Deficiencies in Gut NK Cell Number and Function Precede Diabetes Onset in BB Rats

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Deficiencies in Gut NK Cell Number and Function Precede Diabetes Onset in BB Rats

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Defects in the intestinal immune system may contribute to the pathogenesis of autoimmune diseases. Intraepithelial lymphocytes represent a substantial fraction of gut-associated lymphocytes, but their function in mucosal immunity is unclear. A newly described population of NK cells that spontaneously secrete IL-4 and IFN-γ is present in the intraepithelial lymphocyte compartment of the rat. We hypothesized that defects in the number or function of these cells would be present in rats susceptible to autoimmunity. We report that the number of NKR-P1A⁺CD3⁻ intraepithelial NK (IENK) cells is deficient before onset of spontaneous autoimmune diabetes in diabetes-prone BB (BBDP) rats. The absolute number of recoverable IENK cells was only ∼8% of that observed in WF rats. Bone marrow transplantation from histocompatible WF donors reversed the IENK cell deficiency (and prevented diabetes) in these animals, suggesting a hemopoietic origin for their IENK cell defect. Analysis of diabetes-resistant BB rats, which develop autoimmune diabetes only after perturbation of the immune system, revealed IENK cell numbers intermediate between that of BBDP and WF rats. IENK cells were selectively depleted during treatment to induce diabetes. Prediabetic BBDP and diabetes-resistant BB animals also exhibited defective IENK cell function, including decreased NK cell cytotoxicity and reduced secretion of IL-4 and IFN-γ. IENK functional defects were also observed in LEW and BN rats, which are susceptible to induced autoimmunity, but not in WF, DA, or F344 rats, which are resistant. Defects in IENK cell number and function may contribute to the pathogenesis of autoimmune diseases including type 1 diabetes. The Journal of Immunology, 2004, 172: 5356–5362.

The role of intraepithelial lymphocytes (IELs) in immune regulation at mucosal surfaces remains incompletely understood, and the phenotype and function of IELs with characteristics of NK cells have only recently been described (9–11). Intraepithelial NK (IENK) cells are deficient in humans with celiac disease (9), but there is little or no information concerning the normal number or function of such cells. In mice, IENK cells kill intestinal epithelial cells in vitro in a perforin-dependent manner, an activity that is increased by IL-15 (10). It has been speculated that IENK cells activated by IL-15 may participate in mucosal immunity by killing infected intestinal epithelial cells (10). In the rat, IENK cells comprise a significant fraction of the total IEL compartment, particularly in young animals. The percentage of IENK cells ranges from ∼50% of total IELs at 4 wk of age to ∼17% at 16 wk of age and older (11). We have shown that rat IENK cells are different from splenic NK cells in two respects (11). First, IENK cells express high levels of ADP-ribosyltransferase 2 (ART2) and CD25, which are nearly undetectable on splenic NK cells. Expression of ART2 on peripheral rat T cells is a marker of regulatory capability (12, 13), as is expression of CD25 on a population of peripheral mouse T cells that can prevent diabetes in nonobese diabetic mice (14). Second, unlike splenic NK cells that secrete little or no IL-4 and secrete IFN-γ only in response to stimulation (15), a substantial fraction of unstimulated rat IENK cells spontaneously secrete IL-4 and/or IFN-γ (11). Taken together, these data suggest that IENK cells may contribute to immune regulation at the level of the gut epithelium.

Diabetes-prone BB (BBDP) rats are severely T cell lymphopenic and spontaneously develop autoimmune diabetes (16). Several lines of evidence implicate the gut immune system in the...
pathogenesis of this disorder. For example, hydrolyzed casein-containing diets reduce the incidence of spontaneous diabetes whereas both gluten-rich diets and orally administered insulin appear to accelerate the disorder (17). Interestingly, increased permeability of the gut has been described in BBDP rats (18) as it has in children with type I diabetes (19). Diabetes-resistant BB (BBDR) rats are nonlymphopenic and never become spontaneously diabetic when housed in a viral Ab-free environment (16). However, BBDR rats do become diabetic in response to several immunomodulatory perturbants; these include infection with Kilham rat virus, depletion of ART2.1 regulatory T cells, and/or immune system activation with polyinosinic-polycytidylic acid (poly(I:C)) (16), now known to be a ligand for Toll-like receptor 3 (20). How defects in the gut immune system may contribute to diabetes pathogenesis in BB rats remains poorly understood.

