Transplantation analysis of B cell destruction in (NOD × CBA)F1 mouse bone marrow chimeras

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Summary. F1 hybrids produced by outcross of non-obese diabetic (NOD) mice with diabetes resistant strains are also diabetes resistant. This resistance is abrogated if F1 hybrids are lethally irradiated and then haematopoietically reconstituted with NOD bone marrow. This model was employed to determine whether T lymphocyte recognition and elimination of pancreatic B cells in NOD mice is restricted by the MHC haplotype of the target B cell. Diabetes resistant (NOD/Lt × CBA/J)F1 hybrids were lethally irradiated and reconstituted with NOD/Lt bone marrow. Following haematopoietic reconstitution, donor matched NOD/Lt or CBA/J pancreatic islet and anterior pituitary grafts were grafted under a renal capsule to determine whether effector cells derived from NOD/Lt marrow progenitors would reject islet grafts in a MHC restricted fashion. The H-2^k haplotype expressed by CBA/J mice differs from all known loci of the unique H-2 haplotype of NOD; therefore, if NOD/Lt T lymphocytes eliminate pancreatic B cells in a MHC restricted fashion, NOD islet grafts would be eliminated in these chimeras while CBA islet grafts would be retained. Overt diabetes developed in 80% of the female and 40% of the male F1 hybrids following reconstitution with NOD/Lt marrow, while no hybrids reconstituted with CBA/J marrow became diabetic through a year of age. The retention of CBA/J skin and pituitary grafts in NOD/Lt marrow reconstituted F1 hybrids confirmed that the F1 thymic environment imparted tolerance to CBA/J alloantigens. Nonetheless, responses to a T cell dependent model antigen were restricted to the unique MHC haplotype of NOD. This was associated in the hyperglycaemic chimeras with

rejection (8–21 days post-implantation) of both CBA/J and NOD/Lt islet grafts. This indicated generation of islet specific effectors that were not restricted by the MHC haplotype of the target B cell. Islet specific effectors were less active in those chimeras remaining normoglycaemic following NOD/Lt marrow reconstitution, since both NOD/Lt and CBA/J islet grafts remained intact 21 days post-implantation. Islet and pituitary grafts of each genotype remained free of leucocytic involvement in CBA/J marrow reconstituted F1 hybrids. The generation of B cell specific, but not genotype specific, effectors elicited from NOD/Lt marrow was age-dependent. Following priming with porcine insulin *in vivo*, T lymphocytes from 8-week-old NOD male mice failed to respond to porcine insulin *in vitro*, while primed T lymphocytes isolated from 16-week-old mice proliferated vigorously. This suggested that as pancreatic insulinitis progressed in aging NOD/Lt mice, T lymphocytes capable of responding to B cells in a tissue specific, but not genotype specific fashion, were activated. Thus, we propose that immunological effectors derived from NOD/Lt, but not CBA/J marrow, are capable of eliminating islet grafts in non-MHC restricted fashion. However, this population could be activated as a consequence of B cell erosion initiated in the pancreas by another class of effectors which may be restricted by the MHC haplotype of the target B cell.

Key words: NOD/Lt, CBA/J, diabetes, marrow, transplantation, pancreas, islets, MHC restriction, T cells, insulin.

The development of insulin-dependent diabetes in the non-obese diabetic (NOD) mouse is a T lymphocyte dependent process since treatment of prediabetic NOD mice with antibodies directed against the T cell surface antigens Thy-1 or L3T4 prevented disease onset [1, 2], while disease onset could be accelerated in young prediabetic NOD males by injection of T cells from overtly

diabetic donors [3, 4]. However, the mechanism by which T cells eliminate pancreatic B cells remains controversial. Adoptive transfer studies have indicated that both MHC class II restricted L3T4⁺ and MHC class I restricted Ly-2⁺ T cells are required for pathogenesis since disease was only accelerated in young prediabetic recipients when both T cell subsets were transferred from diabetic donors

[3, 4]. Similarly, T cell cultures derived from isolated NOD islets must contain both T cell subsets in order to accelerate onset of diabetes in young prediabetic recipients [5]. In addition, overtly diabetic NOD mice treated with cyclosporine reportedly retained skin allografts whereas matched islet allografts sharing a MHC class I haplotype with NOD (K^d or D^b respectively) exhibited a greater degree of lymphocytic infiltration than did islet grafts from donors which were incompatible with all known loci of NOD's unique MHC [6]. Collectively, these studies suggested that in NOD mice, pancreatic B cells are recognized and killed by classical MHC class I restricted Ly-2⁺ cytotoxic T cells (CTL) which are dependent on help from MHC class II restricted L3T4⁺ T cells.

