0041-1337/93/5603-0633$03.00/0
TRANSPLANTATION

Vol. 56, 633–638, No. 3, September, 1993
Printed in U.S.A.

EVIDENCE THAT CLONAL ANERGY IS INDUCED IN THYMIC MIGRANT CELLS AFTER ANTI-CD4–MEDIATED TRANSPLANTATION TOLERANCE[1]

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Diabetic (B6) (IE^-) mice treated with a depleting regimen of anti-CD4 monoclonal antibody at the time of transplantation with A/J (IE^s) islets of Langerhans showed indefinite acceptance of their islet allograft, as evidenced by persistent normoglycemia. To address the mechanisms involved in such anti-CD4 induced transplantation tolerance we studied potentially IE-reactive $V_\beta11^+$ T cells from the tolerant allografted mice. Following complete repopulation of the $CD4^+$ cells, both the $CD4^+V_\beta11^+$ and $CD8^+V_\beta11^+$ T cell subsets of the transplanted mice were unresponsive to anti-$V_\beta11$ specific crosslinking. In contrast, lymphocytes tested within the first ten days following transplant were responsive to anti-$V_\beta11$ specific crosslinking; this response decreased as a function of time and reached background levels by day 120 posttransplant. Sorting experiments indicated that the response of lymphocytes to anti-$V_\beta11$ specific crosslinking seen during the initial 120 days posttransplant was confined to the peripheral $CD8^+$ cells; the repopulating $CD4^+V_\beta11^+$ T cells were unresponsive. In addition, administration of r-IL-2 at the time of transplantation induced rejection in anti-CD4-treated animals, again indicating that the peripheral $CD8^+$ cells could respond shortly after transplant if provided with appropriate help. The decreasing response of $CD8^+$ T cells from transplanted animals to anti-$V_\beta11$ stimulation was inversely correlated with the rate of migration of cells from the thymus to the periphery, implying that new thymic migrant $V_\beta11^+$ cells, both $CD4^+$ and $CD8^+$, were rendered anergic upon encountering peripheral alloantigen. These data suggest the possibility that recent thymic migrants are rendered anergic upon encountering antigen in the periphery, a simple model to serve as a "fail-safe" mechanism to prevent autoreactivity.

Anti-CD4 antibodies have been used successfully in mice to treat various murine models of human autoimmune diseases including experimental allergic encephalomyelitis (*1*), systemic lupus erythematosus (*2*), collagen-induced arthritis (*3*), experimental autoimmune myasthenia gravis (*4*), and insulin-dependent diabetes mellitus (*5, 6*). Furthermore, anti-CD4 mAb have been effective in preventing allograft rejection, either alone (*7*) or in combination with anti-CD8 mAb (*8*). Antibodies to CD4 induce a tolerance permissive environment in which unresponsiveness to xenogeneic proteins can be achieved (*9–11*).

We have been studying the mechanisms of anti-CD4-mediated tolerance induction—in particular, the fate of the alloantigen-specific T cells—in an islet allograft model in which donor and recipient mice were selected so that the MHC class II IE antigen was expressed only by the donor tissue. Pancreatic islets of Langerhans were isolated from adult A/J (IE^s) donors and transplanted into streptozotocin-induced diabetic B6 recipients through the portal circulation as previously described (*7*). The recipient B6 mice did not express IE—thus $V_\beta11^+$ T cells that could have responded to IE alloantigens in vivo (*12*) were present in their periphery. All diabetic mice that were treated with a depleting regimen of anti-CD4 mAb at the time they received allogeneic A/J islets showed indefinite acceptance of their graft, as evidenced by persistent normoglycemia (>200 days). In contrast, diabetic recipients that received allografts without anti-CD4 treatment rejected their islet allografts within two weeks (*7*). In addition, anti-CD4-treated, untransplanted mice did not revert to normoglycemia.

To address the mechanisms involved in such anti-CD4-induced transplantation tolerance we have followed the fate of the IE-reactive $V_\beta11^+$ T cells from the tolerant allografted mice. We have previously demonstrated that, following anti-CD4-mediated depletion, the kinetics of repopulation of the $CD4^+V_\beta11^+$ cells in the transplanted mice was comparable to that seen in anti-CD4-treated nontransplanted mice. In addition, there was no significant reduction in the frequency of $V_\beta11^+$ or $V_\beta5^+$ T cells of allografted animals after complete repopulation of the $CD4^+$ cells, indicating that anti-CD4-mediated transplantation tolerance was not due to clonal deletion of potentially IE-reactive T cells. Furthermore, mixing experiments indicated that neither $CD4^+$ nor $CD8^+$ cells from tolerant transplanted mice could suppress the response of cells from normal mice. Instead, following complete repopulation of the $CD4^+$ cells, both $CD4^+V_{\beta11}{}^+$ and $CD8^+V_\beta11^+$ T cell subsets from transplanted mice were found to be anergic; they were unable to respond when stimulated with immobilized mAb to $V_\beta11$ (*13*).

