

T CELL DYSFUNCTION IN THE DIABETES-PRONE BB RAT

A Role for Thymic Migrants That Are Not T Cell Precursors

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Some sublines of Bio-Breeding (BB) rats spontaneously develop type I, insulin-dependent diabetes (1). Various forms of immunosuppression can protect the diabetes-prone BB rats (BB-DP rats)¹ from diabetic disease (2–4). This suggests an immune etiology. BB-DP rats also have several striking abnormalities in thymus-derived (T) lymphocyte immune responses. BB-DP T cells proliferate poorly in response to alloantigen *in vitro* (5) and fail to generate CTL responses (6). These T cell dysfunctions appear to predispose the animals to disease development because when the dysfunctions are reversed the animals are protected from disease (7–9). Other investigators have concluded that the T cell dysfunctions appear to be of bone marrow origin (10), the inference being that they occur at the level of T cell precursors in the bone marrow and are presumably not influenced by the thymus.

The experiments in this report provide evidence for another interpretation, namely, that the defective T cell responses are due to bone marrow-derived cells that (a) are not T cell precursors, and (b) influence the maturation and eventual function of normal thymocyte precursors by residing in the thymus during T cell maturation.

Materials and Methods

Rats. The Barbara Davis Center for Childhood Diabetes (BDCCD) has a colony of specific pathogen-free (SPF) BB-DP rats. They were derived in Basel, Switzerland from a breeding nucleus kindly supplied by Drs. A. Naji and C. F. Barker of the University of Pennsylvania (Philadelphia, PA). The rats have typed as RT 1^u at the MHC. Inbred DA (RT 1^a) and Fisher 344 (RT 1^{lv}) rats were obtained from Trudeau Institute (Saranac Lake, NY) and Taconic Farms, Inc. (Germantown, NY), respectively. Strain PVG (RT 1^c) were purchased from Bantin and Kingman (Freemont, CA). Nondiabetes-prone DA/BB F1 animals were bred at the BDCCD.

Bone Marrow and Thymus Donors. T cell-depleted bone marrow was harvested from adult BB or DA/BB F1 donors 8–10 wk old. These marrow donors had previously been adult thymectomized and treated for 7 d (1 ml intraperitoneally) with OX-19 antibody supernatant. The OX-19 marker is the Lyl equivalent in the mouse and is present on all

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¹ *Abbreviations used in this paper:* atx_{bm}, adult thymectomized, lethally irradiated, and bone marrow reconstituted; DP, diabetes prone; DR, diabetes resistant; dGua, 2-deoxyguanosine; IMDM, Iscove's modified Dulbecco's medium; SPF, specific pathogen free; TDL, thoracic duct lymphocyte.

mature peripheral T cells in the rat. This *in vivo* antibody protocol reduces the percentage of OX-19⁺ cells in the periphery (11). The donors were also thoracic duct cannulated as described (5) and drained for a further 3–5 d before marrow cells were harvested. The thoracic duct drainage procedure is thought to recruit any remaining mature T lymphocytes into the periphery from their residence in the marrow (12).

Fetal thymus was obtained from DA/BB F1 or BB donors of 18–20 d gestation. The tissue was dissected aseptically and freed from adhering tissues. It received 2,000 rad of irradiation before transplantation to delete the tissue of resident DA/BB F1 thymocytes. The equivalent of one whole thymus was transplanted per recipient.

Thymus Culture in 2 Deoxyguanosine (dGua). Thymus tissue was cultured in dGua using a modification of the method first described by Jenkinson et al. (13). Individual thymus lobes were dissected into four equal fragments and placed on the surface of sterile strips of Durapore HVLP filter (Millipore/Continental Water Systems, Bedford, MA). The filter strips were supported on Gelfoam sponge (Upjohn Co., Kalamazoo, MI) soaked in RPMI 1640 culture medium supplemented with 10% FCS, 2 mM glutamine, 10 mM Hepes buffer, 50 μ M 2-ME, 100 U/ml penicillin, and 100 μ g/ml streptomycin. One or two filter/sponge assemblies containing 4–8 thymus lobes were placed in a 10-cm petri dish with 15 ml of culture medium containing 4 mM dGua (Sigma Chemical Co., St. Louis, MO) and cultured for 5–7 d.

T Cell-depleted Recipients. 7-wk-old BB and DA/BB F1 rats were adult thymectomized and allowed to recover for at least 7 d. After this recovery period they were subjected to lethal whole body irradiation (950 and 1,050 rad, respectively). Irradiated animals were immediately reconstituted with 50×10^6 T cell-depleted bone marrow cells given intravenously. Each animal was given syngeneic marrow. 7 d later these adult thymectomized (at), lethally irradiated (x), and bone marrow (bm) reconstituted animals (atxbm) received a transplant beneath the kidney capsule of one whole fetal thymus. At various times after transplantation, the recipients had a cannula placed into the thoracic duct and thoracic duct lymphocytes (TDLs) were collected overnight and tested for surface phenotype and functional activity.

Cell Staining. $\sim 2 \times 10^6$ TDLs were stained with mAb supernatants OX-19 (rat pan-T cell marker), OX-8 (rat CD8 marker for class I MHC antigen-reactive T cells), or W3/25 (rat CD4 marker for class II MHC antigen-reactive T cells) (14). Antibody was derived from cell lines generously provided by Dr. A. Williams (Oxford, England). TDLs were incubated in 1 ml of supernatant at 4°C for 30 min and then washed three times. This was followed by a further incubation with FITC-conjugated sheep anti-mouse (IgG F(ab')₂) antibody diluted 1:100 (Cappel Laboratories, Malvern, PA). For background controls, cells were treated with the second antibody alone. After a further three washes, the cells were fixed in 1% paraformaldehyde and the percentage of positive cells was determined using an EPICS C flow cytometer (Coulter Electronics Inc., Hialeah, FL).