We now report that, in comparison with normal WF rats, the total number of IELs is deficient before diabetes onset in both BBDR rats and in BBDR rats treated to induce the disease. In both cases, the deficiency in IELs was associated with a marked reduction in 1) the number of IENK cells, 2) NK-sensitive YAC-1 cell cytotoxicity, and 3) the number of cells in the intraepithelial compartment that spontaneously secreted IL-4 and/or IFN-γ. The data suggest that defects in gut immunity may predispose to systemic autoimmunity and diabetes in the BB rat.

Materials and Methods

Animals

BBDR/Wor and BBDP/Wor rats were obtained from BRM Incorporated (Worcester, MA). ACI, BN, Copenhagen, DA, F344, LEW, Sprague Dawley, and WF rats were obtained from Harlan Sprague Dawley (Indianapolis, IN). Animals of either sex were used in all experiments. All animals were certified to be serologically free of Sendai virus, pneumonia virus of mice, simian adenovirus, rat corona virus, Kilham’s rat virus, H1 (Toolan’s virus), mouse poliovirus (GD7), Reo-3, Mycoplasma pulmonis, lymphocytic choriomeningitis virus, mouse adenovirus, Hantaan virus, and Encephalitozoon cuniculi. All animals were housed in a viral Ab-free facility until used and maintained in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animals Resources, National Research Council, National Academy of Sciences, 1996) and the guidelines of the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

Antibodies

Fluorochrome- or biotin-conjugated mAbs directed against γδTCR (clone V65), CD3 (clone G4.18), NKR-P1A (clone 10/78), and RT7.2 (clone V65), CD3 (clone G4.18), NKR-P1A (clone 10/78), and RT7.2 (clone H9253/H9254) were prepared for use in flow cytometry as described (21). For measurement of IL-4-secreting cells by ELISPOT assay, paired mAbs (puriﬁed by Torrey Pines Biolabs (La Jolla, CA) and biotinylated as described from Torrey Pines Biolabs (La Jolla, CA) and biotinylated as described (22). Isotype control mouse IgG1, IgG2a, IgG2b, and IgG2b, secondary Ab (anti-rat IgG), and alkaline phosphatase-conjugated streptavidin were purchased from BD PharMingen.

Treatment of BBDR rats with reagents that induce diabetes

BBDR rats 25–28 days of age were treated with i.p. injections of DS4.23 anti-ART2.1 mAb (50 μg five times weekly) plus poly(I:C) (5 μg/100 g body weight three times weekly) as described (23, 24); BBDR rats treated in this way uniformly develop autoimmune diabetes (23, 24). Rats in the present study were treated for only 1 wk, less than the minimum time required for diabetes onset (25), and then killed for experimental analysis. Cell preparation and bone marrow transplantation

IELs were prepared from rat small intestine by Percoll density gradient centrifugation as described (26), with minor modifications. Briefly, rats were killed in an atmosphere of 100% CO2. Single cell suspensions were prepared by gentle extrusion through stainless steel sieves into medium consisting of cold RPMI 1640 medium supplemented with 5% Fetalco I as described (26, 27). Spleen cell preparations were washed in medium and erythrocytes were lysed in a hypotonic buffer (0.15 M NaCl, 1.0 mM KHCO3, 0.1 mM Na2EDTA, pH 7.4).

Cells for bone marrow transplantation were prepared from 6-wk-old donor rats as described (28). Bone marrow recipients 6 wk of age were treated with 7 Gy of gamma radiation (≈0.1 Gy/min) using a 137Cs source (Gammacell 40; Atomic Energy of Canada, Ontario, Canada) and immediately injected with 25 × 106 bone marrow cells via a lateral tail vein.

Flow cytometry

One, two, and three-color flow cytometric analyses were performed as described (11). Cells were analyzed using a FACSscan instrument (BD Biosciences, Sunnyvale, CA). Analyses were gated on lymphoid cells, which were identified by forward and side light-scatter profiles. A minimum of 25,000 events was analyzed for each sample.