How were $CD8^+V_\beta11^+$ cells aneriged? In this report we have studied the kinetics of the appearance of anergy in $CD8^+V_\beta11^+$ and $CD4^+V_\beta11^+$ T cells in relationship to anti-CD4 treatment and transplantation. The data presented here suggest that depletion of $CD4^+$ cells with anti-CD4 mAb in B6 mice that subsequently received an A/J islet allograft led to a gradual

[1] This work was supported by NIH Grants A129796 and DK43711 and by a special grant from the American Diabetes Association.

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decrease in the response of $CD8^+V_\beta11^+$ T cells to anti-$V_\beta11$ stimulation over time. In contrast to the result seen 120 days posttransplant the $CD8^+$ cells present in the periphery at the time of transplant were shown to be responsive since they proliferated in vitro when stimulated with immobilized mAb to $V_\beta11$ and caused rejection of the allograft, in the absence of $CD4^+$ cells, when provided with r-IL-2 at the time of transplant. We propose that these results are consistent with the idea that there was a gradual loss of immunocompetent $CD8^+V_\beta11^+$ T cells from the preexisting peripheral T cell pool and that these cells are replaced by new thymic migrant $CD8^+V_\beta11^+$ (as well as $CD4^+V_\beta11^+$) cells that enter a state of clonal anergy when they come into contact with peripheral alloantigens. Our data on the rate of migration of cells from the thymus to the periphery are in agreement with this hypothesis.

MATERIALS AND METHODS

Mice. Adult A/J and C57Bl/6 (B6)* mice were bred in our departmental animal facility or purchased from Jackson Laboratories (Bar Harbor, ME). Mice were transplanted at 8–12 weeks of age.

Transplantation. The transplantation of pancreatic islets has been described previously (*7*). Briefly, recipient B6 mice were made diabetic by the administration of an intravenous bolus (200 mg/kg) of streptozotocin (Sigma, St. Louis, MO). Plasma glucose values were obtained from retro-orbital blood and analyzed on a Beckman Glucose Analyzer II (Beckman, Fullerton, CA). Mice were considered suitable recipients when plasma glucose readings exceeded 450 mg/dl.

To isolate islets, donor A/J pancreata were perfused in situ with 0.625 mg/ml collagenase P (Boehringer Mannheim, Indianapolis, IN) dissolved in HBSS medium (HBSS, 1% penicillin-streptomycin, 4 mM sodium bicarbonate, 0.22 g/L bovine albumin). Excised A/J donor tissue was incubated for 20 min at 37°C, washed with HBSS medium, and passed through a strainer. Donor islets were separated from digested tissue using 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 picked under a dissecting microscope and cultured overnight at 37°C in CMRL-1066 (Gibco, Grand Island, NY) supplemented with 10% FCS and 1% penicillin-streptomycin. The next day islets were washed and resuspended in HBSS medium, and 800–850 islets were injected into the liver of diabetic recipient mice by portal vein cannulation.

On the day before, the day of, and the day after transplant, recipient B6 mice were injected intraperitoneally with 50 μg anti-CD4 mAb GK1.5 (*14*) or 50 μg anti-CD8 mAb 53.6.7 (*15*) plus 50 μg mAb GK1.5. For the IL-2 treatment protocol, recipient mice were treated with 10,000 U r-IL-2 (Cetus) intraperitoneally starting 8 hr posttransplant, and continuing every 8 hr for the next 5 days.

Cell staining. The relative T cell subset frequencies present in the peripheral blood leukocytes of anti-CD4-treated, anti-CD4-plus-anti-CD8-treated, and control mice were analyzed by FACS analysis. Peripheral blood leukocytes were isolated on the indicated days by separation on a Lympholyte M (Cedarlane, Ontario, Canada) gradient. The cells were washed and stained on ice with FITC-conjugated anti-CD5 (mAb 53.7.3, Becton Dickinson Immunocytometry Systems, San Jose, CA) followed by 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). Cells were analyzed on a modified dual laser FACS with logarithmic amplifiers (Becton Dickinson).