Mixed Leukocyte Reaction. TDLs were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 2 mM glutamine, penicillin/streptomycin, 50 μ M 2-ME, and 5% normal rat serum as described (15). Responder cells (beginning at 3×10^5 per well) were plated at three serial half-dilutions in 96-well U-bottomed plates. Stimulator cells were derived from lymph nodes and irradiated with 2,000 rad. A constant number of stimulator cells (3×10^5 per well) were added to a final volume of 0.2 ml. After 3 d of incubation, 0.025 ml [³H]thymidine (25 μ Ci/ml) was added per well and incubated for a further 18 h. Cells were then harvested onto glass-fiber filters and counted for 1 min in a β -ray scintillation counter. Values are recorded as the mean cpm of triplicate samples; the SE did not exceed 10% of the mean.

Generation of Cytotoxic T Lymphocytes (CTL) and ⁵¹Cr-release Assay. CTLs were generated from TDLs cultured in the presence of either DA/BB F1 or Fisher irradiated lymph node cells as previously described (15). Usually 10×10^6 TDLs were mixed with 25×10^6 stimulator cells and cultured for 6 d in IMDM supplemented with 10% FCS and 5% T cell growth factor (16). The final volume was 25 ml. Day 6 effector cells were then washed and plated in triplicate into V-bottomed plates at four serial half-dilutions. A constant number of ⁵¹Cr-labeled target cells (5×10^3 /well) were added to a final volume of 0.125

TABLE I
 Comparison of Mixed Leukocyte Responses between Diabetes-prone BB
 and Diabetes-resistant Strains

Exp.	Responder TDLs	Responder cells*	Stimulator cells (3×10^5 cells/well)	
1	BB-DP	3.0	<u>DA</u>	<u>None</u>
		1.5	6,958 [‡]	3,298
		0.75	2,636	1,180
	Fisher	3.0	1,998	221
		1.5	41,691	7,298
		0.75	37,415	5,004
2	BB-DP	3.0	<u>DA</u>	<u>None</u>
		1.5	6,074	2,585
		0.75	4,007	938
	PVG	3.0	3,669	385
		1.5	81,751	7,831
		0.75	94,378	2,217
3	BB-DP	3.0	<u>Fisher</u>	<u>None</u>
		1.5	5,110	315
		0.75	1,525	128
	BB-DR	3.0	179	86
		1.5	51,262	10,363
		0.75	103,928	980
	DA	3.0	99,808	249
		1.5	97,552	11,801
		0.75	107,721	4,845
			29,599	258

* Number of responder cells $\times 10^{-5}$ per well.

[‡] Thoracic duct lymphocytes from BB-DP rats were stimulated with populations allogeneic and MHC-incompatible to BB rats as described in Materials and Methods. Values refer to the mean cpm of triplicate cultures when pulsed with [³H]thymidine 3 d after culture initiation. The SE did not exceed 10% of the mean. Strains Fisher, PVG, DA, and BB-DR are diabetes resistant.

ml. Target cells were generated by culturing lymph node cells for 2–3 d in IMDM supplemented with 5 μ g/ml Con A. The plates were incubated for 4 h at 37°C, centrifuged, and half of the supernatant was collected for measurement of radioactivity. The values are recorded as the mean percent specific release from triplicate samples.

Test for Chimerism. TDLs from unmanipulated BB, DA/BB F1, and thymus-transplanted rats were resuspended at 10^7 cells/ml. 25 μ l of cell suspension was added to 50 μ l of twofold serially diluted antiserum in 96-well U-bottomed microtiter plates. (Anti-DA and anti-BB polyclonal antisera were generated by immunizing MHC-incompatible rats with spleen cells.) After the addition of 25 μ l of rabbit C (Cappel Laboratories), the plates were incubated for 1 h at 37°C after which time 10 μ l of trypan blue stain was added and the percentage of dead cells enumerated. Values refer to percentage of dead cells at plateau levels of antibody.

Results

Experimental Approach. We have published previously that TDLs from BB-DP rats proliferate poorly in response to alloantigen in vitro (5). Table I presents

TABLE II
*TDL Surface Phenotype in Normal and Thymus-transplanted
 BBatxbmF1x Rats*

Rats	Positive cells		
	OX-19	OX-8	W3/25
		%	
DA/BB F1 (unmanipulated)	73.7	20.8	45.5
BB-DP (unmanipulated)	19.2	5.6	19.4
BBatxbmF1x No. 1	10.1	0	9.4
2	16.0	7.7	14.8
3	19.4	2.6	19.5

Cells were stained for OX-19 (pan T cell), OX-8 (class I MHC-reactive cell), and W3/25 (class II MHC-reactive cell) surface markers.

Numbers represent the absolute percentage of positive cells (test minus background). The percent background staining for each of the five animals was 5.2, 19.2, 14.2, 14.5, and 15.6, respectively, where background = %-positive cells stained with FITC-labeled second antibody alone.

recent data from three separate experiments that confirm this. In experiments 1 and 2, TDLs from BB-DP rats are compared with TDLs from normal PVG and Fisher rats for their proliferative response to alloantigen in vitro. In experiment 3 the BB-DP response is compared with that of a diabetes-resistant strain of BB (BB-DR) as well as a normal DA rat. In each case the BB-DP response is poor, ranging from 5 to 16% of that from a normal or BB-DR response.

Our first goal was to design experiments to test whether we could influence these characteristic T cell responses of normal and BB-DP rats by transplantation of thymus tissue. To accomplish this first goal we prepared "B" rats; i.e., rats that are devoid of T cell function. BB-DP rats were adult thymectomized, lethally irradiated, and reconstituted with T cell-depleted bone marrow from syngeneic donors as described in Materials and Methods. These T cell-depleted animals (BBatxbm) were transplanted with thymus tissue from F1 animals where one parent was BB-DP and the other was a normal, nondiabetes-prone, strain DA. The DA/BB F1 exhibits T cell functions like those of normal rats and does not develop diabetes.

