: 605.
28. Halttunen J. Failure of rat kidney nephron components to induce allogeneic lymphocytes to proliferate in mixed lymphocyte kidney cell culture. Transplantation 1990; 50: 481.
29. Markmann J, Lo D, Naji A, Palmiter RD, Brinster RL, Herber-Katz E. Antigen presenting functions of class II MHC expressing pancreatic β cells. Nature 1988; 336: 476.
30. Sedgwick JD, Moszner R, Schwender S, ter Meulen V. Major histocompatibility complex-expressing non hematopoietic astroglial cells prime only $CD8^+$ T lymphocytes: astroglial cells as perpetuators but not initiators of $CD4^+$ T cell responses in the central nervous system. J Exp Med 1991; 173: 1235.
31. Risau W, Engelhardt B, Wekerle H. Immune function of the blood-brain barrier: incomplete presentation of protein (auto-)antigens by rat brain microvascular endothelium in vitro. J Cell Biol 1990; 110: 1757.
32. Schwartz RH. A cell culture model for T lymphocyte clonal anergy. Science 1990; 248: 1349.
33. Goldsmith MA, Weiss A. Early signal transduction by the antigen receptor without commitment to T cell activation. Science 1988; 240: 1029.

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EVIDENCE THAT ANTI-CD8 ABROGATES ANTI-CD4–MEDIATED CLONAL ANERGY BUT ALLOWS ALLOGRAFT SURVIVAL IN MICE[1]

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Monoclonal antibodies directed against different T cell subpopulations have been used in several rodent models of transplantation to induce long-term unresponsiveness to allografts by a variety of mechanisms. To investigate whether different mechanisms may be operative when different regimens of mAb therapy are used, we studied the effects of various combinations of anti-T-cell antibody treatment on the induction of tolerance in a mouse islet allograft model. Anti-CD4 mAb alone, anti-CD8 mAb alone, anti-CD4 mAb plus anti-CD8 mAb, and anti-Thy1.2 mAb alone were given at the time of engraftment. Only the anti-CD4 mAb and the anti-CD4 mAb plus anti-CD8 mAb regimens were successful in inducing permanent unresponsiveness to islet allografts. We have previously shown that anti-CD4 mAb alone induces permanent unresponsiveness to islet allografts by a mechanism of clonal anergy, as demonstrated by unresponsiveness of potentially alloreactive T cells to anti-T-cell receptor-specific cross-linking. Interestingly, the potentially alloreactive T cell subsets of recipient mice ($V\beta5^+$ and $V\beta11^+$) made unresponsive to islet allografts by anti-CD4 mAb plus anti-CD8 mAb therapy were not found to be anergic using the same assay. Differences between the repopulation kinetics of $CD8^+$ T cells of anti-CD4 mAb plus anti-CD8

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mAb treated recipient mice, which accepted islet allografts, and anti-Thy1.2 treated recipient mice, which rejected islet allografts despite similar levels of initial T cell depletion, suggest that unresponsiveness to alloantigen may have been induced in anti-CD4 mAb plus anti-CD8 mAb treated recipients by clearance of donor passenger leukocytes during prolonged CD8$^+$ T cell depletion.

Monolonal antibodies directed against various T cell subpopulations have been used to induce long-term unresponsiveness to allografts in several rodent models of transplantation. Depleting regimens of anti-CD4 mAb have been used to induce transplantation tolerance to pancreatic islets of Langerhans allografts in mice (*1*) and rats (*2*). Similar therapeutic regimens have been used to induce tolerance to heart allografts in rats (*3, 4*). Anti-CD8 mAb therapy used in conjunction with anti-CD4 mAb has been used to induce tolerance in mice to islets of Langerhans (*5*), bone marrow (*6*), and skin allografts (*6, 7*). Anti-CD3 mAb therapy has been used to induce permanent unresponsiveness to islets of Langerhans allografts in mice (*8*).

Examination of these models of transplantation has yielded several explanations as to how therapeutic regimens of antibody induce unresponsiveness to allogeneic tissue grafts in treated animals. These explanations to date have emphasized the idea of peripheral tolerance; clonal deletion of potentially reactive T cells has not been demonstrated in the tolerized animals of any of these models. We have demonstrated anergy in the potentially alloreactive T cells of mice tolerized to islets of Langerhans allografts by anti-CD4 mAb therapy. Anergy was demonstrated in these cells by an inability of potentially alloreactive cells to respond to anti-TcR specific crosslinking (*9*). Interestingly, in these studies anergy was seen in both CD4$^+$ and CD8$^+$ T cell subsets. The presence of a suppressor or regulator CD8$^+$ T cell population has also been proposed as a mechanism by which tolerance to heart allografts in rats is achieved by anti-CD4 mAb therapy (*3*). Finally, it has been proposed that anti-CD3 mAb treatment for two weeks following engraftment of allogeneic islets of Langerhans allowed for clearance of donor passenger leukocytes from the graft during the initial period of T cell depletion. At the time of T cell repopulation, the allograft lacked the donor MHC class II$^+$ cells that otherwise would have initiated rejection (*8*).

In order to investigate which of the above mechanisms may be operative during the course of different regimens of mAb therapy, we studied the effects of various combinations of anti-T-cell antibody treatment on the induction of tolerance in an islet allograft model. Donor and recipient mice were selected so that the MHC class II IE product (alloantigen) was expressed only by the donor tissue. Islets of Langerhans were isolated from adult A/J (IE^k) donors and transplanted into streptozotocin-induced diabetic B6 recipients through the portal circulation as previously described (*1*). The recipient B6 mice did not express IE, thus Vβ5$^+$ and Vβ11$^+$ T cells that could have responded to IE alloantigens in vivo were present in their periphery (*10–13*). Indefinite acceptance of allogeneic A/J islets after treatment with tolerizing regimens of antibody was demonstrated by persistent normoglycemia (>100 days). In contrast, diabetic recipients that received allografts without antibody treatment rejected their islet allografts within two weeks (*1*).

