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Natural killer cell depletion and diabetes mellitus in the BB/Wor rat (revisited)

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Summary. The BB/Wor diabetes-prone rat is an animal model of human insulin-dependent diabetes mellitus. In this model of spontaneous autoimmunity, natural killer cells are candidate cytotoxic effector cells, believed to be the mediators of beta-cell cytolysis in vivo. We therefore studied the effects of an anti-natural killer cell monoclonal antibody on the spontaneous development of diabetes in the BB/Wor rat. The 3.2.3 monoclonal antibody recognizes a molecule present on rat natural killer cells and selectively depletes these cells in vivo. Chronic treatment of diabetic-prone rats with 3.2.3 monoclonal antibody cleared circulating phenotypic natural killer cells, depleted in vitro spleen natural killer cell function, and profoundly reduced intra-islet accumulation of $3.2.3^+$ cells, but did not prevent or delay the onset of diabetes. These results indicate that natural killer cells are not necessary for the development of spontaneous diabetes in BB/Wor rats.

Key words: Diabetes mellitus, autoimmunity, natural killer cells.

BB/Wor diabetes-prone (DP) rats develop spontaneous autoimmune diabetes mellitus between the ages of 50–120 days. Pancreatic beta-cell destruction is the culmination of a cell-mediated immune attack. The morphologic hallmark of beta-cell autoimmunity is a destructive lymphocytic insulitis which immediately precedes the onset of hyperglycaemia. It is characterized by the presence of numerous intra-islet Ia^+ (dendritic cells and macrophages), $CD5^+$, and $CD8^+$ cells [1, 2], and the hyperexpression of MHC class I protein in both endocrine and exocrine cells [3, 4]. We previously reported [5] the prevention of spontaneous diabetes by injections of anti-CD5 or anti-CD8 monoclonal antibodies (MoAbs), and suggested that lymphocytes bearing these cell-surface markers are essential components of the autoimmune attack.

In the rat, natural killer (NK) cells are phenotypically characterized as expressing 3.2.3, CD8, and asialoganglioside M1 (ASGM1) cell-surface markers, while lacking CD5 antigen. DP rats have very high numbers of $CD8^+$ NK cells (~12% of peripheral blood mononuclear cells) and a virtual absence of phenotypic and functional $CD8^+$ cytotoxic T lymphocytes (CTL) [6, 7]. The cytotoxic activity of NK ($CD8^+/CD5^-$) cells is also increased [8], and is responsible for lysing Yac-1 and islet cells in vitro [9, 10]. Furthermore, in vivo treatment of DP rats with anti-CD8 MoAb or ASGM1 anti-serum prevents recurrent diabetes [11, 12]. These observations support a direct role for NK cells in BB/Wor diabetes. However, ASGM1 and CD8 are present on both NK cells and CTL, and are thus not selective NK-cell markers. The recently described MoAb 3.2.3 recognizes a molecule present on rat NK, lymphokine-activated killer, and polymorphonuclear cells, but not T lymphocytes, and selectively depletes NK cells in vivo [13, 14]. With the availability of the 3.2.3 MoAb, we re-evaluated the pathogenic role of NK cells in BB/Wor autoimmune diabetes by the immune elimination of $3.2.3^+$ cells in vivo.

Materials and methods

Animals. All BB/Wor rats in this study were raised at the University of Massachusetts Medical School and have been inbred for more than 40 generations. DP rats were raised in a viral-antibody free environment and have an incidence of spontaneous diabetes of 75–95% between the ages of 50 and 120 days, with a mean age at onset of 80 days [15].

Monoclonal antibodies. OX-6 (anti-Ia), OX-8 (anti-CD8) and OX-19 (anti-CD5) MoAbs were produced by hybridoma cell lines from Dr. A.F. Williams and Dr. D.W. Mason (Oxford University, Oxford, UK). The 3.2.3 hybridoma was obtained from Dr. J.C. Hiserodt (Pittsburgh, Pa., USA). The hybridoma cell lines were grown under standard tissue culture conditions, and the undiluted tissue culture supernatants were used for animal injections and flow cytometry.