Cytotoxicity assay

Cytotoxic activity of lymphoid populations was measured by 51Cr-release microcytotoxicity assay as described (11), with minor modifications. Briefly, YAC-1 target cells in growth phase were labeled with 51 Cr as follows: 105 target cells were incubated with 2 μCi of 51Cr (New England Nuclear, Boston, MA), and 1.0 × 103 51Cr-labeled cells were added to each well of a 96-well microtiter plate. Unfractionated IELs were added at E:T cell ratios ranging from 10:1 to 50:1, and the plates were incubated for 4 h at 37°C in a humidified atmosphere of 95% air-5% CO2. All assays were performed in triplicate and averaged. The calculated IENK to target cell ratio was determined by multiplying the IEL to target cell ratio for each data point by the percentage of NKR-PA+CD3+ IELs present in an aliquot of the sample as determined by flow cytometry.

Total releasable radioactivity (“cpmmax”) was determined by incubating an aliquot of 51Cr-labeled target cells with 1% Triton X-100. After incubation, cells were pelleted by centrifugation, and 100-μl aliquots of supernatant were transferred to a separate microtiter plate containing 100 μl of Optiphase Supermix β-scintillation fluid (Wallac, Gaithersburg, MD) and counted (“cpmmax”) using a 1450 Microbeta TriLux instrument (Wallac). In all assays reported here, release of 51Cr in the absence of effector cells (“cpm spontaneous”) was <15% of total 51Cr release. Specific cytotoxicity was calculated as a percentage using the raw cpm and the formula: specific lysis (%) = ([cpmtest - cpm spontaneous]/cpmmaximal - cpm spontaneous) × 100%.

Cytokine analyses

Spontaneous production of IL-4 and IFN-γ by rat IELs was measured by ELISPOT assay as described (11). The optimum number of IELs per well was determined in preliminary experiments and ranged from 2.5 to 20 × 105. The number of discrete spots visible in each well was counted using a light microscope at 2× power. Each cytokine assay was performed using four to six wells, with all wells but one assayed using the cytokine-specific developing Ab. The remaining well was assayed using the irrelevant biotinylated control Ab. For analysis, the number of spots observed in the control well for each assay was subtracted from the number of spots counted in each of the three to five test wells. The maximum number of spots observed in any control well was always ≤3. The adjusted number of spots present in each of the test wells was then averaged.
IEL numbers are reduced in BB rats before onset of diabetes

We first quantified the number of IELs recoverable from 4- to 6-wk-old nondiabetic WF, BBDR, and BBDP rats. The numbers recovered from unmanipulated BBDP rats and WF rats were statistically similar (Table I, groups 1 and 3, p = NS). The number of IELs recovered from BBDR rats was significantly less (Table I, group 2, p < 0.001); the same was true of the number of IELs recovered from BBDR rats that had been treated with anti-ART2.1 mAb plus poly(I:C) for 1 wk (Table I, group 4, p < 0.001). The numbers of IELs recovered from BBDP and treated BBDR rats were statistically similar (p = NS). One week represents approximately half the time required to induce autoimmune diabetes in BBDR animals (23, 24). None of the animals were diabetic, and differences in numbers of IELs among WF, BBDR, and BBDP rats were therefore not a consequence of diabetes per se.

The rat D54.23 anti-rat ART2.1 mAb depletes most peripheral ART2.1+ T cells (24), and nearly 100% of IELs in the rat are ART2+ (26). However, as shown in Fig. 1, most residual IELs in BBDR rats treated with anti-ART2.1 mAb plus poly(I:C) for 1 wk continued to express high levels of ART2.1. By incubating freshly isolated IELs from treated BBDR rats with the secondary FITC-conjugated anti-rat mAb alone, we observed that some anti-ART2.1 mAb from the treatment protocol was residually bound to the surface of IELs recovered from these animals (Fig. 1D). Consistent with previous reports (30), 1 wk of treatment appeared to deplete peripheral (lymph node) ART2.1+ lymphocytes completely; there was no evidence that residually bound Ab was masking the Ag on these cells (Fig. 1, A and C).

IELK cell numbers are selectively reduced in BB rats before onset of diabetes

It has been reported that the number of intestinal γδ T lymphocytes is extremely low in BBDP rats (31). We next tested whether a selective deficiency in γδ T cells fully accounts for the reduced numbers of IELs observed in prediabetic BB rats. As shown in Table II, we confirmed that both the number and percentage of intraepithelial γδ T cells in BBDR rats (group 2) are decreased in comparison to WF rats (group 1) and untreated BBDR rats (group 3). Interestingly, the absolute number of γδ-TCR+ IELs in BBDR rats did not change following 1 wk of anti-ART2.1 mAb plus poly(I:C) treatment (group 4), and the percentage of these cells was actually increased in treated animals. These data suggest that a selective deficiency in an IEL subpopulation other than intraepithelial γδ T cells might better account for the reduced total IEL numbers seen in prediabetic BB rats.