It has also been argued that autoimmune elimination of pancreatic B cells in the NOD mouse occurs in a fashion analogous to DTH (delayed type hypersensitivity) and is not restricted by the MHC haplotype expressed on the B cell. When diabetic NOD mice were grafted with passenger leucocyte-depleted BALB/c islet and pituitary tissue, the islet grafts were eliminated while allogeneic pituitary grafts were retained [7]. It was subsequently reported that diabetic NOD mice would retain leucocyte depleted BALB/c islet allografts if the recipients were depleted of L3T4⁺ T cells [8, 9]. Although NOD and BALB/c both express a common MHC class I $H-2K^d$ allele, these results were interpreted to indicate that the recognition of islet allografts by NOD T cells was not restricted by the MHC class I haplotype of the target B cell. Instead, it was proposed that antigen presenting cells processed a soluble antigen common to all B cells for presentation to L3T4⁺ T cells in the context of the unique I-A molecule of NOD [10], and that these latter cells mediated islet graft destruction [9].

Diabetogenesis in NOD/Lt mice is under polygenic control [11, 12]. Requisite susceptibility genes are expressed at the level of haematopoietic stem cell derived effectors since diabetes developed in nominally resistant F1 hybrids between NOD/Lt and NON/Lt (Non-Obese Normal) or C57BL/10J (B10) mice following lethal irradiation and reconstitution with NOD bone marrow [13, 14]. Both NON/Lt and B10 islet grafts were rapidly rejected in the respective diabetic F1 chimeras, and T cell responses to soluble T dependent antigens were restricted to the MHC haplotype of NOD/Lt in (NOD/Lt × NON/Lt)F1 hybrids reconstituted with NOD/Lt marrow. Thus, the diabetogenic recessive genes in the NOD genome do not code for a B cell specific antigen unique to NOD, but instead create a dysregulated immune system producing T cells autoreactive against an antigen common to all B cells. However, NOD, NON, and B10 mice all express the MHC class I D^b allele [11, 12] which could serve as a common restriction element for the recognition of islet grafts from all three genotypes by B cell specific Ly-2⁺ CTL.

In the present study we attempted to determine if islet grafts were rejected in a MHC restricted fashion by reconstituting diabetes resistant (NOD × CBA)F1 hybrids with NOD/Lt bone marrow and implanting the chimeras with donor matched NOD/Lt or CBA/J pancreatic islet and anterior pituitary grafts. CBA/J mice express the $H-2^k$ haplotype which differs from all known loci of the unique

recombinant H-2 haplotype of NOD (K^d , $I-A^{\text{nod}}$, $I-E^{\text{null}}$, D^b). Thus, if diabetogenesis in NOD mice is mediated by Ly-2⁺ T cells restricted by the MHC class I haplotype of the target B cell, NOD/Lt islet grafts would be eliminated in these chimeras while CBA/J islet grafts would be retained. On the other hand, if islet grafts are eliminated in NOD/Lt mice via presentation by antigen presenting cells of a common soluble B cell antigen to L3T4⁺ T cells in a DTH-like response, both NOD/Lt and CBA/J islet grafts would be eliminated in these chimeras.

Materials and methods

Mice

NOD/Lt (currently at the 50th generation of inbreeding), CBA/J mice (obtained from the Animal Resources Unit of The Jackson Laboratory), and (NOD/Lt × CBA/J)F1 hybrids were housed under specific pathogen free conditions and allowed free access to food (diet 96W, Emory Morse Co., Guilford, Conn., USA) and chlorinated drinking water. Diabetes incidence in the NOD/Lt colony by 30 weeks of age is currently 70% in females and 50% in males.

Bone marrow reconstitution studies

Bone marrow was flushed from the femours and tibias of 4- to 8-week-old NOD/Lt or CBA/J female mice with Hanks' Balanced Salt Solution and treated with anti-Thy 1.2 monoclonal antibody (HO 13-4.9). Four-week-old (NOD × CBA)F1 recipients were irradiated with 1000 rads from a Cs^{137} source and injected i.v. with $2.5-5 \times 10^6$ of the anti-Thy 1.2 treated bone marrow cells. Chimerism was assessed at 4 weeks post-reconstitution by typing erythrocytes from the marrow recipients for expression of the NOD/Lt or CBA/J phenotype at the carbonic anhydrase-2 (*Car-2*) locus as previously described [15]. NOD/Lt mice express the *Car-2^a* allele while the *Car-2^b* allele is expressed by CBA/J mice [13, 15]. Also at 8 weeks of age, the recipients of NOD/Lt marrow (NOD → F1) received CBA/J tail skin grafts while the recipients of CBA/J marrow (CBA → F1) received NOD/Lt tail skin grafts. Tail skin graft survival was then assessed weekly. Starting at 10 weeks of age, plasma glucose levels were determined every two weeks by means of a glucose analyser (Beckman Instruments, Palo Alto, Calif., USA). Mice remaining normoglycaemic through 50 weeks of age following reconstitution with NOD/Lt bone marrow were injected i.p. with cyclophosphamide [300 mg/kg body weight (Mead Johnson, Evansville Ind., USA)], a compound previously demonstrated to accelerate diabetes onset in NOD mice [16]. A mouse was considered diabetic after two successive plasma glucose readings of > 13.9 mmol/l. Upon development of overt diabetes, chimeras were implanted with islets and a piece of anterior pituitary gland from the indicated donors as described below. Chimeras remaining normoglycaemic at 1 year of age also received grafts.

