Insulin Autoantibodies Are Associated With Islet Inflammation But Not Always Related to Diabetes Progression in NOD Congenic Mice

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Susceptibility to diabetes in humans and nonobese diabetic (NOD) mice is believed to arise from the combined effect of multiple genetic loci, resulting in immunemediated destruction of the insulin-secreting β-cells. Insulin autoantibodies (IAAs) are often present in humans for years, and in NOD mice for weeks, before the onset of diabetes. We have evaluated the expression of IAAs in NOD mice and in diabetes-resistant NOD congenic strains to characterize the association of autoantibody expression with insulitis and diabetes. In NOD congenic strains with genes that contribute to protection from insulitis and diabetes (Idd3, Idd5, Idd10, and Idd18), the prevalence of IAAs is reduced relative to NOD mice. In contrast, NOD.B10 Idd9 mice have a high prevalence of IAAs and a high degree of insulitis, despite a nearly complete resistance to diabetes. These data indicate that IAA expression is a phenotype that is associated with insulitis and correlates with overall disease progression in some strains of congenic mice but not in others. It is likely that patients with type 1 diabetes will also show non-major histocompatibility complex genetically determined variation in expression of autoantibodies and progression to diabetes. Diabetes 52:882–886, 2003

Over the past two decades, progress has been made in our understanding of the immunopathogenesis and natural history of type 1 diabetes. Type 1 diabetes is believed to be a chronic disease, since in most cases the disease develops after years of expression of anti-islet autoantibodies and loss of insulin secretion (1). The anti-islet autoantibodies most often measured are reactive to GAD65, ICA512 (IA-2), and insulin. The presence of at least one of these autoantibodies can be found in ~90% of newly diagnosed patients (2), and for insulin autoantibodies (IAAs), high titers and early appearance are associated with an early age of disease onset (3,4).

It is noteworthy that although type 1 diabetes is considered a T-cell-mediated autoimmune disease, both B-cells and transplacent autoantibodies contribute to the pathogenesis (5,6). Although the predictive value of IAAs in humans is high, it is not 100%, and in many cases the progression to diabetes following IAA expression is variable. For example, some individuals develop diabetes in <1 year following the onset of β-cell humoral autoimmunity, whereas others may develop diabetes only after many years or may never progress at all (7). It is not known whether this phenotypic variation in IAA expression is genetically determined or whether environmental influences are responsible for this immunologic response. If certain genetic loci limit the development of diabetes at a stage subsequent to lymphocytic invasion of the islets (termed, insulitis), then anti-islet autoantibodies may be poor immunologic predictors of diabetes in the subset of patients with these protective loci. The availability of animal models carrying diabetes susceptibility and protective alleles has enabled us to test the association of IAA expression with insulitis and diabetes progression.

The NOD mouse is the most extensively studied rodent model of spontaneous type 1 diabetes, and genetic analyses have led to the refinement of >15 susceptibility loci for diabetes. Whereas the greatest genetic influence for type 1 diabetes susceptibility in the NOD mouse and in humans lies in the major histocompatibility complex (MHC), non-MHC genes also play an important role in regulating autoimmunity. A series of NOD genetic loci (e.g., Idd3, Idd10, Idd18, Idd5.1, and Idd5.2) have been reported to regulate susceptibility to development of both insulitis and diabetes, since replacement of these individual susceptibility loci with nondiabetes prone C57BL/6 (B6)- or B10-derived loci limits the degree of insulitis and diabetes incidence (8–11). The replacement of other genetic loci, such as Idd9.1, Idd9.2, and Idd9.3, with B10-derived loci has no major quantitative effect on the development of insulitis at the gross histological level but dramatically restricts the progression to diabetes (12).

Our analysis of IAAs in several well-characterized NOD congenic strains exhibiting various degrees of protection from diabetes suggests that the expression of IAAs is a phenotype that is under complex genetic control and that a high frequency and high level of IAAs may occur in the absence of diabetes progression. The identification of

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Received for publication 16 July 2002 and accepted in revised form 25 November 2002.

IAA, insulin autoantibody; MHC, major histocompatibility complex; mIAA, micro-IAA.
genetic loci capable of limiting diabetes progression from insulitis in humans will be critical for the correct assignment of type 1 diabetes risk.

RESEARCH DESIGN AND METHODS

Mice. NOD/MrkTacIFR (NOD) mice were purchased from Taconic Farms (Germantown, NY). The NOD congenic strains used in this study were derived as previously reported (Table 1) (9, Germantown, NY). The NOD congenic strains used in this study were derived from the NOD.B10 strain (9) and were introgressed into the NOD background (14). Like the previously described NOD/Idd3 Idd5 strain (9), this refined Idd3/Idd5 strain displays a very low frequency of diabetes (2/100 females diabetic by 7 months of age). The NOD congenic strains are abbreviated in the text according to the control locus or loci (either B6 or B10) that is introgressed onto the NOD background (e.g., NOD.B6 Idd9 is abbreviated as Idd9). Mice were evaluated in a cross-sectional fashion at 8–10, 13–15, and 21–34 weeks of age. Only female mice were studied, and all mice were housed under specific pathogen-free conditions. All NOD congenic mice used in this study are available through the Taconic Farms Emerging Models Program.

Histologic analysis. The pancreata of mice were removed and fixed in 10% buffered formalin and processed for paraffin embedding. Tissue sections (5 μm) of each pancreas were stained with hematoxylin and eosin and examined microscopically for the presence of mononuclear cell infiltrates in the islets. Two noncontiguous longitudinal sections of each pancreas were examined. The degree of insulitis for each pancreas sample was recorded as follows: 0, no mononuclear cell infiltrate in the islets; 1, mild insulitis, <20% of islets have infiltrates; 2, moderate insulitis, 20–60% of islets have infiltrates; 3, severe insulitis, affecting most islets; and 4, extensive insulitis, completely infiltrated islets and/or residual islets.