TDL Surface Phenotype. 7 wk after transplantation with γ -irradiated F1 thymus, the BBatxbm rats had a cannula surgically implanted into their thoracic duct and TDLs were collected overnight. The TDLs were stained with mouse monoclonal antibodies specific for rat T cell antigens and compared with those from normal F1 and unmanipulated BB rats. The monoclonals used were OX-19 (pan-T), OX-8 (CD8), and W3/25 (CD4). In Table II it can be seen that DA/BB F1 TDLs have a T cell profile of normal rats. More than three-quarters of the TDLs are OX-19⁺ T cells, and the class II MHC antigen reactive (W3/25⁺) population is roughly twice the class I MHC antigen reactive (OX-8⁺) population. In addition, the sum of OX-8⁺ and W3/25⁺ equals the percentage of OX-19⁺ cells. On the other hand, TDLs from BB-DP rats show a significantly reduced number of OX-19⁺ cells which is roughly equal to the number of W3/25⁺ cells. Thus the small percentage of cells that are OX-8⁺ are probably OX19⁻ and are thought to be NK cells (6). TDLs from the three BBatxbmF1x continued to

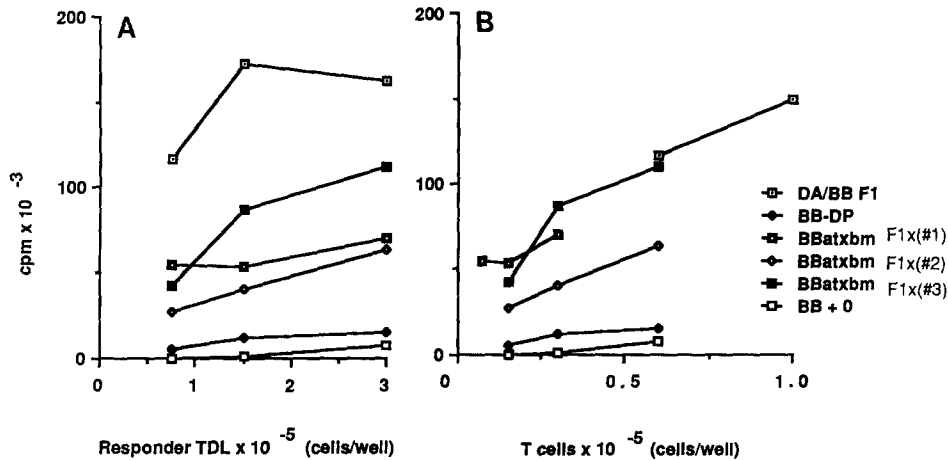


FIGURE 1. (A) TDLs from diabetes-resistant DA/BB F1 and diabetes-prone BB were compared in a mixed leukocyte reaction with TDLs from BB rats that had been adult thymectomized, lethally irradiated, and reconstituted with T cell-depleted syngeneic bone marrow, and grafted with a γ -irradiated DA/BB F1 thymus (BBatxbmF1x). BB+0 refers to the proliferative response of BB TDLs without the addition of allogeneic (Fisher) stimulators. This background response was higher than the background responses of all of the BBatxbmF1x animals. (B) The data from A are replotted to correct for the percentage of responder cells that are OX-19⁺ (pan-T cell marker).

show reduced numbers of OX-19⁺ T cells and few, if any, OX-8⁺ cells. The profile appears remarkably similar to the one observed with unmanipulated BB-DP TDLs. Once again, the W3/25⁺ population roughly equaled the OX-19⁺ population. The higher background staining observed when the cells are labeled with the FITC-tagged sheep anti-mouse IgG (all monoclonals used were raised in mice) is a consistent observation and is not due to Fc binding as the FITC-labeled antibody is an F(ab')₂ fragment. This high background, characteristic of BB-DP rats is also present on TDLs from BBatxbmF1x.

Restoration of MLR in Thymus-transplanted BB-DP Rats. When TDLs from the same BBatxbm animals were tested (7 wk after transplantation with F1 thymus tissue) we observed that all three had an enhanced proliferative response to third-party stimulator cells compared to unmanipulated BB-DP rats (Fig. 1A). Although the proliferative response of the TDLs from these F1 thymus-transplanted animals did not reach the levels observed for TDLs from normal DA/BB F1 rats, it was clearly improved over the response of untreated BB-DP rats. This was despite the fact that all three animals had markedly reduced numbers of OX-19⁺ T cells as assessed by flow cytometry (Table II). Because the absolute number of T cells differed between the populations tested in Fig. 1A (~73% for normal and 19% for BB-DP rats), we replotted the abscissa as the number of OX-19⁺ responder cells per well rather than the number of TDLs per well. When plotted in this manner the curves for two of the three BBatxbmF1x rats appeared to be on the same slope as the curve for T cells from a normal animal (Fig. 1B). The response of the unmanipulated BB animal remained poor. Therefore on a cell-for-cell basis it could be argued that the T cells in the F1 thymus-grafted BBatxbm animals responded like T cells from normal rather

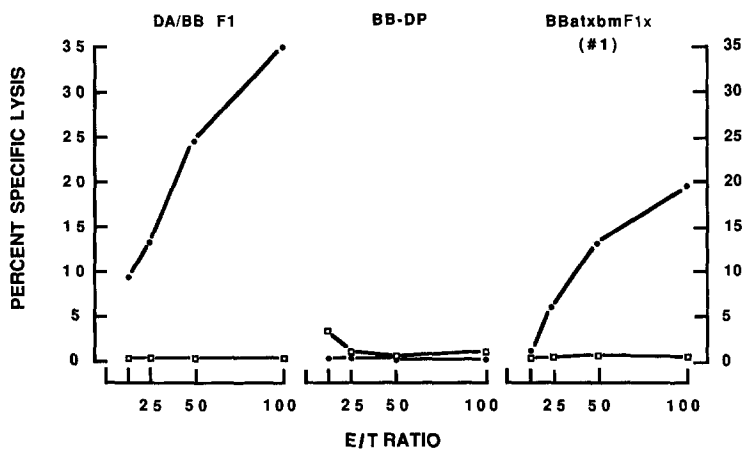


FIGURE 2. Percent specific lysis of Fisher (●) and DA/BB F1 (□) targets by normal and thymus transplanted (#1 from Fig. 1) rats. Each point represents the mean from triplicate samples when TDLs were cultured with Fisher or DA/BB F1 stimulator cells and tested for specific cytolysis of targets from the same haplotype as those used to stimulate the response.

than BB-DP rats. This representation of the data also shows that the poor BB-DP proliferative response is not simply due to a reduction in the absolute numbers of T cells. Even correcting for the relative numbers of T cells per culture does not restore the BB-DP response to the range of normal rats.