Anti-TcR activation assay. T cell proliferation was induced by receptor crosslinking with mAb specific for $V_\beta11$ (RR3-15; [*16*]) or mAb specific for CD3 (1452C11; [*17*]), and $V_\beta2$ (B20.6; [Dr. B. Malissen]), as controls. Antibody (10 μg/ml) was coated onto U-bottomed microtiter plates (Flow Laboratories Inc., McLean, VA) overnight at 4°C. Plates were washed twice and 2.5×10^5 peripheral lymph node or spleen cells were added per well. The plates were incubated for 4 (CD3) or 5 ($V_\beta2$, $V_\beta11$) days with [^{3}H]thymidine added for the final 12–16 hr of culture.

Cell sorting. For assays involving sorted cells we used spleen cells from which red blood cells were lysed by hypotonic shock. Two aliquots of cells were stained with either PE-conjugated anti-CD4 mAb GK1.5 (for anti-CD4 depletion) or FITC-conjugated anti-CD8 mAb 53.6.7 (for anti-CD8 depletion). The cells were then negatively selected by sorting on a FACStar Plus. The resulting CD4-depleted or CD8-depleted populations had less than 1% contamination, as determined by FACS analysis of depleted populations.

Intrathymic labeling. Details of the technique were similar to those previously described (*18*). Briefly, mice were anesthetized with a mixture of ketamine and xylazine, the chest was opened, and 5 μl per thymic lobe FITC (1 mg/ml diluted in PBS) was injected through a 30-gauge needle attached to a 25-μl Hamilton syringe. The chest was closed with surgical clips.

RESULTS

Kinetics of unresponsiveness. Anti-CD4 mAb GK1.5 was given intraperitoneally to streptozotocin-treated diabetic B6 transplant recipients in three doses of 50 μg each on the day before, the day of, and the day following transplant of A/J islets of Langerhans through the portal circulation. Under this treatment regimen 90–95% of the $CD4^+$ cells were depleted from peripheral blood, as determined by FACS analysis. We have previously shown that there was no significant reduction in the frequency of $V_\beta11^+$ T cells in allografted animals relative to untransplanted controls after complete repopulation of the $CD4^+$ cells, 120 days posttransplant (*13*). Instead $V_\beta11^+$ T cells from transplanted animals could not be activated in response to anti–T cell receptor (TcR) specific crosslinking, in contrast to normal activation of the same population of cells from nontransplanted anti-CD4-treated B6 mice. Again, in these current experiments, both $CD4^+V_\beta11^+$ and $CD8^+V_\beta11^+$ T cell subsets from anti-CD4-treated transplant recipients were unresponsive 120 days posttransplant (Fig. 1). In this experiment the percent of $CD4^+V_\beta11^+$ cells relative to total $CD4^+$ cells was again similar in the anti-CD4-treated, transplanted mice (5.2%) when compared with the control mice (4.8%) following repopulation of the $CD4^+$ cells at 120 days.

In the present study we wanted to see when the $V_\beta11^+$ T cells were rendered anergic—immediately after transplant or gradually over time. To do this, anti-CD4-treated B6 mice, transplanted with A/J (IE^s) islets of Langerhans, were sacrificed at various time points posttransplant but before complete repopulation of the $CD4^+$ cells, and their lymph node cells were tested to see if they could respond to anti-$V_\beta11$ specific crosslinking. At each time point the response of lymph node cells from the transplanted mice were compared with that of B6 mice that had been treated with anti-CD4 at the initiation of the experiment but either sham-transplanted or not transplanted (Fig. 2). Repopulation of $CD4^+$ cells was checked in all mice prior to sacrifice and was shown to be similar in transplanted and nontransplanted control mice. The data presented in Figure 2 indicate that the decreasing response to anti-$V_\beta11$ specific crosslinking in cells from the transplanted mice decreased as a function of time. The mean response of $V_\beta11^+$ T cells from mice transplanted ten days prior to assay was approximately 90% that of anti-CD4-treated untransplanted control mice. At 25 days posttransplant the response of $V_\beta11^+$ cells from transplanted animals decreased to 80% that of controls.

* Abbreviations: B6, C57Bl/6; TcR, T cell receptor.