Islet-implantation studies

Islets were isolated from NOD/Lt or CBA/J donors as previously described [13] and aggregated together in a drop of blood from the recipient chimera. The blood clot containing the islets was implanted under the kidney capsule along with a piece of donor matched anterior pituitary as previously described [13]. Islets were intentionally transplanted in numbers insufficient to reverse hyperglycaemia (approximately 100/recipient) but sufficient to be detected histologically. At 8 or 21 days post-implantation, pancreases and the renal subcapsular grafts were fixed with Bouin's fluid and analysed histologically. Pancreases were sampled at 3 different levels of section while the portion of the kidney containing the islet and pituitary grafts was serially sectioned. Granulated B cells were identified by staining with aldehyde fuchsin.

Fig. 1 A and B. Pancreatic histology of NOD/Lt bone marrow reconstituted (NOD $\times$ CBA)F1 hybrids (aldehyde fuchsin staining; haematoxylin and eosin counterstaining). **A** Typical appearance of islet residua in a hyperglycaemic chimera at 28 weeks of age. The islet depicted is virtually devoid of granulated B cells and insulinitis has completely eroded the cytoarchitectural integrity of the islet

($\times 435$). **B** Heavy peri-insular leucocytic infiltration around intact aldehyde fuchsin-positive pancreatic islets in a 1-year-old normoglycaemic chimera. Although there is some erosion of islet structure, the B cell cores remain intact and B cells are heavily granulated ($\times 435$)

MHC restriction of T cells in NOD $\rightarrow$ F1 chimeras

To determine whether peripheral T cell responses in NOD marrow reconstituted F1 hybrids were restricted by the MHC haplotype of NOD or CBA, the ability of antigen presenting cells from NOD and CBA to present the T dependent antigen GAT (poly-L- Glu, L-Ala, L-Tyr; ICN, Lisle, III, USA) to primed T cells from NOD $\rightarrow$ F1 male chimeras was assessed. Two 32-week-old NOD $\rightarrow$ F1 male chimeras (28 weeks post reconstitution) were injected with 20 μ g GAT in complete Freund's adjuvant into a rear foot pad. Two weeks later, T cells from the draining popliteal lymph nodes were purified on nylon wool columns and suspended in culture medium consisting of RPMI 1640 (HyClone Laboratories, Logan, Utah, USA) containing 25 mmol/l HEPES, 2 mmol/l glutamine, 5×10^{-5} mol/l β -mercaptoethanol, 100 U/ml penicillin, and 100 μ g/ml streptomycin, plus 10% heat-treated fetal bovine serum (FBS, HyClone Laboratories). Triplicate cultures of 5×10^5 T cells were seeded in a final volume of 0.2 ml/well in flat bottomed 96 well microtitre plates (Corning Inc., Corning, N. Y., USA) in medium with or without 50 μ g/well GAT in the presence or absence of 5×10^5 irradiated (2000 R) splenic leucocytes from naive 8-week-old NOD or CBA male mice as a source of antigen presenting cells. The cultures were incubated for 3 days at 37°C in a humidified 5% CO₂/95% air atmosphere prior to the addition of 0.5 μ Ci/well [³H] thymidine (New England Nuclear, Boston, Mass, USA). The cultures were incubated an additional 24 h, and then harvested onto glass fiber disks by a cell harvester (Cambridge Technology, Cambridge, Mass., USA) and the disks counted in 3 ml of Aqualol-2 (New England Nuclear). Data are presented as the mean cpm $\pm$ SEM for the triplicate T cell cultures under each assay condition.

Priming and assay of insulin reactive NOD/Lt T lymphocytes

Crystalline porcine insulin (kindly supplied by the Eli Lilly Co., Indianapolis, Ind., USA) was dissolved at 2 mg/ml in 0.85% NaCl and emulsified at a ratio of 1:1 in complete Freund's adjuvant. Fifty μ l of the insulin/adjuvant slurry (50 μ g insulin) was injected into the right rear foot pad of normoglycaemic 8- and 16-week-old NOD/Lt male mice. The mice were boosted 7 days later with a similar dose of por-

cine insulin in incomplete Freund's adjuvant. Mice were killed 7 days after boosting; the draining popliteal lymph nodes from each age group excised and pooled, and the T cells purified on nylon wool columns. The purified T cells were then suspended in the culture medium described above. Triplicate cultures of 5×10^5 T cells were seeded in a final volume of 0.2 ml/well in flat bottomed 96-well microtitre plates in medium with or without 250 μ g/ml porcine insulin in the presence or absence of 5×10^5 irradiated (2000R) splenic leucocytes from naive 8- or 16-week-old NOD/Lt males mice as a source of antigen presenting cells. The cultures were incubated for 3 days at 37°C prior to labeling for 24 h with ³H-thymidine and harvesting as described above.