Experiment protocols. Litters of 25–30-day-old DP rats were randomized into groups and either untreated, or injected i.p. five

Table 1. Effect of monoclonal antibody injections on peripheral blood lymphocyte subset percentages

Group	NK cells[a] ($CD8^+/CD5^-$)	T_h cells[b] ($CD8^-/CD5^+$)	T_c cells[c] ($CD8^+/CD5^+$)	NK cells[d] ($3.2.3^+$)
No treatment ($n=49$)	12.6 ± 0.9	7.3 ± 0.4	0.8 ± 0.0	8.5 ± 0.9
3.2.3 treatment ($n=38$)	2.7 ± 1.7	9.0 ± 0.5	1.0 ± 0.0	0.2 ± 0.0
OX8 treatment ($n=35$)	0.2 ± 0.1	8.2 ± 0.4	0.0 ± 0.0	0.5 ± 0.0

The data were derived from animals bled (orbital) or killed (heart blood) after 1, 1.5, 2, 2.5, 3, 4, 4.5, 5, 6, 6.5 and 8 weeks of tissue culture supernatant injections. The lymphocyte subpopulation data were similar at each time and were therefore pooled. One-way analyses of variance were used to compare treatment groups.

[a] $p < 0.001$, no treatment vs 3.2.3-treated or OX8 treated rats. $p < 0.001$, 3.2.3 treatment vs OX8-treated rats.
[b] $p < 0.05$, no treatment vs 3.2.3-treated rats.
[c] $p < 0.001$, OX8-treatment vs no treatment or 3.2.3-treated rats. $p < 0.05$, no treatment vs 3.2.3-treated rats.
[d] $p < 0.001$, no treatment vs 3.2.3-treated or OX8-treated rats.

Table 2. Effect of monoclonal antibody injections on frequency of diabetes mellitus

No treatment	3.2.3 treatment	OX8 treatment
27 of 32 (84.4%)	19 of 26 (73.1%)[a]	3 of 19 (15.8%)[b]

Experimental animals were diabetes-prone BB/Wor rats of both sexes aged 25–30 days. Rats received daily (Monday–Friday) i.p. injections of monoclonal antibody or were untreated until they reached 120 days of age. Animals were killed when they became diabetic or reached 120 days of age. The data are the combined results of three separate experiments. The Fisher exact test was used to compare treatment groups.
[a] 3.2.3 treatment vs no treatment, NS. [b] OX8 treatment vs 3.2.3 or no treatment $p < 0.01$

times weekly with anti-CD8 (OX-8) or 3.2.3 MoAb tissue culture supernatants as follows: weeks 1 and 2, 1.0 ml i.p. per rat per day; weeks 3 and 4, 1.5 ml per rat per day; weeks 5–14, 2.0 ml per rat per day. Representative non-diabetic animals were killed after 4, 5, 8 and 10 weeks of injections for analysis of spleen lymphocytes. Animals were tested for glycosuria three times weekly. Diabetes was diagnosed on the basis of glycosuria (TesTape; Eli Lilly, Indianapolis, Ind., USA) and a blood glucose of 13.9 mmol/l or more in the tail blood, (Beckman Glucose Analyzer II; Beckman Instruments, Fullerton, Calif., USA). Diabetic animals were killed on the day the diabetes was diagnosed, and the remaining non-diabetic animals were killed at 120 days of age.

Flow cytometry. One and two-colour analysis of peripheral blood lymphocytes was performed as previously described [5].

Cytotoxicity assays. Yac-1 target cells were labelled with ^{51}Cr (Amersham Corp., Arlington Heights, Ill., USA), 0.1 mC/10^6 cells. Spleens were minced, lysed in 0.15 mol/l NH_4Cl, and plated in microtitre plates (flat-bottomed) at effector/target (E/T) ratios of 200:1, 100:1, 50:1 and 25:1 with 10^4 target cells/well. RPMI 1640 was added to target cells for spontaneous lysis and 1% Nonidet P40 for 100% lysis determinations. Assays were incubated for 4 h in a 37°C, 6.5% CO_2 in air incubator. Plates were centrifuged before harvest, and one-half of the supernatant collected for radioactive counting in a Beckman gamma 5500 counter (Beckman Instruments, Palo Alto, Calif., USA). Spontaneous release was always less than 15%. Percent specific ^{51}Cr release = 100 × [(cpm test sample) − (cpm medium control)]/[(cpm NP40 control) − (cpm medium control)].

Morphologic studies. Pancreata were fixed in Bouin's solution and embedded in paraffin. Haematoxylin-eosin stained sections were examined for the presence of lymphocytic insulitis.