NKR-P1A+CD3+ IELK cells are particularly interesting because these cells comprise a major fraction of the IEL compartment in normal WF rat gut and may have an immunoregulatory function (11). Consistent with previous observations (11, 26), ~50% of IELs in 4- to 6-wk-old WF rats expressed the NKR-P1A+CD3+ IELK phenotype (Table II). Percentages of NKR-P1A+CD3− IELK cells in five other rat strains with no known susceptibility to autoimmunity (F344, DA, Sprague Dawley, ACI, and Copenhagen rats) were 53.5 ± 2.5%, 45.1 ± 3.4%, 31.0 ± 2.5%, 28.4 ± 11.2%, and 22.0 ± 4.6%, respectively. In contrast, only ~12% of IELs in age-matched BBDR rats expressed the IELK phenotype (p < 0.02 vs nondiabetic prone strains), and the absolute number of recoverable IELK cells in these animals was only ~8% of that observed in WF rats (Table II).

Both the percentage and number of IELs expressing the IELK phenotype in age-matched, untreated, nondiabetic BBDR rats were less than half that observed in WF controls (Table II). Unexpectedly, both the percentage and number of IELK cells in the IEL

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Table I. IEL numbers in WF and BB rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Strain</th>
<th>Treated</th>
<th>IELs (×10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>WF</td>
<td>No</td>
<td>6.8 ± 1.5 (6)⁵</td>
</tr>
<tr>
<td>2</td>
<td>BBDR</td>
<td>No</td>
<td>2.2 ± 0.8 (4)⁵</td>
</tr>
<tr>
<td>3</td>
<td>BBDR</td>
<td>Yes</td>
<td>8.7 ± 2.3 (8)⁵</td>
</tr>
<tr>
<td>4</td>
<td>BBDR</td>
<td>Yes</td>
<td>3.3 ± 0.4 (4)⁵</td>
</tr>
</tbody>
</table>

¹ IELs from 4- to 6-wk-old WF and BB rats were isolated and counted as described in Materials and Methods. BBDR rats were either untreated or treated for 1 wk with anti-ART2.1 mAb plus poly(I:C). Each data point represents the mean ± SD; the number of independent determinations is indicated in parentheses. No rats were diabetic at 4–6 wk of age. Overall ANOVA was significant at the p < 0.001 level (F₃,₃₈ = 17.9).

⁵ p = NS vs each other and p < 0.001 vs BBDR rats and treated BBDR rats.

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FIGURE 1. Frequency of ART2.1+ peripheral lymphocytes and IELs in BBDR rats following administration of anti-ART2.1 mAb plus poly(I:C). Lymph node lymphocytes and IELs from 4- to 6-wk-old BBDR rats were prepared as described in Materials and Methods. Rats were either untreated (upper panels) or treated for 1 wk with anti-ART2.1 mAb plus poly(I:C) (lower panels). Purified cells were reacted with anti-ART2.1 mAb (rat IgG₂b isotype), secondary FITC-conjugated anti-rat IgG₂b mAb (mouse IgG₂b isotype), and isotype control mAbs and then analyzed by flow cytometry. For each representative histogram shown, fluorescence intensity (horizontal axis) is plotted against cell number (vertical axis). Solid lines indicate fluorescence associated with specific staining by anti-ART2.1 mAb plus secondary FITC-conjugated anti-rat IgG₂b mAb. Dashed lines depict fluorescence associated with staining of control samples as indicated. In D, note that the curves for rat IgG₂b plus FITC anti-rat IgG₂b (dashed line) and FITC anti-rat IgG₂b alone (dotted line) are overlapping, indicative of the presence of residual anti-ART2.1 mAb from the treatment protocol on these cells “masking” their surface ART2.1. Similar results were obtained in at least two additional independent trials for each group.

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compartment of BBDR rats treated with anti-ART2.1 mAb plus poly(I:C) for 1 wk were reduced >90% in comparison to untreated control BBDR rats (Tables II and III).