Results

Assessment of chimerism and immunological tolerance in (NOD $\times$ CBA)F1 mice reconstituted with NOD or CBA bone marrow

Chimerism was confirmed in all irradiated marrow recipients at 4 weeks post-reconstitution, as erythrocytes from all NOD $\rightarrow$ F1 mice (15/15) expressed the NOD *Car-2^a* allele, while erythrocytes from all CBA $\rightarrow$ F1 (6/6) expressed the CBA *Car-2^b* allele. All F1 marrow recipients remained tolerant to the parental MHC haplotype not used for bone marrow reconstitution, since without exception, NOD $\rightarrow$ F1 mice retained CBA tail skin grafts and CBA $\rightarrow$ F1 mice retained NOD tail skin grafts throughout the entire experimental period.

Adoptive transfer of diabetes by NOD marrow

As shown in Table 1, none of the CBA $\rightarrow$ F1 mice (0/3 females and 0/3 males) had become hyperglycaemic by one year of age while 8/10 female and 2/5 male NOD $\rightarrow$ F1

Table 1. Diabetes incidence in chimeric (NOD × CBA)F1 mice

Marrow donor	Recipient	Total no. diabetic mice	Age of onset (weeks)
NOD	F1 female	8/10 ^a	23–56 ^b
	F1 male	2/5 ^a	50–60 ^b
CBA	F1 female	0/3	–
	F1 male	0/3	–

^a One female and one male died of unknown causes at 27 and 33 weeks of age respectively; ^b Three females and one male became hyperglycaemic subsequent to an i.p. injection with 300 mg/kg body weight cyclophosphamide at 51–54 weeks of age. (NOD × CBA)F1 mice were irradiated with 1000 rads from a ¹³⁷Cs source at 4 weeks of age and reconstituted with $2.5\text{--}5 \times 10^6$ T cell depleted NOD or CBA bone marrow cells. Mice were screened bi-monthly for the development of hyperglycaemia starting at 10 weeks of age

mice became diabetic between 23–56 weeks and 50–60 weeks of age respectively. From the total of 10 NOD→F1 diabetic chimeras, 3 females and 1 male became hyperglycaemic following an i.p. injection of 300 mg/kg body weight cyclophosphamide administered between 51–54 weeks of age.

Islets in pancreases of diabetic NOD→F1 mice were atrophic and almost completely devoid of granulated B cells; insulinitis, characteristic of NOD mice was present (Fig. 1A). Islets from normoglycaemic NOD→F1 chimeras were associated with peri-insular leucocytic infiltrates, but for the most part remained structurally intact and B cells remained well granulated (Fig. 1B). Pancreatic islets from CBA→F1 mice remained free of insulinitis and stained strongly with aldehyde fuchsin (data not shown).

Islet implantation studies

From the total of 10 diabetic NOD→F1 chimeras shown in Table 1, four were implanted under a kidney capsule with CBA islet and anterior pituitary grafts, while a fifth received NOD islet and pituitary grafts. A sixth diabetic chimera received both NOD and CBA islet and anterior pituitary grafts under contralateral renal capsules. Graft survival in these NOD→F1 hybrids is summarised in Table 2, with representative histology depicted in Figure 2.

When examined histologically at 8 days post-implantation, 0/2 NOD islet grafts and 1/3 CBA islet grafts had been retained in hyperglycaemic NOD→F1 chimeras. At 21 days post-implantation, two other hyperglycaemic NOD→F1 chimeras had rejected CBA islet grafts. In all cases, rejected islet grafts were associated with an intense leucocytic infiltration, and few or no aldehyde fuchsin-staining B cells remained (Fig. 2A). In contrast to the rapid rejection of islet grafts in hyperglycaemic NOD→F1 chimeras, 5/5 CBA and 2/2 NOD anterior pituitary grafts remained structurally intact when examined at 8 or 21 days post-engraftment (Table 2, Fig. 2B).

Of the five NOD→F1 hybrids described in Table 1 that remained normoglycaemic, two received NOD islet and anterior pituitary grafts, while a third was implanted

Table 2. Anterior pituitary and islet graft survival in NOD bone marrow reconstituted (NOD × CBA)F1 hybrids

	CBA/J Grafts		NOD/Lt Grafts	
	Anterior pituitary	Islets	Anterior pituitary	Islets
Hyperglycaemic recipients 8 days post-implantation				
Recipient no. 2168	0	+	ND	ND
no. 2173	ND	ND	0	+++
no. 2171	+	+++	0	+++
no. 2198	+	+++	ND	ND
Graft survival	3/3	1/3	2/2	0/2
21 Days post-implantation				
Recipient no. 2199	0	+++	ND	ND
no. 2201	+	+++	ND	ND
Graft survival	2/2	0/2	-	-
Normoglycaemic recipients 21 days post-implantation				
Recipient no. 2205	0	++	ND	ND
no. 2172	ND	ND	0	++
no. 2174	ND	ND	0	++
Graft survival	1/1	1/1	2/2	2/2