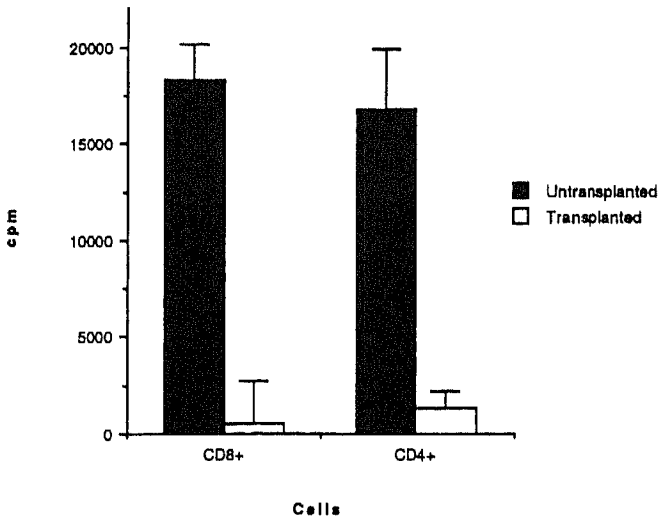

FIGURE 1. The anti-V_β11 response of CD4-depleted ($CD8^+$) and CD8-depleted ($CD4^+$) spleen cells 120 days posttransplant. Separated aliquots of cells from anti-CD4-treated, untransplanted mice (black bar), or anti-CD4-treated, transplanted mice (open bars) were stained with either PE-conjugated anti-CD4 mAb GK1.5 (for anti-CD4 depletion) or FITC-conjugated anti-CD8 mAb 53.6.7 (for anti-CD8 depletion) and negatively selected by cell sorting. The resulting CD4-depleted or CD8-depleted populations contained less than 1% contamination, as determined by FACS analysis. T cell proliferation was induced by receptor crosslinking with mAb specific for anti-V_β11 as described in *Materials and Methods*. Data are expressed as the mean ± SD of triplicate wells (cpm) and represent three similar experiments.

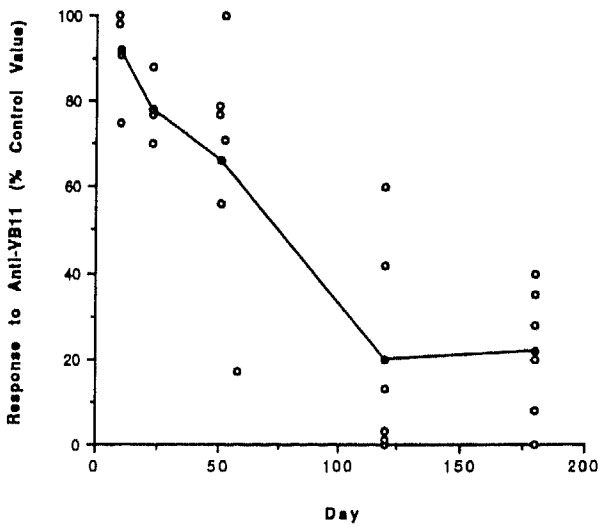

FIGURE 2. The kinetics of unresponsiveness to anti-V_β11 stimulation. T cell proliferation was induced by receptor crosslinking with mAb specific for V_β11 as indicated in *Materials and Methods*. Individual anti-CD4-treated transplanted mice are represented by (O); the means for a given day by (●) and represented by a line. The response is given as a percentage of that of anti-CD4-treated mice that were either sham-transplanted or not transplanted, and assayed at the same time as the transplanted animals.

This response decreased to approximately 65–70% of the control value by day 50 and to background levels by day 120.

Response of separated cell populations. To determine which cells were responding to anti-V_β11 stimulation during the initial 120 days posttransplant, spleen cells from anti-CD4-treated mice transplanted approximately 40 days prior to assay were FACS-sorted into $CD4^+$ (CD8-depleted) and $CD8^+$ (CD4-depleted) populations and analyzed for their ability to respond to anti-V_β11-specific crosslinking. $CD4^+$ cells from the transplanted mice displayed minimal activity to anti-V_β11 stimulation compared with the response of $CD4^+$ cells from anti-CD4-treated untransplanted control mice (Fig. 3, top). The unresponsiveness of $CD4^+$ cells was specific for anti-V_β11, as the response of an aliquot of these same $CD4^+$ cells to anti-V_β2, a TcR expressed at similar levels to V_β11 in B6 mice, was not reduced in transplanted mice (Fig. 3, bottom). In contrast to $CD4^+$ cells and to the results seen with $CD8^+$ cells at day 120, $CD8^+$ cells from the transplanted mice were able to respond on day 40, at approximately 80% of the level seen in the anti-CD4 treated untransplanted mice. As expected, there was no de-