Immunohistochemistry. Immunoperoxidase staining of pancreata with cell-type specific MoAbs was performed as previously described on cryostat sections of pancreata frozen in liquid-nitrogen-cooled isopentane [4].

Statistical analyses

One-way analyses of variance were used to compare the effects of various treatment regimens on peripheral blood lymphocyte subsets and NK-cell cytotoxic function. Comparison of the incidence of diabetes was performed using a Fisher's exact test. Calculations were performed with an IBM personal computer using SPSSx statistical software (SPSS, Inc., Chicago, Ill., USA).

Results

Effect of MoAb injections on peripheral blood lymphocyte subsets

DP rats were either not treated or injected five times weekly with 3.2.3 or anti-CD8 MoAb-containing tissue culture supernatants from the age of 25–120 days. At multiple time points, peripheral blood samples were obtained from all experimental groups and analysed for lymphocyte cell-surface markers by single and two-colour flow cytometry (Table 1). Rats treated with anti-CD8 had markedly reduced percentages of circulating $3.2.3^+$ NK, $CD8^+/CD5^+$ CTL, and $CD8^+/CD5^-$ NK cells. In contrast, 3.2.3-treated rats had less than 0.5% $3.2.3^+$ cells (vs 8.5% for untreated controls) and significantly reduced numbers of $CD8^+/CD5^-$ NK cells (1.0–4.4% vs 11.7–13.5% for untreated controls), but showed no decrease in the number of $CD8^+/CD5^+$ CTLs. $3.2.3^+$ cells were cleared after only 1 week of MoAb injections (data not shown). The concomitant and profound reduction of both $3.2.3^+$ and $CD8^+/CD5^-$ cells indicates that antigenic modulation was not responsible for the absence of circulating $3.2.3^+$ cells. Percentages of helper T cells ($CD8^-/CD5^+$) were not reduced by 3.2.3 or anti-CD8 MoAb in-