(0) Intact grafts with no visible lesions; (+) minimal leucocytic infiltration in region of structurally intact graft; (++) leucocytic infiltrate surrounds structurally intact islets staining strongly with aldehyde fuchsin; (+++) islet structure disrupted by intense leucocytic infiltration; few or no aldehyde-fuchsin positive B cells remain. This score defined a rejected graft. ND = not done

with CBA grafts at one year of age. These grafts were then examined histologically at 21 days post-implantation. Although surrounded by a heavy peri-insular leucocytic infiltrate, both NOD and CBA islets in normoglycaemic NOD→F1 chimeras remained structurally intact and the B cells stained strongly with aldehyde-fuchsin (Table 2, Fig. 2C). It should be noted that the histologic appearance of the ectopic islet grafts in the normoglycaemic NOD→F1 chimeras closely resembled that of the pancreas in the same mice; i.e., structurally intact islets staining strongly with aldehyde-fuchsin were surrounded by peri-insular leucocytic infiltrates (compare Fig. 1B and 2C). Interestingly, the one hyperglycaemic chimera shown in Table 2 (no. 2168) that retained CBA islet grafts through eight days had a plasma glucose concentration of 15.2 mmol/l at the time of engraftment. This chimera returned to normoglycaemia after engraftment. On the other hand, plasma glucose concentrations of 18.3–20.6 mmol/l were observed at the time of engraftment in the hyperglycaemic NOD→F1 chimeras that rejected islet grafts. Thus, it appeared that NOD→F1 chimeras did not acutely reject islet grafts unless B cell necrosis in the in situ pancreas had progressed to the point that overt hyperglycaemia had been permanently established. The normoglycaemic NOD→F1 chimeras probably did not progress to overt diabetes due to insufficient levels of B cell specific

Fig. 2 A–D. Morphology of islet and anterior pituitary grafts in the sub-renal capsule (aldehyde fuchsin staining; haematoxylin and eosin counterstaining). **A** Residium of a CBA/J islet 21 days post-implantation beneath the renal capsule of a 1-year-old hyperglycaemic (NOD $\times$ CBA)F1 chimerized with NOD bone marrow. The core of the islet is completely degenerated and contains inspissated aldehyde fuchsin-positive material. The region around the islet is fibrotic and residual leucocytic infiltrates are apparent ($\times 870$). **B** Intact CBA/J anterior pituitary graft beneath the renal capsule of the same hyperglycaemic chimera described in Figure 2A. A residual islet surrounded by leucocytes is present in the field (arrow) ($\times 435$).

C Aldehyde fuchsin-positive NOD/Lt B cells remaining beneath the renal capsule at 21 days post-transplantation into a (NOD $\times$ CBA)F1 chimerized with NOD bone marrow, but remaining normoglycaemic through 1 year of age. Heavy leucocytic infiltration of the grafted islets indicates that they are in the process of elimination ($\times 870$). **D** The intact nature of both NOD islets (containing numerous, aldehyde fuchsin-positive B cells) and an anterior pituitary graft at 21 days post-implantation into a 1-yr-old normoglycaemic (NOD $\times$ CBA)F1 reconstituted with CBA/J bone marrow. Note that the grafts show minimal leucocytic infiltration ($\times 435$).

immunological effectors. However, these effectors were not absent in these mice as an intense peri-insular leucocytic infiltration could be observed surrounding the ectopic islet grafts as well as the pancreatic islets in situ (Fig. 1B, 2C).

B cell specific immunological effectors were not generated in CBA $\rightarrow$ F1 chimeras since F1 islets in situ and islets of both parental genotypes engrafted into these chimeras remained free of leucocytic involvement (Fig. 2D). This resulted in the retention in this class of chimeras of 2/2 CBA and 3/3 NOD islet and pituitary grafts at 21 days post-engraftment (Table 3). Since NOD islet grafts remained free of leucocytic involvement in the

CBA $\rightarrow$ F1 chimeras, these results also confirmed that CBA/J marrow could not generate diabetogenic effectors and that NOD B cells were not uniquely immunogenic.

MHC Restriction of GAT primed T cells in NOD $\rightarrow$ F1 chimeras

Two 32-week-old (28 weeks post-reconstitution) NOD $\rightarrow$ F1 chimeras not involved in the diabetes incidence and islet grafting studies described above were primed with the T dependent antigen GAT, an antigen to which both NOD/Lt and CBA/J respond. As shown in

Table 3. Anterior pituitary and islet graft survival in CBA bone marrow reconstituted (NOD × CBA)F1 hybrids

	CBA/J Grafts		NOD/Lt Grafts	
	Anterior pituitary	Islets	Anterior pituitary	Islets
Normoglycaemic recipients 21 days post-implantation				
Recipient no. 2195	0	+	ND	ND
no. 2202	0	0	ND	ND
no. 2196	ND	ND	0	+
no. 2203	ND	ND	0	0
no. 2204	ND	ND	0	0
Graft survival	2/2	2/2	3/3	3/3

(0) Intact grafts with no visible lesions; (+) minimal leucocytic infiltration in region of structurally intact graft. This score defined a rejected graft. ND = not done

Table 4. MHC restriction profile of GAT primed T cells in NOD→(NOD × CBA)F1 Chimeras