Several different mAb treatments were given to recipient mice at the time of A/J islet engraftment. As stated above, we have previously shown that depleting regimens of anti-CD4 mAb given alone at the time of engraftment induced tolerance to islet allografts in this model. This tolerance was mediated by a mechanism of clonal anergy among potentially IE-reactive Vβ11$^+$ T cells, as demonstrated by unresponsiveness to anti-Vβ11-specific crosslinking (*9*). In this current study, alternative treatments of anti-CD8 mAb alone, anti-CD4 mAb plus anti-CD8 mAb, and anti-Thy1.2 mAb alone were given at the time of engraftment. Among these alternative treatments, only the anti-CD4 mAb plus anti-CD8 mAb regimen successfully induced permanent unresponsiveness to A/J islet allografts. Interestingly, Vβ5$^+$ and Vβ11$^+$ T cells from B6 recipients that had accepted A/J islets under this regimen were responsive to anti-Vβ5- and anti-Vβ11-specific crosslinking. Thus, the mechanism by which anti-CD4 mAb plus anti-CD8 mAb induced unresponsiveness to alloantigen in this model does not appear to be one of clonal anergy. Instead, differences in the repopulation kinetics of CD8$^+$ T cells between anti-Thy1.2 mAb treated vs. anti-CD4 mAb plus anti-CD8 mAb treated B6 recipients suggest that unresponsiveness to alloantigen in the anti-CD4 mAb plus anti-CD8 mAb treated recipients may have been mediated by clearance of donor passenger leukocytes prior to CD8$^+$ T cell repopulation.

MATERIALS AND METHODS

Mice. Adult A/J and B6 mice were bred in our departmental animal facility or purchased from Jackson Labs (Bar Harbor, ME).

Preparation of monoclonal antibodies. The mAbs used in the treatment regimens for this study were GK1.5, a rat IgG_{2b} directed against mouse CD4 (*14*); 53.6.7, a rat IgG_{2a} directed against mouse CD8 (*15*); and 30-H12, a rat IgG_{2b} directed against mouse Thy1.2 (*15*). The mAb hybridoma cell lines were grown in 50 ml of RPMI tissue culture medium (RPMI 1640, 1 mM sodium pyruvate; Gibco, Grand Island, NY; 5×10^{-5} M2-mercaptoethanol; Calbiochem, San Diego, CA; 10% fetal calf serum; HyClone, Logan, UT) for 3 days. Cells were harvested by centrifugation and grown for another 7 days in RPMI tissue culture medium containing no fetal calf serum. Cell culture supernatant was then harvested and concentrated in an ultrafiltration apparatus (Amicon, Lexington, MA) and titrated for antibody content by FACS analysis. Concentrated supernatant was passed through a 0.22-μ filter (Millipore, Bedford, MA) before being stored at −20°C.

Transplants. Recipient B6 mice were made diabetic by the administration of a single intravenous bolus (200 mg/kg) of streptozotocin (Sigma, St. Louis, MO) (*16*). B6 mice were considered suitable recipients when their plasma glucose values exceeded 450 mg/dl. Plasma glucose values were obtained from retroorbital blood analyzed on a Beckman Glucose Analyzer II (Beckman, Fullerton, CA). No spontaneous remission from streptozotocin-induced diabetes is noted in untreated control animals (*1*).

The transplantation of pancreatic islets has been described previously (*1*). Briefly, each donor A/J pancreas was perfused in situ with 0.625 mg/ml collagenase P (Boehringer Mannheim, Indianapolis, IN) dissolved in HBSS medium (HBSS, 1% penicillin-streptomycin; Whittaker Bioproducts, Inc., Walkersville, MD; 4 mM sodium bicarbonate; J. T. Baker Chemical Co., Phillipsburg, NJ; 0.22 g/L bovine albumin; Sigma). Excised A/J donor tissue was then incubated for 20 min at 37°C and then washed with HBSS medium and passed through a strainer. Donor islets were separated from digested tissue by means of a discontinuous gradient of Ficoll (Sigma) at 27%, 25%, 23%, and 11%. After centrifugation at 1800 rpm for 10 min, islets were removed from the 23–11% interface and washed in HBSS. Free islets were then handpicked under a dissecting microscope and cultured overnight at 37°C with 5% CO_2 in CMRL-1066 (Gibco) supplemented with 10% fetal calf serum (HyClone) and 1% penicillin-streptomycin (Whittaker Bioproducts, Inc.).

The next day, the islets were washed and resuspended in HBSS

medium, and 800–850 islets were injected into the liver of each 8–12-week-old diabetic recipient B6 mouse by portal vein cannulation.

Transplants were done under the cover of anti-CD8 mAb therapy alone, anti-CD4 mAb plus anti-CD8 mAb therapy, and anti-Thy1.2 mAb therapy. On days −1, 0, and +1 relative to islet engraftment, recipient B6 mice were injected intraperitoneally with 50 μg of each mAb in their particular treatment regimen. The mice did not receive any subsequent immunosuppression.

The status of allografted A/J islets was assessed by serial measurement of the plasma glucose of B6 recipients at 3–4-day intervals. After successful transplantation, plasma glucose levels returned to normal (<200 mg/dl) within 7 days.