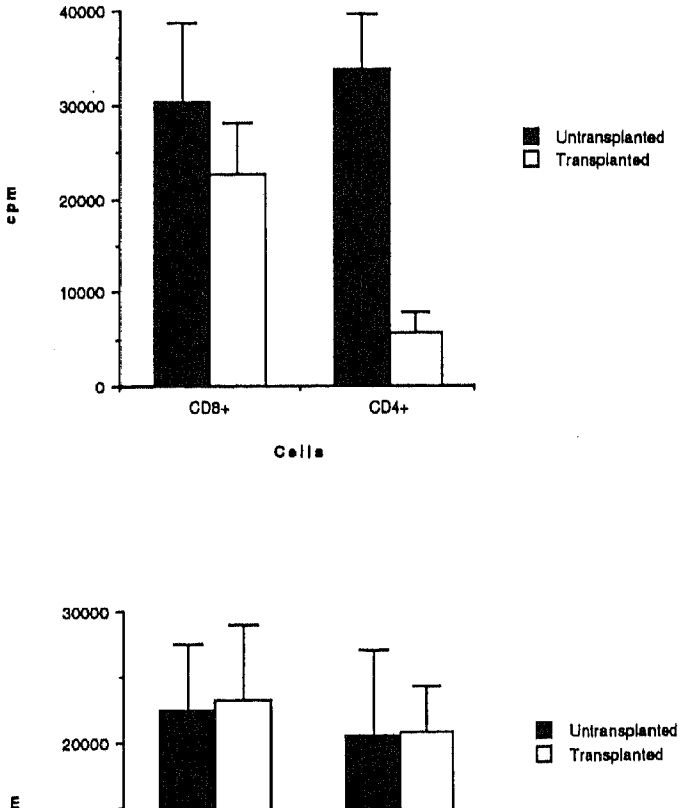

FIGURE 3. Receptor crosslinking assays of sorted cells. Anti-V_β11 (top) or Anti-V_β2 (bottom) response of CD4-depleted ($CD8^+$) and CD8-depleted ($CD4^+$) spleen cells were tested 40 days posttransplant. Separated aliquots of cells from anti-CD4-treated, untransplanted mice (black bar), or anti-CD4-treated, transplanted mice (open bars) were obtained as indicated in Figure 1. T cell proliferation was induced by receptor crosslinking with mAb specific for anti-V_β11 (top) or anti-V_β2 (bottom). Data are expressed as the mean ± SD of triplicate wells (cpm) and represent 2 experiments.

creased response of $CD8^+$ cells from transplanted mice to anti-$V\beta2$ stimulation when compared with anti-CD4-treated untransplanted controls.

Evidence that $CD8^+$ cells can cause rejection when given r-IL-2. Since the results in Figure 3 indicated that $CD8^+$ cells were responsive to anti-$V_\beta11$ shortly after transplant, we asked whether these cells could also cause rejection of the transplant if they were given help in the form of r-IL-2. Mice were treated with anti-CD4 and transplanted as usual. Starting 8 hr posttransplant, and every 8 hr for the next 5 days, mice were given 10,000 U r-IL-2 intraperitoneally. Three of four of the anti-CD4-treated, transplanted mice receiving r-IL-2 rejected their graft within 7 to 10 days (Fig. 4). This was similar to the rejection time seen in mice transplanted in the absence of anti-CD4 treatment. As expected, nontransplanted mice treated with anti-CD4 and r-IL-2 and anti-CD4-treated, transplanted mice not treated with r-IL-2 did not become hyperglycemic. Depletion of $CD4^+$ cells was similar in all groups and averaged 94% in the r-IL-2-treated, anti-CD4-treated mice.

Correlation of loss of response to anti-$V_\beta11$ stimulation with migration of cells from the thymus to the periphery. We have shown that the loss of response to anti-$V_\beta11$-specific crosslinking in $CD8^+$ T cells occured gradually over time (Figs. 2 and 3). The $CD8^+$ cells were responsive in the initial phase posttransplant but became unresponsive by about day 120 posttransplant. Since peripheral $CD8^+$ cells were not anergic shortly after transplant in this model, it was possible that new thymic migrant cells were anergized when they encountered alloantigen in the periphery. To study this possibility, we first examined the repopulation of $CD8^+$ cells in anti-CD4-treated transplanted mice that had additionally been depleted of peripheral $CD8^+$ cells with anti-CD8 mAb. Our data (Fig. 5) demonstrate that repopulation of $CD8^+$ cells was inversely correlated with the response of these cells to anti-$V_\beta11$ stimulation. For example, at two weeks posttreatment approximately 10% of the $CD8^+$ cells had repopulated the periphery, and the anti-$V_\beta11$ response in the tolerant mice was about 90% that of the control value. At day 50, approximately 25% $CD8^+$ cells had repopulated, and the anti-$V_\beta11$ response was about 65–70% that of the control value. When the peripheral $CD8^+$ cells had been replaced by anergic thymic migrant cells, the anti-$V_\beta11$ response was at background levels.