APC source	Response of GAT primed T cells (Mean cpm ± SEM)	
	No GAT	GAT 50 µg/well
None	122 ± 76	58 ± 18
NOD	210 ± 47	2950 ± 235
CBA	1216 ± 414	839 ± 169

20 µg of GAT in complete Freund's adjuvant was injected into the right rear foot pads of 2 NOD→(NOD × CBA)F1 male chimeras at 32 weeks of age (28 weeks post-reconstitution). Two weeks later, pooled T cells isolated from the draining popliteal lymph nodes were cultured in triplicate at 5×10^5 /well in culture medium with or without 50 µg/well GAT in the presence or absence of 5×10^5 irradiated splenocytes from 8 week old NOD/Lt or CBA/J male mice as a source of antigen presenting cells (APC). Data represent the mean ^3H -thymidine incorporation over the final 24 h of a 4-day culture. GAT = poly-L-Glu, L-Ala, L-Tyr

Table 5. Age dependent changes in NOD/Lt T cell responses to porcine insulin

Primed T cell culture conditions	Age in weeks	
	8-week	16-week
Media only	336 ± 23	391 ± 23
APC only	505 ± 21	1248 ± 343
Porcine insulin only	301 ± 126	2634 ± 407
Porcine insulin + APC	452 ± 126	11373 ± 1853

50 µg of porcine insulin in complete Freund's adjuvant was injected into the right rear foot pads of 2 NOD/Lt male mice at 8 or 16 weeks of age. The mice were boosted with a similar dose of insulin in incomplete Freund's adjuvant 7 days later. One week after boosting, pooled T cells isolated from the draining popliteal lymph nodes of each age group were cultured in triplicate at 5×10^5 /well in media with or without 250 µg/ml porcine insulin in the presence or absence of 5×10^5 irradiated splenocytes from naive 8 or 16 weeks NOD/Lt male mice as a source of antigen presenting cells (APC). Data represent the mean ^3H -thymidine incorporation ± SEM over the final 24 h of a 4-day culture

Table 4, GAT primed T cells from NOD→F1 chimeras responded to GAT in vitro in the presence of antigen presenting cells from NOD (2950 ± 235 cpm), but not CBA mice (839 ± 169 cpm). These results indicated that despite maturing in a F1 thymic environment, the peripheral T cells in these chimeras only responded to antigen in the context of the unique MHC haplotype of NOD.

Development of insulin reactive T cells in NOD/Lt mice

The islet grafting results described above indicated that both NOD/Lt and CBA/J islet grafts were acutely rejected in hyperglycaemic NOD→F1 chimeras, while the survival of islet grafts of both genotypes was protracted in normoglycaemic NOD→F1 chimeras. The rapid rejection of islet but not anterior pituitary grafts in the overtly diabetic chimeras suggested the islet grafts were eliminated by a B cell specific DTH-like response that was not restricted by the MHC haplotype of the B cell target. Although insulinitis is present in NOD mice from weaning [17], NOD/Lt mice do not develop high titres of insulin autoantibodies until shortly before the onset of overt diabetes [18, 19], suggesting that immune responsiveness to insulin reflects underlying B cell necrosis and is not its cause. We were therefore curious whether the response of NOD/Lt T cells to B cell specific soluble antigens such as insulin increased as B cell necrosis progressed as a function of age. As shown in Table 5, T cells from 8-week-old NOD/Lt males failed to recognise the epitopes that distinguish porcine insulin from autologous murine insulin since, after priming with porcine insulin, T cells failed to respond when restimulated with porcine insulin in vitro (452 ± 126 cpm). In contrast, T cells from 16-week-old NOD/Lt male mice primed with porcine insulin proliferated strongly (11373 ± 1853 cpm) when restimulated with porcine insulin in vitro (Table 5). The blastogenic response in vitro of GAT primed T cells from 8 week (14759 ± 358 cpm) and 16-week (16537 ± 2290 cpm)-old NOD/Lt male mice was equivalent. These data suggested that the T cell response to porcine insulin found in older NOD/Lt mice may reflect a memory response of T cells previously primed to autologous murine insulin or its precursors (proinsulin, preproinsulin) as a consequence of B cell lysis in the pancreatic islets.

Discussion

The present findings extend previous observations that adoptive transfer of diabetes into a resistant F1 hybrid requires bone marrow from a homozygous NOD donor [13, 14]. In these previous studies employing NOD→F1 marrow chimeras, both NOD islets and semi-allogeneic islets (NON/Lt, B10) were acutely rejected when implanted into diabetic recipients. In the present study, a completely allogeneic strain (CBA/J) was used in hybrid formation with NOD/Lt in order to establish whether NOD/Lt marrow-derived effectors could recognise and eliminate target cells that did not share common MHC antigens. Although irradiated (NOD × CBA)F1 recipients of