Restoration of CTLs in Thymus-transplanted BB-DP Rats. The same BBatxbm animals that expressed improved proliferative responses to alloantigen after transplantation with F1 thymus grafts were also tested for their capacity to generate CTLs. Failure to generate CTLs is the other obvious T cell dysfunction in the BB-DP rat. TDLs from untreated BB-DP and DA/BB rats were compared with F1 thymus-transplanted BB-DP rats. TDLs from all animals were cultured with strain Fisher (allogeneic and MHC-incompatible to both DA and BB) stimulator cells for 6 d as described in Materials and Methods. As expected, TDLs from normal DA/BB F1 rats stimulated with Fisher cells show a significant level of specific lysis of Fisher targets whereas TDLs from unmanipulated BB rats show no detectable cytolysis (Fig. 2). The BBatxbm animals grafted with irradiated DA/BB F1 thymus (F1x) express a significant level of specific cytolytic activity when stimulated with and tested against Fisher targets. Representative data from one thymus-transplanted rat is shown in Fig. 2. No lysis of DA/BB F1 targets was observed when the BBatxbmF1x TDL were stimulated with DA/BB F1 alloantigen.

Test for Chimerism in Thymus-transplanted BB-DP Rats. Although the thymus tissue was irradiated with 2,000 rad before transplantation to presumably kill any F1 thymocytes that were present in the tissue, we tested whether TDLs from BBatxbmF1x were sensitive to lysis with antibodies to DA alloantigen. If they were it would suggest that some of the peripheral lymphocytes present in the TDLs of the grafted animals were of DA/BB F1 rather than BB origin. To test this, we treated the TDLs with polyclonal anti-DA antiserum plus rabbit C. While TDLs from normal DA/BB F1 are all lysed by this procedure, lysis of cells from the unmanipulated and thymus-transplanted rats is roughly equivalent to

TABLE III
Test for Chimerism in Thymus-transplanted BBatxbmF1x Rats

Rats	Dead cells		
	C alone	Anti-DA + C	Anti-BB + C
		%	
DA/BB F1 (unmanipulated)	12.1	100	100
BB-DP (unmanipulated)	16.6	12.5	100
BBatxbmF1x No. 1	9.0	13.6	100
2	13.1	12.6	100
3	22.1	26.0	100

TDLs were treated with either anti-DA or anti-BB polyclonal antiserum in the presence of rabbit C.

the complement-treated alone control (Table III). Therefore the proliferative responses observed in the thymus-transplanted BBatxbm rats would appear to be from cells of the BB haplotype.

Rationale for Subsequent Experiments. Having established that a thymus transplant from a normal rat donor can influence T cell function in BB-DP rats, another goal was to determine if this improved T cell function was mediated by bone marrow-derived cells of the thymic donor haplotype which resided in the thymus at the time of transplant. Our rationale for raising this question was based on two previously published experimental observations. The first was that another laboratory had transplanted BB-DP animals with thymus grafts from a diabetes-resistant line of BB (BB-DR) and concluded that the T cell dysfunction mapped to the marrow rather than the thymus donor (10). The second evolved from recent publications by several other laboratories using the drug dGua. It has been shown that dGua treatment causes selective depletion of lymphoid cells (13) and cells of the macrophage and/or dendritic cell lineages (17). This treatment spares the thymic epithelial cells thus permitting the maturation of T cells restricted to the MHC of the thymic epithelium (17). However, when the marrow-derived thymocytes in these newly reconstituted thymus grafts are tested for function, they are not tolerant to thymic donor alloantigens as assessed by proliferative responses or the generation of cytotoxic T lymphocytes (13, 18, 19). Thymocytes isolated from thymus tissue that was not treated with dGua are tolerant. One conclusion from these studies is that tolerance to self-antigens is critically dependent on a dGua-sensitive population of cells which resides in the thymus.

Our working hypothesis was that the enhanced proliferative response observed in BBatxbm transplanted with F1 thymus tissue was due to the presence of F1 cells which were resistant to the 2,000 rad of irradiation to which the F1 thymus had been subjected before transplantation. These cells were most likely radiation-resistant cells of the macrophage and/or dendritic cell lineages rather than thymocytes. This hypothesis would provide an explanation for the previously published study concluding that the T cell dysfunction in BB-DP rats is mapped to the bone marrow. It would also predict that (a) the T cell response of BB-DP rats transplanted with F1 thymus grafts would deteriorate with time as the thymus became repopulated with BB-DP macrophage and/or dendritic cells; and

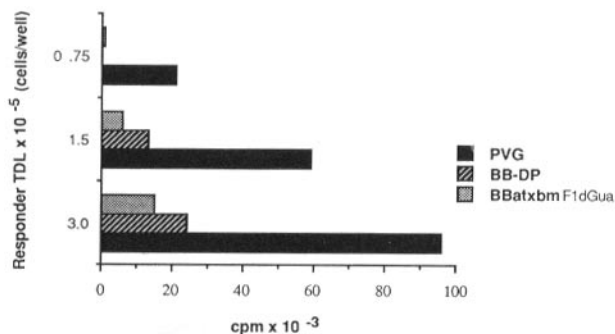


FIGURE 3. Proliferative responses are compared between normal PVG and BB-DP rats with a BBatxbm transplanted with a DA/BB F1 thymus cultured with dGua (BBatxbmF1dGua). Representative data are shown from one animal tested at 7 wk after thymus transplantation.