We next sought to determine whether the dramatic reduction in the absolute number of NKR-P1A<sup>+</sup>CD3<sup>+</sup> IENK cells observed in BBDR rats following 1 wk of treatment with anti-ART2.1 mAb plus poly(I:C) was restricted to this population or occurred in other lymphocyte populations as well. As shown in Table II, the number of intraepithelial γδ T lymphocytes is similar in untreated and treated BBDR rats. As shown in Table III, the numbers of splenic NKR-P1A<sup>+</sup>CD3<sup>+</sup> NK cells in untreated and treated BBDR rats are also similar to each other (p = NS). In addition, the number of NKR-P1A<sup>+</sup>CD3<sup>+</sup> intraepithelial T lymphocytes in BBDR rats was unchanged by 1 wk of anti-ART2.1 mAb plus poly(I:C) treatment, and NKR-P1A<sup>+</sup>CD3<sup>+</sup> splenic T cells were reduced by ~40% (Table III).

NKR-P1A<sup>+</sup>CD3<sup>+</sup> NK-T cells comprise a relatively small fraction of the total IEL population in rats (11). Consistent with this observation, the percentage of intraepithelial NK T cells in 4- to 6-wk-old untreated animals was 5.2 ± 0.7% in WF rats (n = 4), 2.6 ± 0.8% in BBDR rats (n = 12, p < 0.001), and 1.4 ± 0.2% in BBDP rats (n = 4, p < 0.001 vs both WF and BBDR). These differences in percentage were paralleled by differences in the absolute number of NKT cells present in the IEL preparations, but these numbers were all very low, ranging from 330 × 10<sup>3</sup> cells in WF rats to 210 × 10<sup>3</sup> in BBDR rats to 30 × 10<sup>3</sup> in BBDP rats.

**Intraepithelial NK cell cytotoxic activity is reduced in BB rats before onset of diabetes**

We next tested the hypothesis that the residual population of IENK cells in prediabetic BB rats would exhibit reduced functionality. To do so, we measured specific lysis of NK-sensitive YAC-1 cells incubated with unfractionated IELs freshly isolated from 6-wk-old WF, BBDR, and BBDP rats. Consistent with previous reports (11), IELs from WF rats exhibited efficient YAC-1 cell killing (Fig. 2A).

YAC-1 specific lysis was consistently less in IELs from BBDP rats, untreated BBDR rats, and BBDR rats treated for 1 wk with anti-ART2.1 mAb plus poly(I:C).

Essentially all YAC-1 cell killing activity in the IEL compartment of rats is associated with the IENK cell subpopulation (11). Using flow cytometry, we determined that NKR-P1A<sup>+</sup>CD3<sup>+</sup> IENK cells comprised 36% of IELs in WF rats, 11% in BBDP rats, 14% in untreated BBDR rats, and 2% in treated BBDR rats. With this information, we reanalyzed the data in Fig. 2A using calculated IENK:target cell ratios based on these percentages (see Materials and Methods). The analysis (Fig. 2B) suggests that, on a per cell basis, the YAC-1 cell killing activity of IENK cells from WF rats is high, that of BBDP rat IENK cells is low, and that of IENK cells from untreated and treated BBDR rats is intermediate.

**Spontaneous secretion of IL-4 and IFN-γ is reduced in BB rats before onset of diabetes and in other rat strains susceptible to autoimmunity**

Unstimulated rat IELs spontaneously produce IL-4 and/or IFN-γ, an activity entirely attributable to the NKR-P1A<sup>+</sup>CD3<sup>+</sup> IENK cell subpopulation (11). We next tested the hypothesis that the residual population of IENK cells in prediabetic BB rats would exhibit reduced cytokine production. To do so, we performed ELISPOT analyses. As shown in Fig. 3 (*upper panel*), we confirmed that unstimulated IELs isolated from 4- to 6-wk-old WF rats spontaneously produce both IL-4 and/or IFN-γ. In contrast, very few IELs from age-matched BBDP rats or untreated BBDR rats produced either cytokine. Treatment of BBDR rats with anti-ART2.1 mAb plus poly(I:C) further reduced the number of IL-4 and/or IFN-γ-producing IELs in these animals.