Cell staining. The relative T cell subset frequencies present in the PBLs of anti-CD4 mAb treated, anti-CD8 mAb treated, anti-CD4 mAb plus anti-CD8 mAb treated, anti-Thy1.2 mAb treated, and control mice were analyzed by FACS analysis. PBLs were isolated on the indicated days by separation on a lympholyte M (Cedarlane, Ontario, Canada) gradient. $CD5^+CD8^-$ and $CD5^+CD4^-$ T cells were analyzed by staining with FITC-conjugated anti-CD5 (mAb 53.7.3, Becton Dickinson Immunocytometry Systems, San Jose, CA) and either PE conjugated anti-CD4 (mAb GK1.5; Becton Dickinson) or biotinylated anti-CD8 (mAb 53.6.7; Becton Dickinson) followed by avidin-PE (Caltag Laboratories, South San Francisco, CA). $CD4^+V\beta11^+$ T cells were analyzed using mAb-to-Vβ11 (RR3-15, [12]), followed by fluoresceinated goat anti-rat (mouse-adsorbed) Ig (Caltag Laboratories). The cells were washed, incubated in 50% normal rat serum to bind remaining anti-rat Ig, and stained with PE-conjugated anti-CD4 (mAb GK1.5; Becton Dickinson). For $CD8^+V\beta11^+$ T cells, biotinylated anti-CD8 (mAb 53.6.7; Becton Dickinson) was used followed by avidin-PE (Caltag Laboratories). Cells were analyzed on a modified dual laser FACS with logarithmic amplifiers (Becton Dickinson, Mountain View, CA).

Anti-T-cell receptor activation assay. T cell proliferation was induced by receptor crosslinking with mAb specific for Vβ5 (MR9-4, [17]) and Vβ11 (RR3-15; [12]), or mAb specific for Vβ2 (B20.6; (Dr. B. Malissen)), as a control. Anti-Vβ11 and anti-Vβ2 antibodies (10 μg/ml) were coated onto U-bottom microtiter plates (Flow Laboratories Inc., McLean, VA) overnight at 4°C. Plates were washed twice, and 2.5×10^5 peripheral LN cells were added per well. For the anti-Vβ5 assay, goat anti-mouse IgG (Caltag Laboratories) at 20 μg/ml was coated onto U-bottom microtiter plates overnight at 4°C. These plates were washed twice and coated with 50 ml undiluted MR9-4 cell culture supernatant for 2 hr at room temperature. The plates were washed twice again, and 2.5×10^5 LN cells were added per well. All plates were incubated for 5 days with [^{3}H] thymidine added for the final 12–16 hr of culture.

RESULTS

Depletion and repopulation of $CD4^+$ and $CD8^+$ cells. Initial depletion of $CD4^+$ and $CD8^+$ PBLs in all recipient B6 mice was measured by FACS analysis. These data are presented in Figure 1. We have previously shown that anti-CD4 mAb treated recipients have only 7% of $CD4^+$ cells remaining in their periphery on the sixth day after islet engraftment, as determined by staining for cells that were $CD5^+CD8^-$ (9). Here, anti-CD8 mAb-treated recipients were shown to have only 9% of $CD8^+$ cells remaining in their periphery on day 10, as determined by staining for cells that were $CD5^+CD4^-$. Anti-CD4 mAb plus anti-CD8 mAb treatment depleted both $CD4^+$ and $CD8^+$ cells to 11% by day 15. Anti-Thy1.2 mAb treatment depleted $CD4^+$ and $CD8^+$ cells to levels of 5.6% and 8.2% by day 8.

Over the months following the initial period of T cell depletion, we measured the return of $CD8^+$ cells in the periphery of anti-CD8 mAb treated B6 recipients and the return of $CD4^+$ and $CD8^+$ cells in the periphery of anti-CD4 mAb plus anti-CD8 mAb treated and anti-Thy1.2 mAb treated recipients in order to compare the repopulation kinetics of the cell populations targeted by these three regimens. The $CD4^+$ cells of

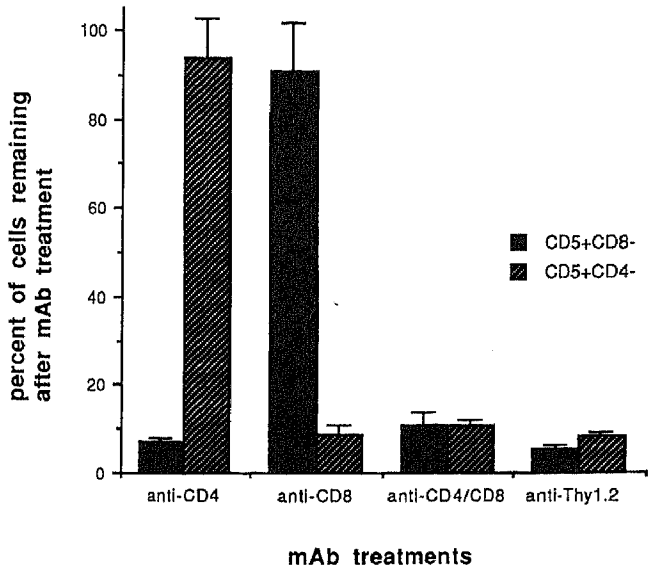

FIGURE 1. Initial depletion of $CD5^+CD8^-$ and $CD5^+CD4^-$ T cells in B6 recipients treated with anti-CD4 mAb alone, anti-CD8 mAb alone, anti-CD4 mAb plus anti-CD8 mAb, and anti-Thy1.2 mAb alone. Mice were injected with 50 μg of each mAb in their particular treatment regimen on days −1, 0, and +1 relative to transplant. Depletion was measured on day 6 relative to transplant for anti-CD4 mAb treated recipients, day 10 for anti-CD8 mAb treated recipients, day 15 for anti-CD4 mAb plus anti-CD8 mAb treated recipients, and day 8 for anti-Thy1.2 mAb treated recipients. PBLs were isolated on these days, and T cells were analyzed by staining with FITC-conjugated anti-CD5 and either PE-conjugated anti-CD4 or biotinylated anti-CD8 followed by avidin-PE. Data for each T cell subpopulation are expressed as mean percentages of untreated B6 control mice ±SD. Data for anti-CD8 mAb and anti-CD4 mAb plus anti-CD8 mAb treated B6 recipients represent the mean depletion of 4 recipients in each group. Data for anti-Thy1.2 mAb treated B6 recipients represent the mean depletion of 6 recipients. Depletion of anti-CD4 mAb treated recipients represents historical control data.