(b) that the F1 thymus graft would be ineffective in reversing the T cell dysfunction if treated with dGua before transplant. The experiments that follow provide evidence that supports these predictions. The four main experimental groups tested were: (a) BBatxbm rats transplanted with γ -irradiated F1 thymus (BBatxbmF1x; data shown above); (b) BBatxbm rats transplanted with dGua-treated F1 thymus (BBatxbmF1dGua); (c) DA/BBatxbm rats transplanted with γ -irradiated BB thymus (F1atxbmBBx); and (d) DA/BBatxbm rats transplanted with dGua-treated BB thymus (F1atxbmBBdGua).

Response of BBatxbmF1dGua at 7 wk. According to our working hypothesis, the enhanced proliferative response of BBatxbmF1x is due to the presence of radioresistant bone marrow-derived cells which migrate to the thymus. These cells do not appear to be T cell precursors as there was no evidence of chimerism in the peripheral T cell pool (Table III). If these cells are poisoned by culture in dGua then transplantation of dGua-treated F1 thymus tissue should not have the same effect as γ irradiation-treated F1 thymus tissue because the F1 thymus should be repopulated with BB-DP marrow-derived cells. To test this, BBatxbm were prepared as before and transplanted with an F1 thymus. Instead of γ irradiation the thymus tissue was cultured for 5–7 d in the presence of dGua as described in the Materials and Methods. At the dose used in this experiment we observed that the dGua reduces the in vitro thymocyte proliferation assay to background (data not shown). When examined histologically there was no evidence of thymocytes present in the dGua-treated tissue. At 7 wk after transplanting BBatxbmF1dGua, we tested for proliferative response to alloantigen. The response was similar to that observed for untreated BB-DP (Fig. 3). The weak responses were not changed by data manipulations which corrected for the percentage of OX-19⁺ cells although OX-19⁺ cells were clearly present (data not shown).

Response of F1atxbmBBx and F1atxbmBBdGua at 7 wk. The results thus far suggest that an F1 thymus graft can lead to an improved T cell response and that this enhanced response is due to the presence in the F1 thymus of a γ radiation-resistant, dGua-sensitive population of cells which are not T cell precursors. When this population is replaced with cells of the BB-DP lineage, the T cells that mature in this thymus exhibit the functional characteristics of the BB-DP marrow donor rather than the F1 thymus donor. Therefore F1 T cell precursors maturing in a BB thymus containing BB marrow-derived cells should

TABLE IV
Comparison of Mixed Leukocyte Responses between FlatxbmBBx and FlatxbmBBdGua at 7 wk after Transplantation

Responder TDL	Responder cells*	Stimulator cells (3×10^5 cells/well)		
		Fisher	DA	None
PVG (unmanipulated)	3.0	137,242 [‡]	81,751	7,831
	1.5	130,269	94,378	2,217
	0.75	90,300	57,720	1,886
BB-DP (unmanipulated)	3.0	4,281	6,074	2,585
	1.5	1,131	4,007	938
	0.75	685	3,669	385
FlatxbmBBdGua	3.0	117,990	18,589	20,963
	1.5	113,377	11,308	14,544
	0.75	53,281	3,167	1,125
FlatxbmBBx	3.0	24,713	2,585	4,686
	1.5	19,303	657	896
	0.75	15,058	150	338

* Number of responder cells $\times 10^{-5}$ per well.

[‡] Values refer to the mean cpm of triplicate cultures; the SE did not exceed 10% of the mean.

exhibit the T cell functions of the BB-DP thymus donor. Conversely, if the BB thymus is treated with dGua, the T cells that mature should exhibit the functions of the F1 marrow donor.

To test this, DA/BB F1 animals were made into atxbm animals as described in Materials and Methods. Animals were transplanted with γ -irradiated or dGua-treated BB thymus grafts. Representative data from an animal in each group tested at 7 wk after thymus transplant are shown in Table IV. TDLs from the FlatxbmBBdGua animal proliferated comparably to those from a normal PVG rat in response to third-party strain Fisher stimulator cells. FlatxbmBBx proliferated poorly in response to Fisher. Neither thymus-transplanted F1 animal responded to strain DA stimulator cells when their proliferative responses were compared with those without any stimulators in the cultures.

This experiment shows that otherwise normal F1 T cell precursors proliferate in a manner similar to BB-DP T cells if they mature in a BB-DP thymus which contains dGua-sensitive BB-DP cells. Elimination of these dGua-sensitive BB-DP cells results in the return of normal T cell function. The return of normal T cell function is presumably mediated by the repopulation of the BB thymus with dGua-sensitive F1 cells.

Response of Thymus-transplanted Animals at 16 wk. Longo and Schwartz were the first to propose that T cell specificity was influenced by the phenotype of thymic APCs (20). They examined turnover of thymic APCs and concluded that APCs of the marrow donor haplotype could be detected in the thymus by 2 mo and that the new T cells emerging after that time behaved like the marrow rather than the thymus donor.

We tested whether T cells from thymus-transplanted animals exhibited the T cell functions of the marrow donor 16 wk after thymus transplantation when it

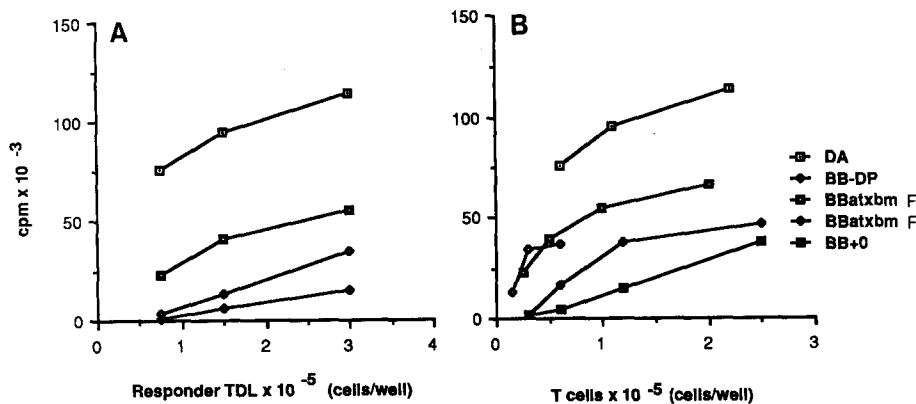


FIGURE 4. (A) Mixed leukocyte proliferative responses were compared between normal DA and thymus-transplanted BBatxbmBBx and BBatxbmF1x rats. TDLs were tested 16 wk after thymus transplantation. (B) The data from A are replotted to correct for the percentage of responder cells that are OX-19⁺ (pan T cell marker). BB+0 refers to the proliferative response of BB TDLs without the addition of allogeneic (Fisher) stimulators.

might be expected that the thymus transplants would be repopulated with marrow-derived radiation-resistant, dGua-sensitive cells.