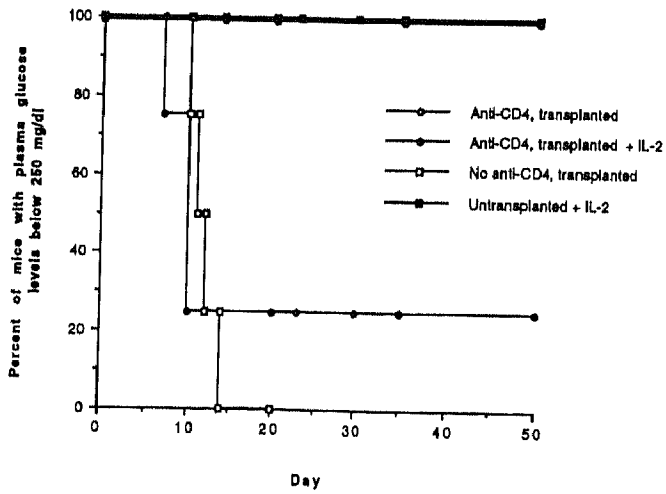

FIGURE 4. $CD8^+$ cells cause rejection of the allograft when given r-IL-2. B6 recipients were treated at the time of transplant with three 50 μg doses of GK1.5 intraperitoneally. IL-2 treatment was started 8 hr posttransplant and was continued every 8 hr for 5 days, with 10,000 units given at each injection. Data for IL-2 transplants (●) represent 4 mice; for IL-2 controls (■) data represent 2 mice. Allograft survivals for untreated (□) and anti-CD4-treated (○) transplant recipients represent historical data.

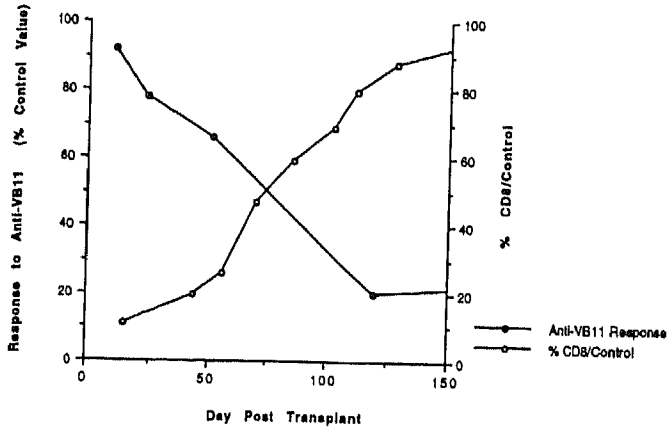

FIGURE 5. Repopulation of $CD8^+$ cells is inversely correlated with the response to anti-$V_\beta11$ stimulation. Repopulation of $CD8^+$ cells in mice treated with three doses of 50 μg anti-CD4 and 50 μg anti-CD8 is shown by (○). Unresponsiveness to anti-$V_\beta11$ in anti-CD4 treated transplanted mice (●) is taken from Figure 2.

TABLE 1. Quantitative analysis of new thymic migrant cells[a]

Organ	% Migrants (in lymphocyte population)	% T cells	% Migrants (in T cell population)	Turnover (days)
LN	0.45±0.06	58.2±1.9	0.8±0.1	129±17
Spleen	0.3±0.01	28.7±1.1	0.9±0.3	119±41

[a] Thymocytes were labeled by intrathymic injection of FITC. Then 24 h later mice were sacrificed and single-cell suspensions of lymph node and spleen cells were made. Cells were counted and stained with PE-conjugated anti-CD5 for 30 min on ice. The percentage of cells staining with anti-CD5-PE that were $FITC^+$ was quantitated by FACS analysis. Percentages represent the mean ± SD from 3 mice.

In a second series of experiments we directly quantitated new thymic migrant cells by using intrathymic injection of FITC to label thymocytes in situ, followed by quantitation of $FITC^+$ cells in the periphery by FACS analysis. This method has been shown to give random labeling of the thymocyte population and thus can be used to quantitate the migration of cells from the thymus to the periphery (*18*). The quantitation of total thymic migrants to lymph node and spleen of B6 mice 24 hr after intrathymic injection of FITC is given in Table 1. Approximately 0.45% of total peripheral lymph node cells and 0.3% of spleen cells were $FITC^+$, as determined by FACS analysis. These cells were double-stained with phycoerythrin conjugated anti-CD5 to exclude counting background $FITC^+$ staining. Accounting for the percentage of T cells in each organ examined, the percent of new thymic migrant T cells was approximately 0.8% in the lymph node and 0.9% in the spleen within a 24-hour period. From these figures the turnover rate for peripheral T cell was estimated to be about 120–130 days (Table 1).