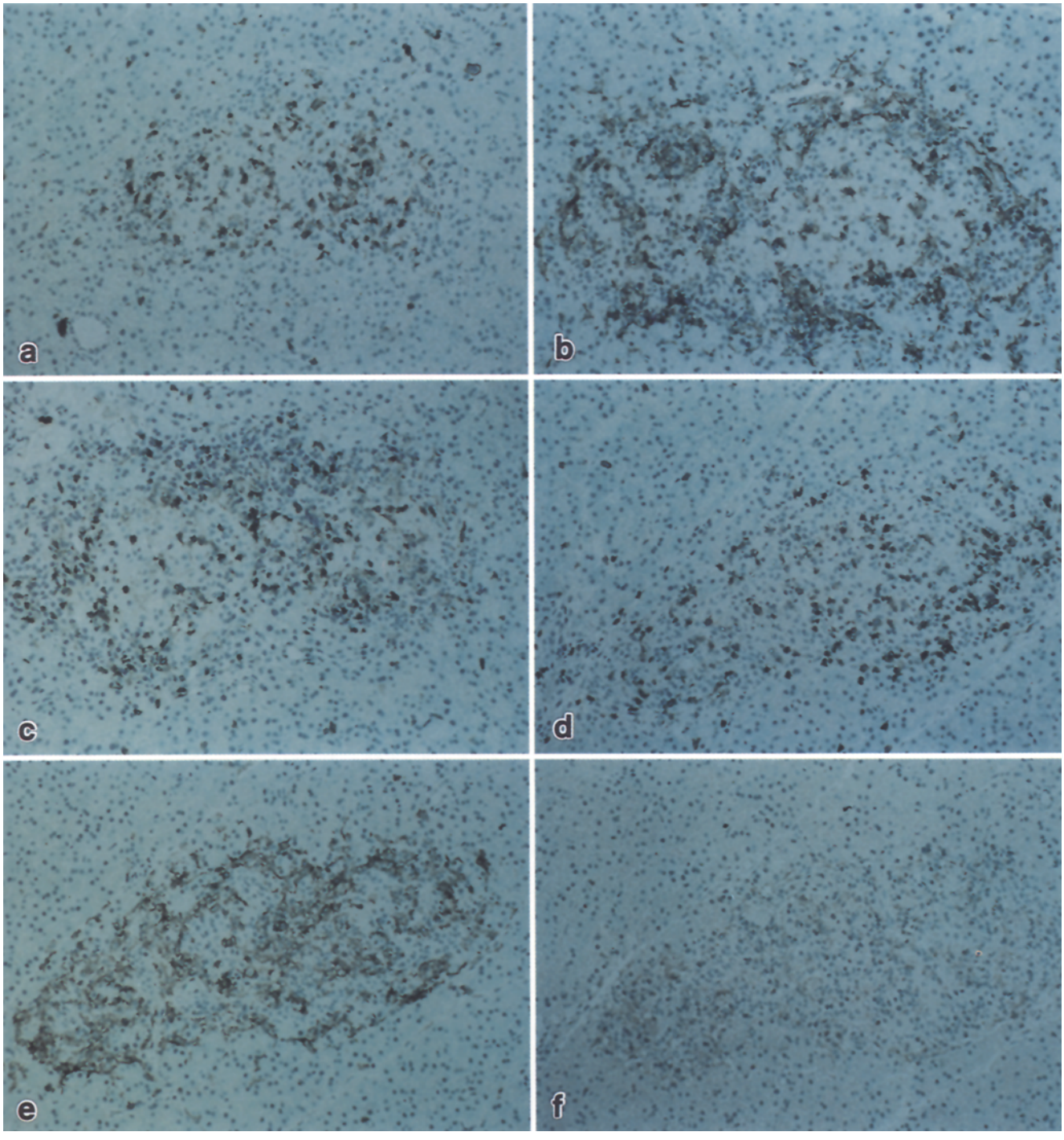

Fig. 1 a–f. Sections of pancreatic islets from acutely diabetic BB/Wor rats stained for CD5, CD8 and 3.2.3 cell-surface antigens. **a–c** Adjacent sections of an islet with acute insulitis from an untreated control rat. Numerous infiltrating lymphocytes are stained after incubation with CD5 (**a**), CD8 (**b**) and 3.2.3 (**c**). **d–f** Adjacent sections of an islet with acute insulitis from a 3.2.3-injected rat. Numerous infiltrating lymphocytes stain with CD5 (**d**) and CD8 (**e**), however, virtually no cells are stained after incubation with 3.2.3 (**f**). Magnification × 64 for all micrographs.

jections at any time point. Thus, chronic treatment with both 3.2.3 and anti-CD8 tissue culture supernatants depleted DP rats of phenotypic NK cells ($3.2.3^+$ and $CD8^+/CD5^-$). However, only anti-CD8 depleted treated rats of phenotypic CTLs ($CD8^+/CD5^+$).

Effect of MoAb injections on frequency of diabetes

The cumulative incidence of diabetes (at age 120 days) was 84% (27 of 32) in untreated controls, 73% (19 of 26) in 3.2.3-treated rats, and 16% (3 of 19) in anti-CD8 treated animals (Table 2). These data show that depletion