Because these data were obtained from total IEL populations, we also measured the percentage of IENK cells present in the IEL populations tested (Fig. 3, *lower panel*). Inspection of the two panels of the figure shows that, as would be expected from previous

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**Table II. IEL subpopulations in WF and BB rats**

<table>
<thead>
<tr>
<th>Group</th>
<th>Strain (n)</th>
<th>Treated</th>
<th>% of recoverable IELs</th>
<th>Absolute number (×10&lt;sup&gt;6&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>WF (4)</td>
<td>No</td>
<td>14.5 ± 2.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.0 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>BBDR (4)</td>
<td>No</td>
<td>5.3 ± 2.0</td>
<td>0.1 ± 0.02&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>BBDR (12)</td>
<td>No</td>
<td>29.0 ± 5.7</td>
<td>2.5 ± 1.0&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>BBDR (8)</td>
<td>Yes</td>
<td>46.3 ± 6.2</td>
<td>2.1 ± 0.6&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> IELs from 4- to 6-wk-old WF and BB rats were isolated and prepared for flow cytometry as described in Materials and Methods. BBDR rats were either untreated or treated for 1 wk with anti-ART2.1 mAb plus poly(I:C), and cells were analyzed for surface expression of γδ TCR, NKR-P1A, and CD3 as described in Materials and Methods. Data are expressed as the mean ± SD. The number of independent determinations is indicated in parentheses.

<sup>b</sup> All groups differ from each other at the 0.05 level. 

<sup>c</sup> All groups differ from each other at the 0.001 level.

<sup>d</sup> NS vs untreated group.

<sup>0.01</sup> p = 0.001 vs untreated group.

<sup>e</sup> p < 0.002 vs all other groups.

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**Table III. IEL and splenic lymphocyte numbers in BBDR rats treated with anti-ART2.1 mAb plus poly(I:C)**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treated</th>
<th>NKR-P1A&lt;sup&gt;+&lt;/sup&gt;CD3&lt;sup&gt;+&lt;/sup&gt; IELs (×10&lt;sup&gt;6&lt;/sup&gt;)</th>
<th>NKR-P1A&lt;sup&gt;+&lt;/sup&gt;CD3&lt;sup&gt;+&lt;/sup&gt; Splenic Lymphocytes (×10&lt;sup&gt;6&lt;/sup&gt;)</th>
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<tr>
<td></td>
<td></td>
<td>NKR-P1A&lt;sup&gt;+&lt;/sup&gt;CD3&lt;sup&gt;+&lt;/sup&gt;</td>
<td>NKR-P1A&lt;sup&gt;+&lt;/sup&gt;CD3&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>BBDR</td>
<td>No</td>
<td>1.58 ± 0.64</td>
<td>0.08 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>BBDR</td>
<td>Yes</td>
<td>5.73 ± 2.03</td>
<td>4.16 ± 1.43&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.05 ± 0.23</td>
<td>0.90 ± 0.54&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>34.26 ± 16.07</td>
<td>20.81 ± 5.35&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> IELs and splenic lymphocytes from 4- to 6-wk-old BBDR rats were isolated and prepared for flow cytometry as described in Materials and Methods. Rats were either untreated or treated for 1 wk with anti-ART2.1 mAb plus poly(I:C), and lymphocytes were analyzed for surface expression of NKR-P1A and CD3 as described in Materials and Methods. Data are expressed as the mean ± SD. The number of independent determinations for each group was at least four.

<sup>b</sup> p < 0.001 vs untreated group.

<sup>c</sup> p = NS vs untreated group.
reports (11), the number of cells producing IL-4 and/or IFN-γ correlated with the percentage of IENK cells present.

To determine whether the apparent deficiencies in IENK cell numbers and cytokine production are unique to BB rats, we studied four other rat strains. IELs were isolated from 4- to 6-wk-old F344, BN, DA, and LEW rats and analyzed for both the percentage of IENK cells present and the number of cytokine-producing cells (Fig. 3). WF, F344, and DA rats were similar with respect to the percentage of IENK cells (45–54%) and the relatively high number of IELs that spontaneously produced IL-4 (47–119 spots/10^5 IELs) and/or IFN-γ (33–94 spots/10^5 IELs). Both the percentage of IENK cells and the number of cytokine-producing IELs in these three strains were much greater than in any of the BB rat groups. Fig. 3 also shows that BN and LEW rats were similar to each other with respect to the number of IELs producing IL-4 and/or IFN-γ. These numbers were much lower than in WF, F344, and DA rats and comparable to the numbers observed in BB rats. The percentage of LEW IELs that were IENK cells (18%) was similar to that in untreated BBDR rats (17%), whereas the percentage of BN IELs that were IENK cells (33%) was higher, but still not as high as that observed in WF, F344, or DA rats.