recipient B6 mice treated with either anti-CD4 mAb plus anti-CD8 mAb therapy or anti-Thy1.2 mAb therapy repopulated to control levels with similar kinetics (Fig. 2). In mice treated with either antibody regimen, 50% of $CD4^+$ cells were repopulated within 50 days. $CD8^+$ cells of recipient B6 mice treated with anti-CD8 mAb therapy and anti-Thy1.2 mAb therapy repopulated over a similar time course. However, the repopulation of $CD8^+$ cells of recipient B6 mice treated with anti-CD4 mAb plus anti-CD8 mAb appeared to be delayed by comparison. In recipient B6 mice treated with anti-CD4 mAb plus anti-CD8 mAb, repopulation of 50% of $CD8^+$ cells did not occur until day 75 (Fig. 2).

Acceptance and rejection of islet allografts. The status of allografted A/J islets was assessed by serial measurement of the plasma glucose of B6 recipients at 3–4-day intervals. An allograft was considered rejected when the plasma glucose value of the recipient exceeded 200 mg/dl on two consecutive bleedings.

We had previously shown that recipient B6 mice depleted of $CD4^+$ cells at the time of A/J islet engraftment remained unresponsive to their allograft indefinitely, as evidenced by persistent normoglycemia (1). Untreated control diabetic B6 mice that received A/J islets rejected their allografts within two weeks (1). Among the alternative mAb therapeutic regi-

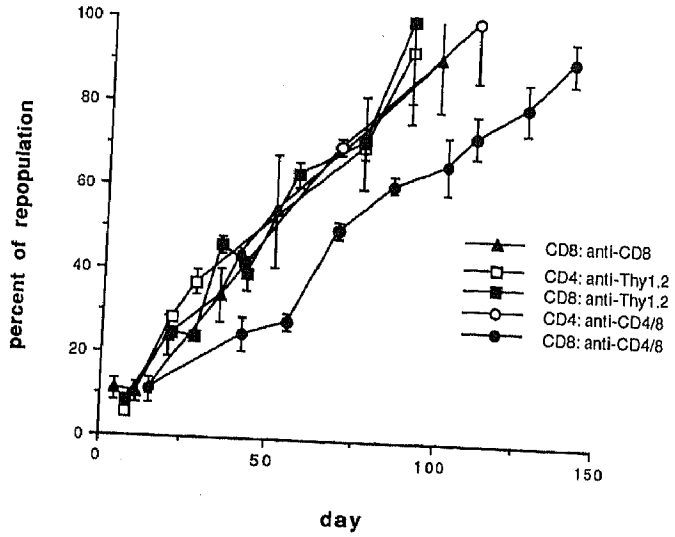

FIGURE 2. Repopulation of $CD4^+$ and $CD8^+$ cells in B6 recipients treated with anti-Thy1.2 mAb alone, anti-CD8 mAb alone, and anti-CD4 mAb plus anti-CD8 mAb. Staining was done as in Figure 1, and data for each T cell subpopulation are again expressed as the percentage of untreated B6 control mice. Repopulation data for anti-Thy1.2 mAb treated recipients at each time point represent the mean ±SD repopulation of 6 recipients. Repopulation data for anti-CD8 mAb treated and anti-CD4 mAb plus anti-CD8 mAb treated recipients at each time point represent the mean ±SD repopulation of 4 recipients.

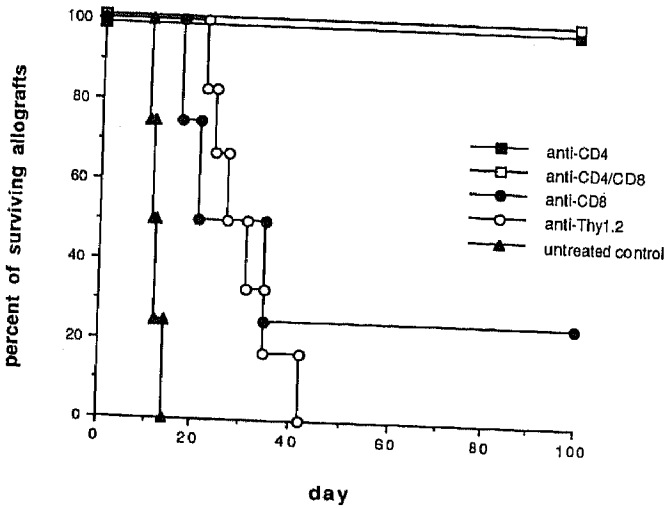

FIGURE 3. Effects of various regimens of mAb therapy on the survival of allografted A/J islets in diabetic B6 recipients. B6 recipients were treated at the time of engraftment with 3-day regimens of anti-CD4 mAb alone, anti-CD8 mAb alone (4 recipients), anti-CD4 mAb plus anti-CD8 mAb (4 recipients), or anti-Thy1.2 mAb (6 recipients). The daily dose of each mAb was 50 μg administered intraperitoneally. Allograft survival of untreated and anti-CD4 mAb treated recipients represents historical data (*1*, *9*).

mens tested in this study, only the anti-CD4 mAb plus anti-CD8 mAb treatment was successful in inducing indefinite (>100 days) unresponsiveness to A/J islet allografts in all B6 recipients (Fig. 3). Anti-Thy1.2 mAb treatment was only successful in delaying the onset of rejection (mean time of rejection=30 days). Anti-CD8 mAb treatment alone yielded similar results, with three of four B6 recipients rejecting their A/J islet grafts within 35 days (Fig. 3).