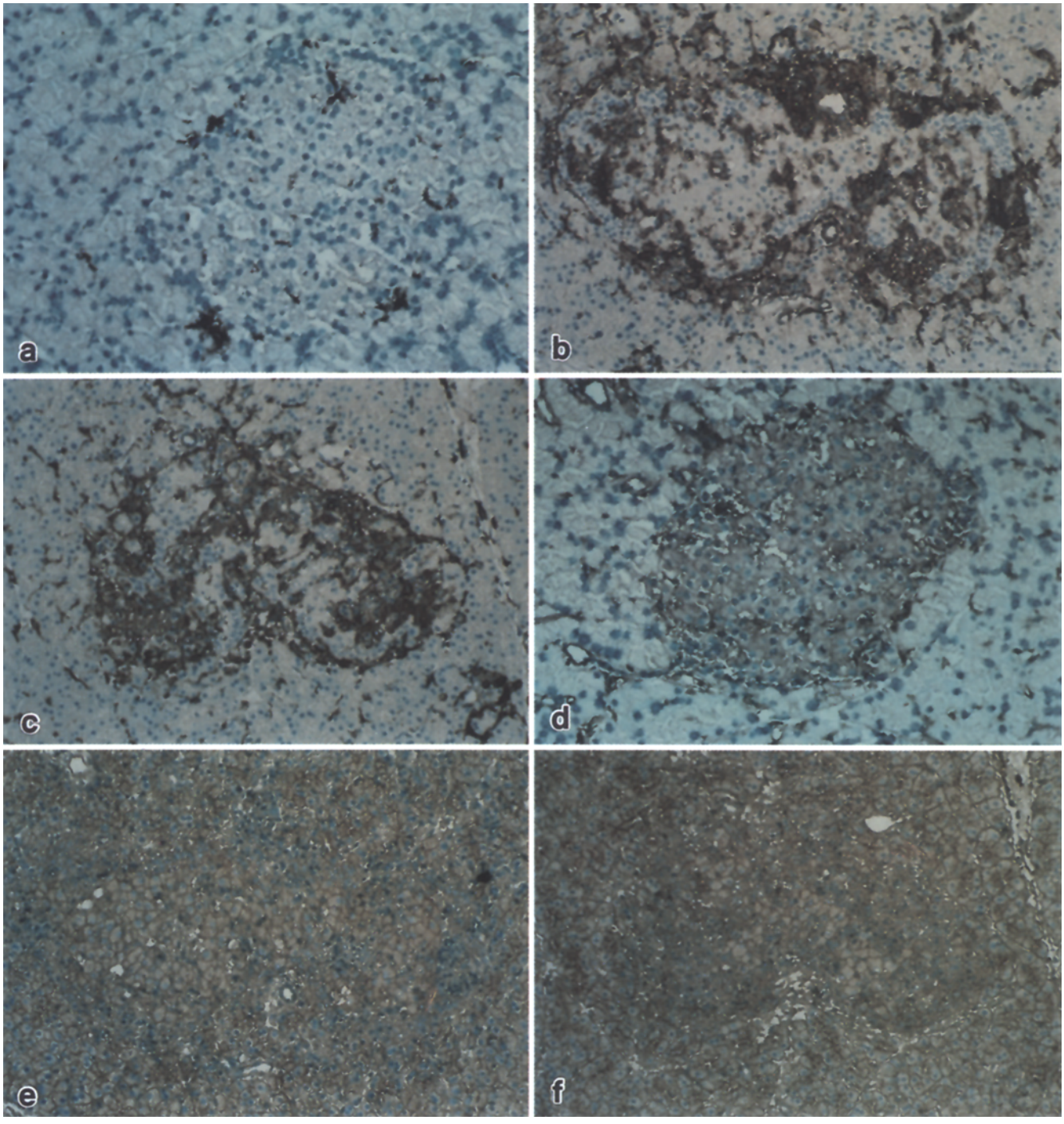

Fig. 2a–f. Pancreatic islet sections from normal and acutely diabetic BB/Wor rats stained for class II and class I MHC antigens with OX6 and OX18. **a** MHC class II antigen expression in a normal islet from a non-diabetic rat. Islets contain occasional OX-6 staining interstitial dendritic cells. **b** and **c** MHC class II antigen expression in islets with acute insulitis after incubation with OX6. (**b**) from untreated control; (**c**) from 3.2.3-treated rat. Islets are infiltrated with Ia^+ macrophages and dendritic cells which stain intensely after exposure to OX6. Islet and exocrine cells are negative for Ia. **d–f** MHC class I antigen expression after incubation with OX18. (**d**) illustrates MHC class I antigen expression in a normal islet from a non-diabetic rat. Low level of class I expression are present in islet cells, but not in exocrine cells. Class I antigen expression is markedly enhanced in islet cells and induced in surrounding exocrine cells of both untreated control (**e**) and 3.2.3 (**f**) treated rats. Magnification × 64 for all micrographs. MHC class I and II antigen expression was not altered by 3.2.3 antibody injections.

of $3.2.3^+$ cells by MoAb injections did not decrease the frequency of diabetes in DP rats. 3.2.3 treatment also did not delay the mean age at onset of diabetes (data not shown). In contrast, anti-CD8 injections significantly ($p < 0.01$) reduced the incidence of diabetes.