The **IELN** cell deficiency in BBDDP rats is the result of a hemopoietic defect

We next sought to determine whether reduced IENK cell number and function in BBDDP rats results from defects in hemopoietic progenitors or defects in the microenvironment of the host. To do so, we performed reciprocal bone marrow transplantations between 6-wk-old BBDDP and WF rats and analyzed the animals 6 wk later. It is already known that transplantation of WF bone marrow prevents diabetes in BBDDP rats (32). The RT7 allotypic marker was used to distinguish donor-origin cells from residual recipient cells. We have previously reported that the percentage of NKR-P1A^+/CD3^− IENK cells present in the WF rat IEL compartment declines with age, decreasing from 50% in 4-wk-old animals to 17% in animals 16 wk of age and older (11). In the present study, we observed that WF bone marrow appeared to generate an IEL compartment in BBDDP rats similar to that which would be expected (11) for a 12-wk old WF rat with respect to both the number of IELs and the percentage of IENK cells (Table IV, group 5). The number and percentage were comparable to those observed in both irradiated WF rats transplanted with syngeneic WF bone marrow (group 2) and in unmanipulated 12-wk-old control WF rats (group 1). In contrast, transplantation of BBDDP bone marrow into irradiated WF recipients failed to generate a normal IEL compartment of BB rat origin (group 6). Although the number of IELs recovered was high, most of these IELs were of WF origin (RT7.2^+/CD3^−), and the percentage of IENK cells was similar to that in control BBDDP rats (Table IV, groups 3 and 4).
Table IV. Lymphocyte populations in bone marrow transplanted rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Donor</th>
<th>Recipient</th>
<th>Intestinal IELs</th>
<th>Bone Marrow Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Yield (×10⁶)</td>
<td>% IENK</td>
</tr>
<tr>
<td>1</td>
<td>None</td>
<td>WF</td>
<td>7.6 ± 2.8</td>
<td>10.6 ± 4.6</td>
</tr>
<tr>
<td>2</td>
<td>WF</td>
<td>WF⁺</td>
<td>8.0 ± 1.4</td>
<td>9.0 ± 1.3</td>
</tr>
<tr>
<td>3</td>
<td>None</td>
<td>BBDP</td>
<td>1.4 ± 0.7</td>
<td>1.0 ± 0.6</td>
</tr>
<tr>
<td>4</td>
<td>BBDP</td>
<td>BBDP⁺</td>
<td>1.4 ± 0.7</td>
<td>1.4 ± 0.5</td>
</tr>
<tr>
<td>5</td>
<td>WF</td>
<td>BBDP</td>
<td>5.0 ± 1.4</td>
<td>8.2 ± 1.3</td>
</tr>
<tr>
<td>6</td>
<td>BBDP</td>
<td>WF</td>
<td>5.9 ± 1.9</td>
<td>2.1 ± 0.5</td>
</tr>
</tbody>
</table>

* WF (RT7.2⁺) and BBDP (RT7.1⁺) rats 6 wk of age were randomized and either not treated further (groups 1 and 3) or irradiated and transplanted with either WF or BBDP bone marrow as described in Materials and Methods (remainder of groups). When 12 wk of age, all rats were killed, and IELs and bone marrow lymphocytes were isolated, quantified, and analyzed by flow cytometry. Each data point represents the mean ± 1 SD for four individual animals.

* No paired comparisons between the syngeneic WF→WF transplanted hosts and nontransplanted WF controls are statistically significant.

* No paired comparisons between the syngeneic BBDP→BBDP transplanted hosts and nontransplanted BBDP controls are statistically significant.

* p < 0.05 vs BBDP→WF chimeras. No other paired comparisons between the two allochimeric groups are statistically significant.

Discussion

These data document major deficiencies of intraepithelial NK cell number and function before the onset of diabetes in spontaneously diabetic BBDP rats and in BBDR rats that are treated to induce the disease. A less pronounced but nonetheless significant reduction in IENK cell number and function was also observed in unmanipulated BBDR rats but not in WF rats. Differences in IENK cell number and function among BBDP, BBDR, and WF rats were not a consequence of diabetes per se because none of the rats were diabetic when studied.