DISCUSSION

Clonal anergy was induced in $CD8^+V_\beta11^+$ as well as $CD4^+V_\beta11^+$ T cells during transplantation tolerance induction

in which an IE^+ islet allograft was transplanted into a CD4-depleted diabetic B6 (IE^-) recipient (*13*). In this report we present data showing that the clonal anergy seen following anti-CD4 treatment and transplant developed gradually over time in $CD8^+$ cells; the response of the $V_\beta11^+$ T cells was 90% of the control value 10 days posttransplant and did not reach background levels until about 120 days posttransplant (Fig. 2). That the anti-$V_\beta11$ response was predominantly due to the response of peripheral $CD8^+V_\beta11^+$ cells during this initial 120 days posttransplant was supported by the data obtained using sorted cells (Fig. 3); the slight response of the $CD4^+V_\beta11^+$ cells was presumably due to the non-depleted $CD4^+V_\beta11^+$ cells remaining following anti-CD4 treatment.

How were $CD8^+V_\beta11^+$ (and $CD4^+V_\beta11^+$) cells rendered anergic by depletion regimens of anti-CD4? We favor the idea that depletion of $CD4^+$ cells with anti-CD4 mAb allowed transplanted allografts to behave as self tissue to a subset of T cells that increased with time, most likely new thymic migrant cells, and that these migrant cells were rendered anergic when they came into contact with peripheral alloantigens. In this model the anti-$V_\beta11$ response seen in the first few weeks posttransplant would represent the response of the residual $CD8^+V_\beta11^+$ cells present in the periphery at the time of anti-CD4 treatment plus the small population of nondepleted $CD4^+V_\beta11^+$ cells. Repopulation kinetics is consistent with the hypothesis that the gradual diminution of response seen in the peripheral $CD8^+V_\beta11^+$ population during the course of normal T cell turnover was due to repopulation of peripheral responsive $CD8^+V_\beta11^+$ cells by thymic migrant $CD8^+V_\beta11^+$ cells that became anergic upon encounter with alloantigen in the periphery.

In support of this hypothesis, we have observed an inverse correlation between the kinetics of repopulation of $CD8^+$ cells in the anti-CD4-treated mice (treated with anti-CD8 to deplete peripheral $CD8^+$ T cells) and the decreasing anti-$V_\beta11$ response in the anti-CD4-treated tolerant mice (Fig. 5). For example, at two weeks posttreatment, approximately 10% of the $CD8^+$ cells had repopulated the periphery, and the anti-$V_\beta11$ response in the tolerant mice was about 90% that of the control value. At day 50, approximately 25% $CD8^+$ cells had repopulated, and the anti-$V_\beta11$ response was about 65–70% that of the control value. When the peripheral $CD8^+$ cells had been replaced by thymic migrant cells, the anti-$V_\beta11$ response was at background levels. Furthermore, on day 40, the response of sorted $CD8^+$ cells from the anti-CD4-treated transplanted animals was approximately 80% that of anti-CD4-treated untransplanted controls (Fig. 3). These data and those presented in Figure 5 (20% repopulated $CD8^+$ cells seen at day 40), correlated well with our hypothesis that new thymic migrants should be anergic. The small anti-$V_\beta11$ response seen in $CD4^+$-sorted cells is presumably due to the $V_\beta11^+$ population contained within the small $CD4^+$ population not depleted by GK1.5 treatment. The observation that all $V_\beta11^+$ cells are anergic at day 120 posttransplant, and the repopulation kinetics of CD4 and CD8 depleted mice (Fig. 5), suggested a normal turnover of approximately 120 days for $CD8^+$ and $CD4^+$ thymic migrants.

To address this possibility by another technique, we also looked at migration of FITC-labeled thymocytes from the thymus to the periphery in B6 mice aged-matched to our transplant recipients. Our data (Table 1) again suggest that replacement of peripheral T cells in the lymph node and spleen of these mice takes about 120–130 days. This figure is close to the estimate from repopulation kinetics of the anti-CD8-treated mice (Fig. 5) and is similar to the time at which lymph node and spleen cells from the transplanted mice show no (background) anti-$V_\beta11$ response (Fig. 2). Thus these data are consistent with our hypothesis that the new thymic migrant $V_\beta11^+$ cells were rendered anergic when they came into contact with peripheral alloantigens.