Effect of 3.2.3 MoAb treatment on insulitis

Pancreatic tissue sections from diabetic rats treated with 3.2.3 MoAb were compared with sections from untreated diabetic controls after staining with a panel of cell-type specific MoAbs (Fig. 1). Spontaneous diabetes in the

Table 3. Effect of monoclonal antibody injections on splenic natural killer cell activity

Group	200:1[a]	100:1[b]	50:1[a]	25:1[b]
No treatment ($n = 24$)	50.58 ± 2.66 %	39.14 ± 2.63 %	24.77 ± 1.90 %	13.29 ± 1.90 %
3.2.3 treatment ($n = 26$)	13.35 ± 1.88 %	9.42 ± 1.40 %	5.63 ± 0.87 %	3.19 ± 0.87 %
OX8 treatment ($n = 18$)	21.87 ± 2.53 %	15.6 ± 2.12 %	9.41 ± 1.22 %	5.32 ± 1.22 %

Spleen cells from untreated and monoclonal-antibody-treated non-diabetic rats were assayed for NK-cell activity after 2, 2.5, 4, 4.5, 5, 6, 6.5, 8 and 10 weeks. Activity was measured in a 4-h ^{51}Cr release assay against Yac-1 target cells. Results are illustrated as the percentage of specific ^{51}Cr release at effector:target ratios of 200:1, 100:1, 50:1, and 25:1. The results at each time period were similar and were therefore pooled. One-way analyses of variance were used to compare treatment groups.

[a] $p < 0.001$, no treatment vs 3.2.3 treated or OX8-treated rats. $p < 0.01$, 3.2.3 treatment vs OX8-treated rats.

[b] $p < 0.001$, no treatment vs 3.2.3-treated or OX8-treated rats. $p < 0.05$, 3.2.3 treatment vs OX8-treated rats.

BB/Wor rat is characterized, at the onset of hyperglycaemia, by a mononuclear cell infiltrate (insulitis) surrounding and infiltrating the pancreatic islets of Langerhans. The infiltrate is comprised of Ia^+ macrophages and dendritic cells, $CD5^+$ T cells, $CD8^+$ cells, and is accompanied by markedly enhanced MHC class I expression on islet and exocrine cells [3, 4]. In control untreated diabetic rats, the insulitis lesion consisted of Ia^+ macrophages and dendritic cells, $CD8^+$ cells, $3.2.3^+$ NK cells, and $CD5^+$ T cells (listed in order of decreasing frequency) (Fig. 1 a–c and Fig. 2 a). The inflammatory islet infiltrate of animals injected with 3.2.3 MoAb however, contained very few or no $3.2.3^+$ cells (Fig. 1 f). In contrast, 3.2.3-treated rats appeared to have normal numbers of Ia^+, $CD5^+$, and $CD8^+$ cells (Fig. 1 d, e and Fig. 2 b). These results demonstrate that 3.2.3 MoAb treatment greatly reduced the number of $3.2.3^+$ NK, but not $CD8^+$ cells, within the islet infiltrate. Both untreated and 3.2.3-treated diabetic rats showed a similarly amplified expression of MHC class I protein in the insulitis lesion and the surrounding exocrine tissue (Fig. 2 c, d). This is a morphologic hallmark of the autoimmune process in BB/Wor Type 1 diabetes.

Effect of MoAb injections on spleen NK-cell activity

Levels of splenic NK-cell lysis against Yac-1 targets were compared in non-diabetic animals from all treatment groups (Table 3). Assays for all groups were performed concurrently, under identical conditions, to facilitate comparisons. Splenic mononuclear leucocytes from untreated DP rats had characteristically high NK lytic activity, and mediated approximately 40% killing at E/T ratios of 100:1. Both 3.2.3 and anti-CD8-treated rats had significantly lower levels of splenic NK-cell killing at all E/T ratios ($p < 0.001$) and at all time points tested. The geometric mean lysis of Yac-1 targets at an E/T ratio of 100:1 was 9.4% for 3.2.3-treated rats, and 15.6% for anti-CD8-treated rats. Levels of NK-cell lytic activity in 3.2.3-treated rats were also significantly lower than those in anti-CD8-treated rats at all E/T ratios, and were similar to those seen in the diabetes-resistant (DR) line of BB/Wor rats. Spleen NK-cell killing in DR rats, at an E/T ratio of 100:1, is typically approximately 5% (data not shown).

Discussion

Chronic treatment of DP rats with 3.2.3 MoAb removed circulating phenotypic ($3.2.3^+$, $CD8^+/CD5^-$) NK cells, reduced in vitro spleen NK-cell function, and greatly diminished intra-islet accumulation of $3.2.3^+$ cells, while failing to protect against diabetes or delay its onset. These results demonstrate that spontaneous autoimmune diabetes in the BB/Wor rat can develop in the presence of greatly reduced numbers of $3.2.3^+$ cells. In contrast, anti-CD8-treated rats were profoundly depleted of both phenotypic NK cells and $CD8^+/CD5^+$ CTL, and were significantly protected against diabetes. Neither 3.2.3 nor anti-CD8 tissue culture supernatant injections reduced the percentages of circulating $CD8^-/CD5^+$ T-helper cells. Since 3.2.3-treated rats were not depleted of CTLs and did develop diabetes, the combined data suggest that $CD8^+$ CTL, rather than $CD8^+$ NK cells, are the beta-cell cytotoxic effectors in this animal model of Type 1 diabetes.