In the BBDP rat, autoimmune diabetes occurs spontaneously but the animals are T cell lymphopenic due to a mutation in the *Iaα* gene (33, 34). Our data reveal that the lymphopenia observed in the periphery of BBDP rats extends to the IEL compartment in general and to the IENK subpopulation in particular. Our analysis of bone marrow chimeras demonstrates that the BBDP rat’s deficiency in IELs and IENK cells, like its deficiency in peripheral T cells (35), is intrinsic to hemopoietic stem cells.

However, lymphopenia is not a characteristic of human autoimmune diabetes, calling into question the suitability of the BB rat as a model for this disease. For this reason, we performed parallel analyses in the BBDR rat, which does not share the lymphopenia mutation of the BBDP rat but remains susceptible to the induction of diabetes as a function of other shared genetic loci (25, 36, 37). We document that these animals have normal numbers of IELs, but even before treatment to induce diabetes, they appear to be deficient in IENK cell number and function. YAC-1 cytotoxic activity, IL-4 production, and IFN-γ production in BBDR rat IELs are lower than in normal WF rat IELs, although higher than that in BBDP rat IELs. Based on our previous analyses of the IEL compartment of the rat (11), it is likely that these deficiencies are largely, if not entirely, due to deficiencies in the IENK subpopulation of IELs. Treatment to induce diabetes in BBDR rats using the well-characterized combination of anti-ART2.1 mAb plus poly(I:C) to perturb the immune system (16) was associated with further reductions in IENK cell number, cytotoxic activity, and cytokine production. Interestingly, the treatment protocol appeared to reduce NK cells in the IEL compartment but not the spleen. These data are all consistent with the hypothesis that abnormal IENK cell activity may be one of the underlying immunological defects in BB rats.

The constellation of NK-like phenotype and function, together with an unusual cytokine profile—constitutive expression of IL-4 and IFN-γ in the local microenvironment of the gut—indicate that intraepithelial NK cells are specialized cells that may subserve gut-specific functions. Given the present data, it is plausible to speculate that ART2.1⁺CD25⁺ rat IENK cells could play a role in the processes that regulate both the “physiologic inflammation” that is characteristic of the gut generally (1) and the genetic predisposition to autoimmunity in the BB rat specifically. Consistent with this hypothesis, we have also documented the existence of relatively deficient IENK cell number and cytokine production in two unrelated rat strains, LEW and BN, which are also susceptible to autoimmune diabetes (BN, susceptible to HgCl₂-induced nephritis (38), and LEW, susceptible to experimental allergic encephalomyelitis (39)). These deficiencies were not observed in three strains, WF, F344, and DA, which have little or no predisposition to autoimmunity. The specific genetic and/or environmental factors that contribute to the deficiency of IENK cell number and function in rats susceptible to autoimmunity are unknown.

BBDP rats are reportedly deficient in intraepithelial γδ TCR⁺ lymphocytes (31), but the relationship of this deficiency to diabetes pathogenesis is unclear. We have confirmed the earlier observation in BBDP rats, but additional data from the BBDR rat suggest that a deficiency in the γδ TCR⁺ IEL subpopulation is not associated with diabetes expression. BBDR rats treated to induce diabetes have similar absolute numbers of γδ TCR⁺ IELs as do untreated animals, suggesting that the prediabetic state in BBDR rats is not characterized by reduced numbers of γδ TCR⁺ IELs. That the percentage but not the absolute number of IELs expressing the γδ TCR in treated BBDR rats is increased likely results from the selective loss of IENK but not other IEL populations.

Based on the data presented here and the well-recognized polygenic nature of type 1 diabetes in both humans (40) and BB rats (25, 36), we hypothesize that diabetes in the BB rat is a multistep process. The process begins with a genetically determined bone marrow-derived defect in regulatory gut IENK cells. The ensuing defect in immunoregulation at the level of the intestinal mucosa leads to abnormalities in gut-draining mesenteric lymph node cells, an inference supported by preliminary studies in which we have documented that cells capable of the adoptive transfer of autoimmunity in BBDR rats appear in mesenteric lymph nodes before their appearance elsewhere in the periphery (R. Bortell, unpublished observations). Finally, in animals like the BB rat, in which additional susceptibility genes are present, the abnormal mesenteric lymph node populations proceed to induce insulitis and β cell destruction. This hypothesis, if proven correct, could help explain the linkage of environmental perturbation in the gut with abnormal peripheral immune responses against β cells in BB rats (16), nonobese diabetic mice (41), and humans (42) with autoimmune diabetes.
Acknowledgments

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References