Although the kinetics of anergy induction in $CD8^+V_\beta11^+$ cells and the estimate of rate of thymocyte migration is consistent with the idea that anergy was induced in recent thymic migrant cells, it was also formally possible that a necessary signal normally provided by $CD4^+$ cells to allow activation of $CD8^+$ cells could not be provided in the absence of $CD4^+$ cells. Lacking this signal the $CD8^+$ cells gradually became unresponsive. This does not seem likely since the peripheral $CD8^+V_\beta11^+$ cells were responsive soon after transplant, a time when the majority of $CD4^+$ cells were depleted by administration of anti-CD4 mAb. Another alternative might be that the repopulating anergic $CD4^+$ cells induced unresponsiveness in the peripheral $CD8^+$ T cells. However, our earlier data (*13*) showing that $CD4^+$ cells from the transplanted mice could not suppress the response of $CD8^+$ cells, as well as the kinetics of unresponsiveness shown here, would argue against this.

These data support the hypothesis that recent thymic migrant cells are rendered anergic or unresponsive when they encounter alloantigen in the periphery. Although these data were obtained studying alloreactivity, they could explain peripheral unresponsiveness to self by suggesting that recent thymic migrants are rendered unresponsive or anergic if they encounter self antigen (as well as imposed alloantigen) in the periphery. This hypothesis further suggests that thymic migrants that do not encounter self as an "antigen" further differentiate with time to allow subsequent encounters with nonself antigen to trigger an immune response, as opposed to anergy.

Acknowledgments. The authors gratefully acknowledge L. Jerabek for intrathymic injections, P. Raju for expertise in cell sorting, C. Taylor-Edwards for technical support, and I. Weissman for a critical review of the manuscript.

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Received 14 July 1992.
Accepted 8 December 1992.

0041-1337/93/5603-0638$03.00/0
TRANSPLANTATION

Vol. 56, 638–642, No. 3, September, 1993
Printed in U.S.A.

PREVENTION OF OVERT DIABETES AND INSULITIS BY INTRATHYMIC INJECTION OF SYNGENEIC ISLETS IN NEWBORN NONOBESE DIABETIC (NOD) MICE[1]

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Based on previous studies showing that allogeneic islets transplanted into the thymus can induce donor-specific unresponsiveness, we investigated in the nonobese diabetic (NOD) mouse the effect of intrathymic islet isografts on preventing autoimmunity directed against pancreatic islet antigens. Islets prepared from newborn NOD pancreata were injected into one lobe of the thymus of 10- to 11-day-old female NOD mice (experimental group) with no immunosuppression. PBS alone was used for injection into age- and sex-matched litter mates (control group). Thirty of 32 (94%) experimental mice remained normoglycemic for over 30 weeks. Well-formed islets with no indication of insulitis were found in the thymus of these 30 mice, whereas no grafted islets were found in the 2 mice that became diabetic at 17 and 19 weeks, respectively (technical failures). In the control group, 10 of 32 (31%) mice became diabetic between 20 and 29 weeks. This diabetic incidence was, however, lower than that in our colony female mice. In the pancreas of experimental mice, 90.9% of islets were free of infiltrates, whereas only 13.1% of islets were intact in control mice. The spleens of 30-week-old experimental mice contained a slightly higher percentage of CD8+ T cells ($P<0.05$) than those of control mice. Cyclophosphamide injections at 30 weeks induced diabetes in 4 of 9 experimental mice. The 2 lines of evidence, (1) marked reduction in insulitis of intrathymic islet-grafted mice and (2) induction of diabetes after treatment with cyclophosphamide, suggest that both thymic clonal deletion and peripheral tolerance may play a role in preventing diabetes.

Type I, insulin-deficient diabetes mellitus is considered as an autoimmune disease in which T lymphocytes play a critical role in the destruction of the pancreatic islets of Langerhans (*1*). After onset of the disease, transplantation of islets in the form of a whole pancreas or isolated islets can control blood glucose, but it is preferable to prevent the disease by blocking the autoimmune process before destruction of the islets. Treatment of patients with immunosuppressive drugs, such as CsA (*2*), AZA (*3*), or FK 506 (*4*), at the early phase of disease onset has been attempted with little success.

In the NOD mouse, an animal model for type I diabetes, the defect that causes abnormal T cell responses, is found at the bone marrow cell level (*5*). In BB rats, a bone marrow-derived non-T cell population in the thymus appears to be responsible for the development of autoreactive T cells (*6*). The thymus of the NOD mouse also exhibits several histological abnormalities

[1] This work was supported by a grant from the Nora Eccles Treadwell Foundation and NIH grant DK20827.

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