Characterization of the unresponsive state induced by anti-CD4 mAb plus anti-CD8 mAb treatment. We next investigated some of the qualitative aspects of the unresponsive state induced in recipient B6 mice to A/J islets by anti-CD4 mAb plus anti-CD8 mAb treatment. In order to determine whether transplantation tolerance induced under this treatment regimen resulted from clonal deletion of potentially IE-reactive T cells, we assayed PBLs from long-term tolerant B6 recipients for the expression of a TCR Vβ that would have undergone deletion in IE^+ mice by using FACS analysis to assay the number of peripheral T cells expressing TcR Vβ11. These recipients were analyzed 6 months after being transplanted. Data presented in Table 1 indicate that the presence of an islet allograft from an IE^+ donor did not influence the eventual repopulation of $V\beta11^+$ cells in long-term tolerant B6 recipients. $CD4^+V\beta11^+$ and $CD8^+V\beta11^+$ T cells from these mice were repopulated to the same extent as in anti-CD4 mAb plus anti-CD8 mAb treated control mice. The levels following repopulation were comparable with those found in an untreated B6 control mouse.

Finally, we asked whether T cells of long-term tolerant B6 recipients expressing the relevant Vβ gene segments could be activated in response to anti-TcR-specific crosslinking. We have previously shown that in recipient B6 mice tolerized to A/J islets by anti-CD4 mAb treatment, LN cells were unresponsive to stimulation by immobilized mAb to Vβ11 (*9*). This result suggested that transplantation tolerance induced by anti-CD4 mAb treatment was mediated by a mechanism of clonal anergy among potentially IE-reactive T cells. Our results from similar experiments on recipient B6 mice made tolerant to A/J islets by anti-CD4 mAb plus anti-CD8 mAb treatment indicate that clonal anergy was not the underlying mechanism in this case (Table 2). Long-term tolerant B6 recipients showed equal proliferation, compared with the untreated B6 control mouse, to anti-Vβ11 and anti-Vβ5 specific crosslinking. The mAb specific for Vβ2, a TcR expressed at similar levels to Vβ11 in B6 mice, was used as a positive control in this experiment. In a second experiment, we used as a control a recipient B6 mouse made tolerant to A/J islets by anti-CD4 mAb treatment alone (Fig. 4). This recipient had the expected previously demonstrated diminished response to anti-Vβ11 specific crosslinking (*9*), whereas two additional anti-CD4 mAb plus anti-CD8

TABLE 1. Repopulation of $CD4^+V\beta11^+$ and $CD8^+V\beta11^+$ cells in two B6 recipients treated with anti-CD4 mAb plus anti-CD8 mAb therapy[a]

	Mouse			
	Normal B6 control	Anti-CD4/CD8 treated B6 control	Anti-CD4/CD8 treated B6 recipient A	Anti-CD4/CD8 treated B6 recipient B
$\%CD4^+V\beta11^+$	3.2	3.2	3.2	3.1
$\%CD8^+V\beta11^+$	3.2	2.7	3.0	3.2

[a] B6 mice transplanted with A/J islets coincident with anti-CD4 mAb plus anti-CD8 mAb administration were analyzed 6 months after being transplanted. Age-matched untreated and anti-CD4 mAb plus anti-CD8 mAb-treated untransplanted B6 mice served as positive controls. PBLs were isolated, and $CD4^+V\beta11^+$ cells were analyzed using mAb to Vβ11, followed by fluoresceinated goat anti-rat Ig and PE-conjugated anti-CD4. $CD8^+V\beta11^+$ cells were analyzed similarly, except that for CD8 staining, biotinylated anti-CD8 mAb was used, followed by avidin-PE. Percentage of cells staining with the indicated antibodies is expressed relative to total $CD4^+$ plus $CD8^+$ cells.

TABLE 2. Normal response of LN cells from two B6 recipients treated with anti-CD4 mAb plus anti-CD8 mAb therapy to anti-Vβ5 and anti-Vβ11 specific crosslinking[a]

	Proliferation (cpm)		
	Vβ2	Vβ5	Vβ11
Normal B6 control	46,950±136	44,617±2189	30,872±2397
Anti-CD4/CD8 treated B6 recipient A	55,609±3299	38,211±5870	28,691±2478
Anti-CD4/CD8 treated B6 recipient B	63,690±2254	56,561±4689	29,732±2987

[a] LN cells from B6 recipients were analyzed 6 months after islet engraftment. T cell proliferation was induced by receptor crosslinking with mAb specific for Vβ2 (positive control), Vβ5, and Vβ11. Peripheral LN cells (2.5×10^6) were added to each well, and the plates were incubated for 5 days with [^{3}H] thymidine added for the final 12–16 hr of culture. Data are expressed as the mean cpm±SD of triplicate wells. Background proliferation of LN cells incubated in assay medium alone without TcR crosslinking was <15%.