When the same experiment as described in Fig. 1 was repeated in two BBatxbmF1x animals at 16-wk after thymus grafting, their TDL proliferative response remained enhanced. However, it was obviously less than that of TDLs from normal diabetes-resistant strains (Fig. 4A). In this experiment we also included a BBatxbm transplanted with a γ -irradiated BB thymus (BBatxbmBBx). The response of the TDLs from this animal mirrored that of the unmanipulated BB-DP rats showing that the experimental procedures of adult thymectomy, lethal irradiation, and bone marrow reconstitution are not themselves factors in the improved proliferative response. Data manipulations to correct for the percentage of OX-19⁺ cells did not lead to the suggestion that the responses of T cells from these animals was comparable to normal rats on a cell-for-cell basis (Fig. 4B). Therefore the response with time after thymus transplantation appears to be moving towards that of the BB-DP marrow donor.

Similarly, F1atxbmBBx have responses in the normal range and mirror those observed for F1atxbmBBdGua at 16 wk (Table V). Again, the proliferative response of the T cells from these animals approximates that observed for the F1 marrow donor.

Two BBatxbmF1dGua were also tested at 16 wk after thymus transplant. The collected TDLs were tested for proliferative responses to alloantigen. The results, shown in Fig. 5 were the same as those of identically treated animals tested at 7 wk. The proliferative responses were in the range observed for unmanipulated BB-DP rats.

Summary of Data. Fig. 6 attempts to collate the data from all four groups tested at 7 and 16 wk after thymus transplantation. Plateau cpm values from each experimental animal are compared as a percentage of the cpm values of the normal rats used as controls in the same experiment. The closer the value approaches 100% the more the experimental animals response approximates

TABLE V
Comparison of Mixed Leukocyte Responses between FlatxbmBBx and FlatxbmBBdGua at 16 wk after Transplantation

Responder TDL	Responder cells	Stimulator cells (3×10^5 cells/well)			
		Fisher	DA	None	
PVG (unmanipulated)	3.0*	160,124 [‡]	142,584	251	
	1.5	61,847	49,687	90	
	0.75	18,527	6,447	88	
FlatxbmBBdGua No. 1	3.0	160,788	19,935	14,439	
	1.5	84,893	1,501	8,760	
	0.75	18,746	353	2,029	
	2	3.0	138,855	8,973	9,919
		1.5	92,926	543	7,010
		0.75	34,346	109	973
FlatxbmBBx No. 1	3.0	154,541	22,858	25,769	
	1.5	94,379	8,386	7,943	
	0.75	30,413	803	1,746	
	2	3.0	131,959	12,710	12,233
		1.5	92,627	1,316	2,327
		0.75	47,472	240	506

* Number of responder cells $\times 10^{-5}$ per well.

[‡] Values refer to the mean cpm of triplicate cultures; the SE did not exceed 10% of the mean.

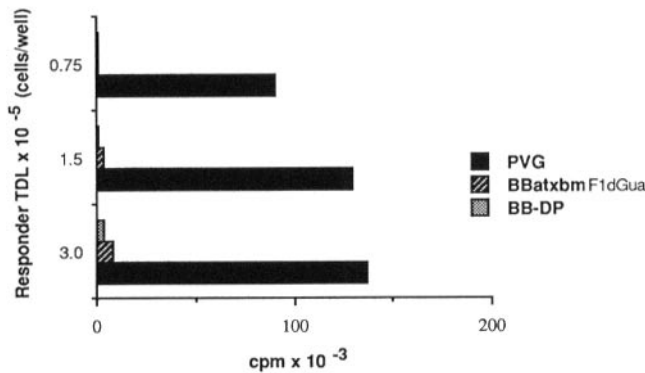


FIGURE 5. Mixed leukocyte proliferative responses are compared between TDLs from normal PVG, BB-DP, and BBatxbm rats transplanted with dGua-treated DA/BB F1 thymus (BBatxbm-F1dGua). TDLs were tested 16 wk after thymus transplantation.

that of a normal nondiabetes-prone rat. As can be observed diagrammatically, the BBatxbmF1x response approaches the range of normal rats at 7 wk after thymus transplant and is deteriorating by 16 wk. Conversely, the FlatxbmBBx responses are in the range of untreated BB-DP at 7 wk and improve to normal levels by 16 wk. BBatxbmF1dGua remain constant at the levels observed for BB-DP and FlatxbmBBdGua remain at levels observed for normal DA/BB F1 rats.