The efficacy of anti-CD8 treatment in the prevention of BB/Wor diabetes suggested a cardinal role for $CD8^+$ NK cells in the pathogenesis of this autoimmune syndrome [5]. In the lymphopoenic DP rat, $CD4^+$ T-helper cells are markedly reduced and the $CD8^+$ compartment is almost entirely comprised of phenotypic NK cells: 12.6% $CD8^+/CD5^-$ vs 0.8% $CD8^+/CD5^+$ cells. DP rats also appear to be entirely lacking in functional CTL [7]. The DR BB/Wor rats double their $CD8^+/CD5^+$ spleen-cell population 1 week post-infection with lymphocytic choriomeningitis virus (LCMV); the CTLs are virus-specific and restricted to the BB/Wor $RT1^u$ haplotype. LCMV-infected DP rats, however, do not generate increased numbers of spleen CTLs or detectable cytotoxic activity to LCMV-infected targets in vitro. DP rats also fail to generate CTL responses to Pichinde and vaccinia viruses [16]. The greater ability of DP spleen cells, as compared to DR BB/Wor spleen cells, to lyse Yac-1 and islet cells in vitro [9, 10] therefore suggest a direct role for NK cells in spontaneous BB/Wor diabetes.

The results reported here, however, demonstrate that NK cells are not a necessary element of the diabetogenic process: immune elimination of $3.2.3^+$ NK cells did not prevent or delay the onset of the clinical syndrome. Recently, other investigators reported similar findings after in vivo depletion of $3.2.3^+$ NK cells in BB/Wor rats [17]. It has previously been reported that anti-CD5 treatment of

DP rats completely prevents diabetes and insulitis [5]. Anti-CD5 treatment reduced numbers of both T-helper cells ($CD8^-/CD5^+$) and CTL, but did not have any effect on the levels of phenotypic NK cells. Similarly, an anti-CD2 MoAb (OX-34) prevents spontaneous diabetes in DP rats [18]. OX-34 injections profoundly deplete $CD4^+$ T cells, while sparing both $CD8^+$ NK cells and CTL. The combined data indicate that T lymphocytes, but not NK cells, mediate autoimmune diabetes in the BB/Wor rat.

The identity of the cytotoxic effector responsible for beta-cell cytolysis in vivo remains to be determined. Prevention of diabetes by anti-CD8 treatment indicates that the effector cell may be a classic CTL. In the DP rat, CTL are present in low, barely measurable percentages. They appear to be expanded within the insulitis lesion, however, and are present in 3.2.3-treated and NK-cell-depleted diabetic rats. Anti-CD8-treated rats, although protected from diabetes, have widespread islet infiltration, ranging from early-stage insulitis (~64% of rats), to advanced insulitis (~36% of rats). Significantly, those animals with early to moderate insulitis have no detectable $CD8^+$ cells within the lesion, while those rats with advanced insulitis have less than 5% $CD8^+$ cells within the infiltrate (M. Kurrer, A. A. Like, unpublished observation). Anti-CD8-treated rats, however, have normal numbers of islet-infiltrating $CD5^+$ cells and increased numbers of macrophages. The paucity of $CD8^+$ cells in the insulitis lesion of anti-CD8 protected rats suggests that $CD8^+$ CTL's are necessary for beta-cell cytolysis in vivo.

Since rodent beta cells do not express detectable MHC class II antigen [3, 4, 19, 20], but do express cell-surface MHC class I molecules, a $CD8^+$ CTL is most likely responsible for beta-cell cytolysis in vivo. Islet-cell MHC class I expression is also greatly enhanced during the course of autoimmune insulitis which further supports a cardinal role for $CD8^+$ CTL in BB/Wor diabetes. Although $CD4^+$ T cells appear to be involved in the inductive phase of diabetogenesis [21, 22], we do not believe that they would serve as the final effector cell in the absence of MHC class II-bearing target cells.

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