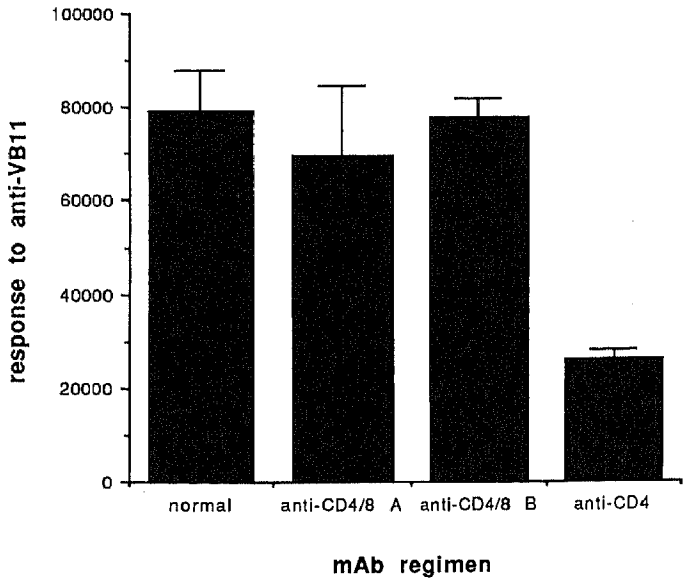

FIGURE 4. Normal response to anti-Vβ11 specific crosslinking of LN cells from 2 B6 recipients tolerized to A/J islets by anti-CD4 mAb plus anti-CD8 mAb vs. a diminished response of LN cells from a B6 recipient tolerized by anti-CD4 mAb alone. LN cells from B6 recipients were analyzed 6 months after islet engraftment. T cell proliferation was induced by receptor crosslinking with mAb specific for Vβ11. Peripheral LN cells (2.5×10^5) were added to each well, and the plates were incubated for 5 days with [^{3}H] thymidine added for the final 12–16 hr of culture. Data are expressed as the mean cpm±SD of triplicate wells. Background proliferation of recipient B6 LN cells incubated in assay medium alone without TcR crosslinking was <15%.

mAb treated B6 recipients continued to have a response similar to that of the untreated control (Fig. 4).

DISCUSSION

We have begun to investigate the mechanisms by which tolerance is induced to allografts by different regimens of mAb therapy. We have previously shown that anti-CD4 mAb therapy induces tolerance in B6 (IE^-) mice to A/J (IE^k) islet allografts by a mechanism of clonal anergy, as demonstrated by unresponsiveness to T cell receptor-specific crosslinking (*9*). In this current study, we have demonstrated that both anti-CD4 mAb plus anti-CD8 mAb therapy and anti-Thy1.2 mAb therapy were equally effective in depleting $CD4^+$ and $CD8^+$ T cells from the periphery of B6 recipients (Fig. 1); however, only the anti-CD4 mAb plus anti-CD8 mAb treatment was successful in reproducibly inducing permanent unresponsiveness to A/J islet allografts (Fig. 3). Interestingly, the repopulation kinetics of T cells in the periphery of B6 recipients treated with anti-CD4 mAb plus anti-CD8 mAb therapy differed from that of B6 recipients treated with anti-Thy1.2 mAb therapy. The repopulation of $CD8^+$ cells was delayed in recipients treated with anti-CD4 mAb plus anti-CD8 mAb when compared with the repopulation of $CD8^+$ cells in recipients treated with anti-CD8 mAb alone or anti-Thy1.2 mAb (Fig. 2).

Next, we studied two potential mechanisms by which anti-CD4 mAb plus anti-CD8 mAb therapy could have induced permanent unresponsiveness to islet allografts. Allograft tolerance was not due to clonal deletion as analyzed by FACS analysis of PBLs from long-term tolerant B6 recipients (Table 1). Also, since potentially IE-reactive $V\beta5^+$ and $V\beta11^+$ T cells from long-term tolerant B6 recipients treated with anti-CD4 mAb plus anti-CD8 mAb were shown to respond to T cell receptor-specific crosslinking (Table 2, Fig. 4), the mechanism of tolerance induction in anti-CD4 mAb plus anti-CD8 mAb treated B6 recipients was not the induction of clonal anergy. Thus the mechanism of long-term unresponsiveness to IE^+ allografts in the anti-CD4 mAb plus anti-CD8 mAb treated recipients seems to differ fundamentally from the mechanism of clonal anergy proposed to be operative in anti-CD4 mAb treated animals.

The lack of clonal anergy or clonal deletion in B6 recipients treated with anti-CD4 mAb plus anti-CD8 mAb prompted us to consider other mechanisms by which unresponsiveness to the A/J islet allografts may have been achieved. It is possible that anti-CD4 mAb plus anti-CD8 mAb treatment at the time of islet engraftment allowed clearance or inactivation of donor passenger leukocytes from the grafts during the initial period of T cell depletion. Upon T cell repopulation, the allografts therefore lacked MHC class II^+ cells that otherwise would have initiated rejection. Our data showing that anti-Thy1.2 mAb treated B6 recipients uniformly reject A/J islet allografts support this hypothesis (Fig. 3). These B6 recipients were shown to have been at least as effectively depleted of $CD4^+$ and $CD8^+$ cells at the time of engraftment as were B6 recipients treated with anti-CD4 mAb plus anti-CD8 mAb therapy (Fig. 1). The relatively rapid repopulation of $CD8^+$ cells in anti-Thy1.2 mAb treated B6 recipients compared with those in anti-CD4 mAb plus anti-CD8 mAb treated mice suggested the possibility that the $CD8^+$ cells of anti-Thy1.2 treated B6 recipients recovered rapidly before donor passenger leukocytes were "cleared" (Fig. 2). Thus mere prolongation of islet allograft survival resulted from anti-Thy1.2 mAb treatment compared with universal tolerance to the same donor islet combination using anti-CD4 mAb plus anti-CD8 mAb treatment.