Discussion

Adult thymectomized, lethally irradiated, and bone marrow-reconstituted BB-DP rats were transplanted with thymus tissue from DA/BB F1 fetal donors. The thymus tissue was subjected to γ irradiation before transplantation to kill resident F1 thymocytes. The thoracic duct lymphocytes from these thymus-transplanted

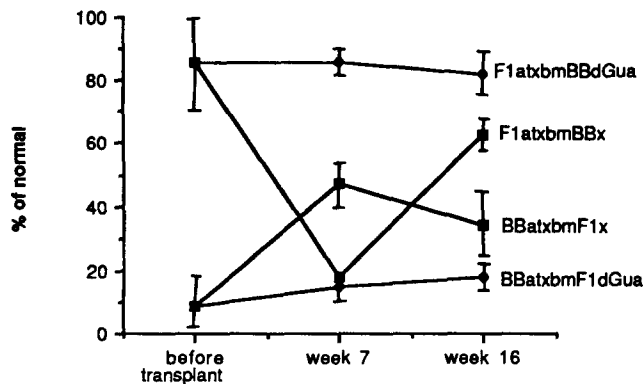


FIGURE 6. On the x axis, BB-DP TDLs are compared with BB and DA/BB F1 animals transplanted with thymus tissue 7 or 16 wk before testing. The y axis (% of normal) was derived by dividing the maximum cpm observed for the BB-DP or thymus-transplanted animals by the maximum cpm of the normal, non-diabetes-prone animal used as a control in each experiment. Maximum cpm is the [^3H]thymidine incorporation of lymphocytes from each animal stimulated with the same MHC-incompatible populations for 4 d as described in Materials and Methods.

animals (BBatxbmF1x) were compared with those from normal DA/BB F1 and unmanipulated BB-DP rats for their proliferative responses to alloantigen. Whereas TDLs from the unmanipulated BB-DP animals displayed the typical T cell dysfunction (i.e., a very weak proliferative response to alloantigen; Table I), TDLs from the thymus-transplanted BBatxbmF1x animals generated proliferative (Fig. 1A) responses which far exceeded responses of BB-DP. On a cell-for-cell basis the OX-19⁺ T cell responses of the normal and thymus-grafted animals appeared comparable in two of three examples tested (Fig. 1B). This first experiment performed at 7 wk after thymus transplant was repeated at 16 wk. By this time, the responses, though obviously better than those from normal BB-DP rats, appeared to be diminishing compared with normal diabetes-resistant rats (Fig. 4A) regardless of any corrections made for the percentage of OX19⁺ T cells (Fig. 4B).

When the same experiment was performed with TDLs from animals that had been transplanted 7 wk previously with F1 thymus tissue treated with dGua, the proliferative responses of the BBatxbmF1dGua thymus-grafted animals remained poor (Fig. 3) and in the range observed for BB-DP TDLs. It did not improve in animals tested at 16 wk after thymus transplant. It has been shown by others that dGua is toxic for bone marrow-derived cells but spares the thymic epithelial cells.

We conclude from these experiments that the proliferative defect to alloantigen observed in the BB-DP rat is not preprogrammed at the level of T cell precursors in the bone marrow. It would appear instead that BB T cell precursors can function normally if they mature in a thymus containing a radiation-resistant, dGua-sensitive population of thymic immigrants from normal strains. The literature suggests these immigrants are APCs (20) that are most likely macrophage and/or dendritic cells (17-19), hereafter referred to as thymic APCs. If this population of cells is destroyed and replaced with thymic immigrants of BB-DP origin, then the T cell precursors that mature in the chimeric-thymic environment express the functional phenotype of the marrow donor.

The results with T cell-depleted F1 animals transplanted with BB tissue are

consistent with this hypothesis. F1atxbm transplanted with a dGua-treated BB thymus graft have normal T cell proliferative responses (Table IV). This shows that dGua does not destroy the capacity of the tissue to provide the thymic environment necessary for T cell maturation. It is also consistent with the hypothesis that BB thymus tissue appears to perform normally if repopulated with F1 thymic APCs (a bone marrow-derived, radiation-resistant, dGua-sensitive thymic immigrant). If the BB thymus contains BB thymic APCs, the results appear to be different. We observed a weak proliferative response in F1atxbm rats transplanted with BB γ -irradiated thymus tissue (Table IV) but this response returns to normal by 16 wk after transplantation (Table V). Our explanation for the rapid recovery of T cell function is that the BB thymus becomes repopulated with F1 thymic APCs. To prevent this we are presently repeating these experiments with the addition of F1atx injected with BB bone marrow before BB thymus transplantation. In this latter case, the T cell dysfunction should be evident because the BB thymus will be repopulated with BB thymic APCs.

The BBatxbm thymus-transplanted animals remained lymphopenic with reduced numbers of OX-19⁺ mature T lymphocytes and class II MHC reactive (W3/25⁺) T cells, and few, if any, class I MHC reactive (OX-8⁺) T cells (Table II). Therefore, the lymphopenia appears to be nascent in the bone marrow and is not influenced by the thymus. Conversely, we have observed that F1atxbm with BB tissue are not lymphopenic (data not shown).

In other studies it can be concluded that the T cell dysfunction in the BB-DP rat is separable from the lymphopenia (8) and that the lymphopenia has its origins in the marrow (10). Our results are in agreement on these points. However, we also conclude that the T cell dysfunction is influenced by the thymus and on this issue our results appear to be at odds with another study (10). In that study BBatxbm were transplanted with BB diabetes-resistant thymus grafts. When these animals were tested later (at least 12 wk after transplantation), the TDLs functioned like the diabetes-prone marrow donor, i.e., the proliferative dysfunction remained. There are several possible explanations for the differences between their results and ours. The one we prefer is that, in the former study, there was an influence of the diabetes-resistant thymus graft but that it was not observed due to the time of assay after grafting. It would appear that the longer one waits to test for T cell function after transplantation the more the response appears to express the phenotype of the marrow donor. A similar conclusion was made earlier by Longo and Schwartz who studied the MHC-restricted T cell profile of thymic chimeras with time after transplantation (20). Other possible explanations for the differences in results are as follows: (a) we transplanted semiallogeneic F1 thymus grafts while they used an MHC-compatible diabetes-resistant strain. Perhaps the differences at the MHC are important to correct the dysfunction; (b) our animals are SPF while theirs were conventionally reared. Conceivably some immunosuppressive effect of pathogen infection may have influenced their proliferation data.

It seems unlikely that the proliferative responses observed after transplantation of an F1 thymus is due to F1 thymocyte contamination. The most direct evidence is that the transplanted animals do not appear to be chimeric for F1 lymphocytes (Table III). One must also hypothesize that such F1 thymocytes are resistant to

2,000 rad of irradiation to which the thymus tissue was subjected before transplantation.