NOD/Lt bone marrow developed overt diabetes characterized by severe insulinitis, the time of disease onset (19–56 weeks post-reconstitution) was greatly protracted when compared to the time of disease onset previously reported for NOD marrow reconstituted (NOD $\times$ NON)F1 and (NOD $\times$ B10)F1 hybrids (16–28 weeks and 12–28 weeks post-reconstitution respectively). This difference could be explained if a B cell specific CTL derived from NOD marrow was H-2D^b restricted. Since NOD, NON, and B10 mice all share a common H-2D^b antigen [11, 12], while CBA/J mice express H-2D^k antigen, the restriction element would presumably be expressed at a higher density on B cells of (NOD $\times$ NON)F1 or (NOD $\times$ B10)F1 hybrids than on B cells of (NOD $\times$ CBA)F1 mice. However, the acute rejection of CBA/J islet grafts, but not anterior pituitary grafts, in hyperglycaemic NOD $\rightarrow$ (NOD $\times$ CBA)F1 mice suggested activation of a population of B cell specific cytotoxic effectors not restricted by MHC molecules expressed on B cells. This conclusion is supported by the finding that at an age where diabetes was observed in NOD $\rightarrow$ (NOD $\times$ CBA)F1 chimeras, the response of peripheral T cells to the T dependent antigen GAT was restricted to the MHC haplotype of NOD and not CBA. Since T cells in these chimeras matured in an F1 thymic environment, it is possible that a population of T cells capable of responding to antigen in the context of the MHC haplotype of CBA would be present. However, at the age the chimeras were primed with GAT, the only type of antigen presenting cell present in the periphery was of NOD marrow donor origin. Thus, even if a peripheral T cell population had been positively selected in the F1 thymus by the *H-2^k* haplotype of CBA, these T cells would not respond following antigenic priming due to the absence of antigen presenting cells of the *H-2^k* haplotype in the periphery.

The findings of significant damage to CBA/J islets mediated by NOD marrow-derived effectors would appear to contradict an earlier study which indicated NOD mice rejected islet grafts through a MHC-restricted recognition mechanism [6]. Despite the variable survival of CBA islet grafts in cyclosporine-treated NOD mice, a MHC class I restricted pathogenesis was nevertheless proposed by Terada et al. [6] on the basis of less severe inflammation associated with the CBA vs NOD islet grafts. Although we observed ectopic islet grafts to be eliminated in a non-MHC restricted fashion, immunological effectors capable of eliminating islets in a MHC restricted fashion may still have initiated pathogenesis in the NOD $\rightarrow$ (NOD $\times$ CBA)F1 chimeras. While NOD/Lt and CBA/J islet grafts were acutely rejected in hyperglycaemic NOD $\rightarrow$ (NOD $\times$ CBA)F1 chimeras, islet graft survival was protracted if the recipients were normoglycaemic at the time of implantation. Interestingly, high glucose exposure increases autoantigen levels in islet cells in culture [20]. This could account for the observation in the current study of differential survival of CBA islet grafts in hyperglycaemic compared to normoglycaemic NOD $\rightarrow$ (NOD $\times$ CBA)F1 recipients.

It is possible that B cell necrosis may be initiated in the pancreas by NOD marrow derived effectors restricted by the MHC of the target B cell. The progression of B cell

necrosis initiated by these MHC restricted effectors may then result in the activation of additional effector population(s) capable of rejecting subsequent islet grafts by DTH-like responses, including cytokine-mediated damage, that are B cell specific but not genotype specific. Thus, the immunological mechanism(s) responsible for rejection of transplanted islets may differ from the mechanism by which B cell necrosis was initiated in the pancreas.

Although B cell specific DTH like effectors are not restricted by the MHC molecules expressed on the surface of the B cell target, the unique I-A gene product of NOD may be capable of presenting soluble B cell antigens that are not normally presented by the MHC class II gene products of other haplotypes. There is evidence for differential presentation of B cell specific antigens by different murine MHC haplotypes; for example, the response to heterologous insulin has been demonstrated to be under I-region gene control [21]. Interestingly, T cells from 8-week-old NOD/Lt mice that were primed with porcine insulin failed to respond when restimulated with porcine insulin *in vitro*, while T cells from 16-week-old NOD/Lt mice primed with the same antigen demonstrated strong proliferative responses following restimulation *in vitro*. Our data clearly demonstrate that the development of insulin reactive T cells in older mice is not simply due to a general increase in immune response levels since the responses to the T dependent antigen GAT were equivalent in 8- and 16-week old NOD/Lt male mice. Thus, the failure of T cells from young (8-week-old) NOD/Lt mice to respond following priming with porcine insulin suggests that T cells from NOD mice are incapable of recognizing the epitopes that distinguish porcine insulin from the structurally similar murine insulin molecule. Alternatively, the unique I-A gene product of NOD may not be able to present these particular epitopes to T cells. However, as B cell necrosis progresses in aging NOD mice, the data presented herein indicate that insulin-reactive T cells are generated that proliferate when presented with porcine insulin. The most likely explanation for this age-dependent phenomenon is that the responding T cells are actually memory T cells that have been primed *in situ* to autologous mouse insulins or their precursors as a consequence of the destruction of the pancreatic B cells potentially initiated by other mechanisms. These could include macrophage mediated cytotoxicity as well as CTL involvement [22]. The increased ability of NOD/Lt T cells to respond to porcine insulin with aging could also reflect a breakdown in suppressor activity. It has been demonstrated that insulin specific suppressor T cells (Ts) are activated in strains of mice non-responsive to porcine insulin, and that elimination of these Ts unmasked helper T cell activity to porcine insulin as well as autologous murine insulin [21]. NOD/Lt mice are defective in Ts activation [23]. However they are elicited, these insulin reactive T cells in turn probably provide the help required for the generation of insulin autoantibodies which also increase with aging in NOD mice [18, 19]. Thus, a T cell population which is B cell specific, but not genotype specific, could be activated as pancreatic B cell necrosis progressed.