It should be emphasized that the kinetics of repopulation of $CD4^+$ cells of anti-CD4 mAb plus anti-CD8 mAb treated B6 recipients did not differ significantly from the kinetics of repopulation of $CD4^+$ cells of anti-Thy1.2 mAb treated mice. We must therefore conclude that the repopulated $CD4^+$ cells of anti-CD4 mAb plus anti-CD8 mAb treated B6 recipients that were present before the repopulation of $CD8^+$ cells and also before clearance of donor passenger leukocytes were not sufficient to reject the islet allografts in the absence of $CD8^+$ cells.

This reasoning is supported by a previous study that reported that neither $CD8^+$ nor $CD4^+$ cells alone were capable of mediating rejection of islet allografts, and that interaction between the two T cell subpopulations was necessary for rejection to occur (*5*). Supporting the concept that $CD8^+$ cells are involved in allograft rejection is our observation that anti-CD8 mAb treatment alone succeeded in prolonging A/J islet allograft survival in all anti-CD8 mAb treated B6 recipients beyond the two-week survival time seen in untreated allografted controls (Fig. 3), and in one of the mice, succeeded in inducing permanent unresponsiveness (Fig. 3).

The idea that the presence of donor passenger leukocytes is required for rejection to occur is supported by work in other models of islet transplantation. For instance, it has been shown that mouse or rat islets cultured in 95% oxygen for one week are accepted indefinitely by untreated allogeneic recipients (*18*). Loss of tissue immunogenicity by this technique is thought to be the result of the sensitivity of passenger leukocytes to oxygen toxicity (*18*). Our model of anti-CD4 mAb plus anti-CD8 mAb induced unresponsiveness may differ from that model only in that during the initial period of T cell depletion our therapeutic mAb regimen allowed the depletion of donor passenger leukocytes to occur in vivo rather than in vitro.

Diabetic mice treated with anti-CD3 mAb therapy have also been shown to accept islet allografts indefinitely (*8*). Although data describing the depletion and repopulation of $CD4^+$ and $CD8^+$ cells are not available from that experiment, we suspect that the regimen of anti-CD3 mAb employed there also depleted recipient $CD8^+$ cell populations for an extended period of time. The fact that all recipients made unresponsive to islet allografts in that experiment were treated with anti-CD3 mAb for the first 15 days after engraftment makes this a reasonable prediction.

The lack of clonal anergy found in B6 recipients made unresponsive to A/J islets by anti-CD4 mAb plus anti-CD8 mAb treatment also has implications regarding the mechanism of induction of clonal anergy in B6 recipient mice treated with anti-CD4 mAb alone. Our previous results in rats suggested that the presence of a $CD8^+$ regulator cell was necessary during the initial period of $CD4^+$ cell depletion for allograft tolerance to be achieved (*2*). We believe that there may be a similar role for a $CD8^+$ regulator cell in the model of mouse islet transplantation. Although data describing the depletion and repopulation of $CD4^+$ and $CD8^+$ cells are not available from the rat study, we propose that allogeneic islets engrafted under the cover of anti-CD4 mAb plus anti-CD8 mAb in the rat model failed to be accepted on two counts. First, the anti-CD8 mAb therapy depleted recipient rats, at the time of engraftment, of a $CD8^+$ regulator cell whose presence was required for induction of clonal anergy. Second, subsequent repopulation of $CD8^+$ cells occurred too quickly for donor passenger leukocytes in the grafts to become depleted. Islet allografts were rejected after a slight prolongation of survival time in that study.

In this current study, we have demonstrated that there are qualitative differences in the unresponsive states induced to allogeneic tissue by different regimens of anti-T-cell mAb therapy. Our data suggest that these qualitative differences are the result of distinct underlying mechanisms by which these therapies induce unresponsiveness. We have previously shown that a mechanism of clonal anergy mediates anti-CD4 mAb induced unresponsiveness. Here we propose that anti-CD4 mAb plus anti-CD8 mAb induced unresponsiveness is not mediated by the induction of clonal anergy but rather by clearance of donor passenger leukocytes during T cell depletion. Further study is required to determine the specific requirements of T cell depletion and kinetics of clearance of donor passenger leukocytes that are necessary for the induction of unresponsiveness to alloantigen to occur by either mechanism. This information will allow us to determine which mechanisms are more or less clinically feasible as we move closer to the ultimate goal of using analogous therapeutic regimens in human transplant recipients.