Of interest to this report is the observation that BB rats lack the OX-19⁺/OX-8⁺ class I-reactive T cell subset. The absence of this population provides a likely explanation why CTLs are difficult to generate in the BB-DP rat (6). Because the BBatxbmF1 thymus-grafted animals remained lymphopenic with few, if any OX-8⁺ cells, it is conceivable that the CTL response observed is due to the W3/25⁺ subset. Alternatively, the very few OX-19⁺/OX-8⁺ cells may expand sufficiently over the 6 d of culture to account for the response. This is our preferred explanation for the CTL activity. We have observed that normal thymectomized and T cell-depleted rats, while having essentially background levels of OX-8⁺ cells at three wk after transplantation of a syngeneic thymus, generate good CTLs on a cell-for-cell basis in vitro (our unpublished data). By 12 wk these animals have ratios of W3/25 to OX-8 that approach the normal (2:1) range. Experiments have been designed to test the phenotype of the CTLs in BBatxbmF1x.

For technical reasons we were only able to test one of the BBatxbmF1dGua animals for CTL activity. TDLs from this animal could generate detectable CTL activity to third-party alloantigens. Interestingly, TDLs from this animal also generated CTLs to "thymic self" DA alloantigens but only when the cultures were supplemented with exogenous sources of IL-2 (unpublished data). We observed no proliferation with stimulators of the DA haplotype in any of the animals transplanted with an F1 thymus whether the thymus was subjected to γ irradiation or dGua treatment. Perhaps the T cells that proliferate early in response to alloantigen provide the necessary lymphokines required to activate the CTL precursors (15). Apparently these cells are tolerized in the F1dGua thymus which would explain why CTLs to DA are only generated in the presence of exogenous sources of help. If confirmed with more animals the result with the dGua-treated thymus supports the conclusions in the murine model that the class I-reactive T cells are not tolerized when the class I antigen is expressed only on the thymic epithelium (19).

The deterioration of normal T cell function in BBatxbm with γ -irradiated F1 thymus grafts (Fig. 4, A and B) has interesting implications. It could simply mean that the early functional T cells become diluted by abnormal "diabetes-prone" T cells. It is also possible that BB-DP T cells are dominant and can influence the function of normal T cells. The evidence thus far is mostly against this. Addition of BB-DP T cells to normal T cell proliferation assays has not resulted in suppression (5). Also, normal mature T cells present in a bone marrow inoculum can function normally in the BB-DP environment (7, 8), which argues against a suppressive influence of the BB-DP environment on mature T cells. However, bone marrow inocula containing T cell precursors from normal, diabetes-resistant donors does not prevent diabetes if it is depleted of mature T cells before injection into BB-DP neonates (8) suggesting a potent influence of the BB-DP environment on T cell precursors.

T cell function may be influential in determining the susceptibility of BB rats to diabetes. The best experiments to support this statement are those in which BB-DP animals are protected from disease development after inoculation with

cells from nondiabetes-prone donors (7–9). Prevention of disease in BB-DP animals inoculated with normal cells correlates with a return of normal T cell function (7, 8). Interestingly, the diabetes-resistant BB-DR rat (4, 21) also appears to have normal T cell proliferative responses (Table I). So far, the only animals in our thymus transplant study which have developed diabetes are those which express the T cell dysfunction.

The prevailing view at the moment is that two important roles of the thymus, imprinting MHC restriction and the generation of self-tolerance are orchestrated by different cells in the thymus. Tolerance is heavily influenced by marrow-derived cells which are not T cell precursors and MHC restriction can occur when the MHC-restricting elements are presented only on the thymic epithelium. Because the T cell dysfunction is dependent on the presence of BB-DP radiation-resistant cells, this may have important implications for the development of diabetes. It could suggest that self-tolerance induction is aberrant in the BB-DP rat and this in some way leads to the destruction of pancreatic islets. However it appears, based on the reduced numbers of T cells, the weak MLR, and non-existent CTLs that if there is an aberration in tolerance induction, it leans toward increased rather than decreased tolerance. The increased tolerance could result from hyperexpression of MHC antigen in the thymus mediated by BB-DP marrow-derived cells. The increased tolerance could explain the absence of class I-reactive T cells and the reduced MLR. Casualties of this overzealous tolerance might also include T cells important in regulatory or suppressor function.

Summary

Diabetes-prone BB (BB-DP) rats express several T cell dysfunctions which include poor proliferative and cytotoxic responses to alloantigen. The goal of this study was to determine the origin of these T cell dysfunctions. When BB-DP rats were thymectomized, T cell depleted, and transplanted with neonatal thymus tissue from diabetes-resistant and otherwise normal DA/BB F1 rats, the early restoration of T cell function proceeded normally on a cell-for-cell basis; i.e., peripheral T cells functioned like those from the thymus donor. Because the thymus in these experiments was subjected to γ irradiation before transplantation and there was no evidence of F1 chimerism in the transplanted BB-DP rats, it appeared that the BB-DP T cell precursors could mature into normally functioning T cells if the maturation process occurred in a normal thymus. If the F1 thymus tissue was treated with dGua before transplantation, the T cells of these animals functioned poorly like those from untreated BB-DP rats. dGua poisons bone marrow-derived cells, including γ radiation-resistant cells of the macrophage/dendritic cell lineages, while sparing the thymic epithelium. Therefore, the reversal of the T cell dysfunction depends on the presence in the F1 thymus of γ radiation-resistant, dGua-sensitive F1 cells. Conversely, thymectomized and T cell-depleted F1 rats expressed T cell dysfunction when transplanted with γ -irradiated BB thymus grafts. T cell responses were normal in animals transplanted with dGua-treated BB thymus grafts. With increasing time after thymus transplantation, T cells from all animals gradually expressed the functional phenotype of the bone marrow donor. Taken together these results suggest that BB-DP

bone marrow-derived cells that are not T cell precursors influence the maturation environment in the thymus of otherwise normal BB-DP T cell precursors.

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