The findings that the adoptive transfer of diabetes to young NOD mice requires both L3T4⁺ and Ly-2⁺ T cells from overtly diabetic mice [3, 4], while the elimination of L3T4⁺ T cells alone is sufficient to protect islet allografts in overtly diabetic NOD mice [8, 9] also supports the concept that the mechanism by which B cell necrosis is initiated in the pancreas of NOD mice may differ from the mechanism by which islet grafts are rejected. It was subsequently shown that the adoptive transfer of diabetes to young NOD mice with L3T4⁺ and Ly-2⁺ T cells from overtly diabetic mice could be blocked if the recipients were treated with either anti-L3T4 or Ly-2 monoclonal antibodies [24]. However, following diabetes induction by adoptive transfer of both T cell subsets from diabetic donors, islet allografts could be protected by treating the diabetic hosts with anti-L3T4, but not anti-Ly-2 monoclonal antibodies [24]. In view of the finding that both MHC class II restricted L3T4⁺ T cells and MHC class I restricted Ly-2⁺ T cells were required to initiate B cell destruction in an accelerated transfer model [3–5], it is tempting to speculate that NOD B cells may well be eliminated in a MHC restricted fashion *in situ*. However, once these effectors elicit sufficient levels of B cell necrosis, a population of L3T4⁺ T cells may be engendered that are capable of eliminating ectopic islet grafts of any genotype in a DTH-like fashion. As discussed above, if B cell necrosis in the pancreas of NOD mice is initiated by a MHC class I restricted effector population, this could explain the protracted disease onset in NOD→(NOD × CBA)F1 compared to NOD→(NOD × NON)F1 or NOD→(NOD × B10)F1 chimeras. This mechanism could also account for the present observation that insulin reactive T cells could not be generated in young NOD/Lt mice, but could be observed in older mice as B cell necrosis progressed.

It does not appear, however, that pancreatic B cell necrosis must proceed to the point that overt hyperglycemia is established in order to activate this population of B cell-specific but not genotype-specific immunological effectors. LaFace and Peck [25] observed peri-insular leucocytic infiltrates in 100% of B10.BR/cd (*H-2^k*) mice reconstituted with T cell-depleted NOD marrow. However, only 17% of these animals became overtly diabetic. These results were taken to indicate that a common B cell antigen could elicit an immune response from NOD marrow derived T cells, but that this response in itself is usually insufficient to result in overt diabetes [25]. This is consistent with the finding that although insulin autoantibody titres increase with aging, not all NOD mice developing insulin autoantibodies become overtly diabetic [18, 19]. Also, in the present study, the 16-week-old NOD/Lt males from which the insulin reactive T cells were isolated were not yet diabetic. This could account for our observation that although both NOD/Lt and CBA/J islet grafts remained structurally intact and stained strongly with aldehyde fuchsin following transplantation into normoglycaemic NOD→F1 chimeras, they were still associated with leucocytic infiltrates. The generation of a critical mass of effectors mediating acute islet graft elimination in a non-MHC restricted fashion may require that the destruction of B cells in the pancreas progress to the point

that overt hyperglycaemia is established. As described above, this may be initiated by an effector restricted by the MHC haplotype of the pancreatic B cell. This would imply that hyperglycaemic NOD→F1 chimeras treated with anti-L3T4 monoclonal antibodies should retain CBA/J, but not NOD/Lt islet grafts. The MHC class I restricted Ly-2⁺ T cell population that possibly initiated pancreatic B cell destruction should eventually eliminate NOD/Lt islets grafted into anti-L3T4 treated hyperglycaemic chimeras. This hypothesis is currently under investigation.

In conclusion, once pancreatic B cell necrosis has progressed to establishment of overt hyperglycaemia, NOD marrow derived immunological effectors, capable of eliminating subsequent islet grafts in a non-MHC restricted, but tissue specific fashion, are present. However, the data do not eliminate the possibility that effectors initiating pancreatic B cell necrosis *in situ* may be MHC restricted, whereas the elimination of ectopic islet grafts may reflect the consequences of their emplacement into a hyperimmune environment. Thus, the mechanism by which islet grafts are rejected may not necessarily reflect the mechanism by which B cells in the pancreas of NOD mice are eliminated.

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D. V. Serreze et al.: B cell destruction in NOD bone marrow chimeras

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