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REFERENCES

1. Shizuru JA, Gregory AK, Chao CTB, Fathman CG. Islet survival after a single course of treatment of recipient with antibody to L3T4. Science 1987; 237: 278.
2. Seydel K, Shizuru JA, Grossman D, Wu A, Alters S, Fathman CG. Anti-CD8 abrogates effect of anti-CD4–mediated islet allograft survival in rat model. Diabetes 1991; 40: 1430.
3. Herbert J, Roser B. Strategies of monoclonal antibody therapy that induce permanent tolerance of organ transplants. Transplantation 1988; 46: 128S.
4. Shizuru JA, Seydel KB, Flavin TF, et al. Induction of donor-specific unresponsiveness to cardiac allografts in rats by pretransplant anti-CD4 monoclonal antibody therapy. Transplantation 1990; 50: 366.
5. Yamamoto H, Monden M, Kawai M, et al. The role of $CD8^+$ and $CD4^+$ cells in islet allograft rejection. Transplantation 1990; 50: 120.
6. Cobbold SP, Martin G, Qin S, Waldmann H. Monoclonal antibodies to promote marrow engraftment and tissue graft tolerance. Nature 1986; 323: 164.
7. Cobbold SP, Martin G, Waldmann H. The induction of skin graft tolerance in major histocompatibility complex–mismatched or primed recipients: primed T cells can be tolerized in the periphery with anti-CD4 and anti-CD8 antibodies. Eur J Immunol 1990; 20: 2747.
8. Mackie JD, Pankewycz OG, Bastos MG, Kelley VE, Strom TB. Dose-related mechanisms of immunosuppression mediated by murine anti-CD3 monoclonal antibody in pancreatic islet cell transplantation and delayed-type hypersensitivity. Transplantation 1990; 49: 1150.
9. Alters SE, Shizuru HA, Ackerman J, Grossman D, Seydel KB, Fathman CG. Anti-CD4 mediates clonal anergy during transplantation tolerance induction. J Exp Med 1991; 173: 491.
10. Bill J, Appel VB, Palmer E. An analysis of T-cell receptor variable region gene expression in major histocompatibility complex disparate mice. Proc Natl Acad Sci USA 1988; 85: 9184.
11. Gao E, Kanagawa O, Sprent J. Capacity of unprimed $CD4^+$ and $CD8^+$ T cells expressing $V\beta11$ receptors to respond to IE alloantigens in vivo. J Exp Med 1989; 170: 1947.
12. Bill J, Kanagawa O, Woodland D, Palmer E. The MHC molecule I-E is necessary but not sufficient for the clonal deletion of $V\beta11$-bearing cells. J Exp Med 1989; 169: 1405.
13. Woodland D, Happ M, Bill J, Palmer E. Requirement for cotolerogenic gene products in the clonal deletion of IE reactive T cells. Science 1990; 247: 964.
14. Dialynas DP, Quan ZS, Wall KA, et al. Characterization of the murine T cell surface molecule designated L3T4, identified by monoclonal antibody GK1.5: similarity of L3T4 to the human Leu3/T4 molecule. J Immunol 1983; 131: 2445.
15. Ledbetter JA, Herzenberg LA. Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. Immunol Rev 1979; 47: 63.

16. Brosky G, Logothetopoulos J. Streptozotocin diabetes in the mouse and guinea pig. Diabetes 1969; 18: 606.
17. Kanagawa O, Utsunomiya Y, Bill J, Palmer E, Moore MW, Carbone FR. Conformational difference of T cell antigen receptors revealed by monoclonal antibodies to mouse Vβ5 T cell receptor for antigen determinants. J Immunol 1991; 147: 1307.
18. Lafferty KJ, Prowse SJ, Simeonovic CJ. Immunobiology of tissue transplantation: a return to the passenger leukocyte concept. Annu Rev Immunol 1983; 1: 143.

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THE EFFECT OF CYCLOSPORINE TREATMENT ON THE EXPRESSION OF GENES ENCODING GRANZYME A AND PERFORIN IN THE INFILTRATE OF MOUSE HEART TRANSPLANTS[1]

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Following activation of cytotoxic T cells and NK cells several genes encoding proteins putatively involved in cell-mediated cytotoxicity become expressed. The expression of genes encoding the cytotoxic T cell associated serine protease granzyme A and perforin was analyzed in cellular infiltrates of MHC mismatched (H-2^d→H-2^k) heterotopic heart transplants both in immunosuppressed recipients treated with cyclosporine and in untreated recipients. Heart transplants were completely rejected by untreated animals on day 10 posttransplantation, whereas CsA treatment generally prolonged survival of the transplants beyond 30 days.

In untreated recipients the number of granzyme A– and perforin-expressing cells in heart transplants increased from approximately 10 granzyme A–positive cells/mm^2 and 1 perforin-positive cell/mm^2 on day 2 posttransplantation to over 80 positive cells for both genes on day 5 posttransplantation. In contrast, these values remained always below 15 positive cells/mm^2 for both genes between day 5 and day 30 posttransplantation in CsA-treated recipients. Comparison of the frequency of CD8$^+$ T cells in the infiltrates showed that lower numbers of perforin and granzyme A–positive cells were mainly due to the immunosuppressive action of CsA rather than to reduced infiltration of transplants.

The present study shows that expression of granzyme A and perforin gene can be used to discriminate between quiescent and activated cytotoxic cells also in immunosuppressed animals and further confirms that these can be used as sensitive markers for monitoring the fate of a transplant.

Cytoplasmic granules of cytotoxic T lymphocytes and NK cells contain several proteins that may be involved in cell-mediated cytotoxicity. These molecules constitute the pore-forming protein perforin, also known as cytolysin (*1–3*), which shows structural and functional similarities to the C9 component of the complement system, as well as a family of highly homologous serine proteases, also termed "granzymes" (*4*). A direct role for perforin in cell-mediated cytotoxicity has been suggested by experiments in which antisense oligonucleotides of the perforin gene were used to downregulate perforin expression and to simultaneously reduce the cytotoxicity of stimulated cells (*5*). The exact role of granzymes in cell-mediated cytotoxicity is still unknown. However, a key role for serine proteases in the cytolytic process was postulated some years ago when protease inhibitors were found to inhibit CTL-mediated cytolysis in vitro (*6, 7*). More recently, Hayes et al. (*8*) suggested that granzyme A may be involved in the induction of DNA release from target cells. A good correlation between expression of granzymes and perforin and signs of cellular destruction in vivo has been reported both in human and experimental animal systems (*9*).

We previously demonstrated activation of the genes for granzyme A and perforin in cytotoxic cells during rejection of secondarily vascularized transplants in mice (*10*) and during the elimination of virus containing cells in mice infected with lymphotropic choriomeningitis virus (*11*). Comparison of granzyme A gene expression in rejected allogeneic transplants and tolerated syngeneic heart transplants, respectively, revealed a striking correlation of the presence of granzyme A mRNA–

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