Micro-IAA assay. IAA analysis was performed using a competitive protein A/G based radioimmunoassay (micro-IAA [mIAA] assay) as described previously (4). In brief, human 125I-insulin (Amersham) of 20,000 cpm was incubated with 5 μl serum (in the presence or absence of cold human insulin) in a 1:5 dilution of serum overnight at 4°C. Immunoprecipitation of the anti-insulin autoantibodies was performed by adding 50% protein A and 8% protein G-Sepharose (Pharmacia) on a MultiScreen-NOB 96-well filtration plate (Millipore), and radioactivity was counted on a TopCount 96-well plate β-counter (Packard). All of the serum samples were measured in duplicate, and a mean counts per minute was calculated. The final result, expressed as an index, was calculated based on the difference in counts per minute (Δ cpm) between the well without cold insulin and the well with cold insulin: index = (sample Δ cpm – negative control Δ cpm)/(positive control Δ cpm – negative control Δ cpm). A positive IAA measurement is set at the upper limit of normal (0.01) chosen as the 99th percentile in 106 healthy control subjects. The inter-assay coefficient of variation was 11% (n = 8).

Statistical analysis. Fisher’s exact test was used to compare the cumulative frequencies of IAA positivity among the NOD congenic mice with control NOD mice. Comparisons resulting in P < 0.05 were considered to be significant. The Wilcoxon’s rank-sum test was used to compare the positive IAA indexes and the Student’s t test was used to compare insulitis severity.

RESULTS

IAA-positive diabetes-resistant congenic mice have levels of autoantibody that are equivalent to NOD mice. Figure 1 summarizes the incidence of IAs in all of the mice examined in the study and also details the IAA index of the NOD and NOD congenic mice at 8–10 weeks of age. Although the incidence of IAs in the congenic strains varied widely (Figs. 2 and 3), there was no statistically significant difference in the positive IAA indexes between NOD mice and the diabetes-resistant strains at 8–10 weeks of age (Fig. 1) or at 13–15 and 21–34 weeks of age (data not shown). These data suggest that the absolute level of IAs is not associated with protection from diabetes.

The prevalence of IAs is associated with islet inflammation. Anti-islet autoantibodies are believed to be important markers of immune cell activation against

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**TABLE 1**

<table>
<thead>
<tr>
<th>Strain (genes in interval)</th>
<th>% Type 1 diabetic</th>
<th>Chromosome</th>
<th>Candidate genes</th>
<th>Reference (strain name in reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOD</td>
<td>80</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Idd9 (Idd9.1/9.2/9.3)</td>
<td>3.7</td>
<td>4</td>
<td>Cd30, Tnfr2, Cd137</td>
<td>(12) NOD.B10 Idd9R28</td>
</tr>
<tr>
<td>Idd5</td>
<td>40</td>
<td>1</td>
<td>Cd28, Cd152 (Cita4), 1cos1</td>
<td>(9) NOD.B10 Idd5R44</td>
</tr>
<tr>
<td>Idd3/Idd5 (Idd3/5.1/5.2)</td>
<td>&lt;3</td>
<td>1 and 3</td>
<td></td>
<td>Unpublished (see RESEARCH DESIGN AND METHODS)</td>
</tr>
<tr>
<td>Idd3/Idd10/18</td>
<td>5–9</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Idd3</td>
<td>25</td>
<td>3</td>
<td>Il2, Il21, Fgf2</td>
<td>(13) NOD.B6 Idd3R450</td>
</tr>
<tr>
<td>Idd10/18</td>
<td>50</td>
<td>3</td>
<td>Csfm, Cd53, Kcna3, Rap1α</td>
<td>(11) NOD.B6 Idd10R62</td>
</tr>
</tbody>
</table>
of diabetes (4). In agreement with this, age) was strongly associated with the early development of IAA expression in NOD mice (at 8 weeks of age was associated with the prevalence of insulitis in animals with diabetes-resistant genes that protect from both insulitis and diabetes. To test the hypothesis that IAA expression is associated with the prevalence of insulitis in animals with genetic susceptibility to diabetes, we measured IAAs in the sera of NOD and NOD congenic mice and examined their genetic susceptibility to diabetes, we measured IAAs in the pancreatic islets of NOD and NOD congenic mice at 8 weeks of age was strongly associated with the early development of diabetes (4). In agreement with this finding was a highly significant reduction in IAA prevalence (relative to NOD mice) at 8–10 weeks of age for five of six of the diabetes-resistant NOD congenic strains having genes that protect from insulitis and diabetes (Fig. 2A). At this time point, only the Idd5 strain had a prevalence of IAAs that was not statistically significantly lower than that in NOD mice (48% positive [n = 21] vs. 70% positive for NOD, P = NS). Interestingly, the IAA prevalence declined with time in the Idd5 strain and was markedly reduced relative to NOD mice by 13–15 weeks (17%, P = 0.0002) and 21–34 weeks (23%, P = 0.01) (Fig. 2A). The majority of NOD mice at 8–10 weeks of age were positive for IAAs (70%, n = 20) compared with only 24% of Idd3 mice (n = 38, P = 0.002), 0% of Idd3/5 mice (n = 12, P = 0.0001), 27% of Idd10/18 mice (n = 15, P = 0.02), and 17% of Idd3/10/18 mice (n = 6, P = 0.05). With the exception of Idd3 and Idd10/18 mice, there were similar statistically significant reductions in the percent IAA positive among the NOD congenic strains at 13–15 and 22–34 weeks of age. In the Idd3 and Idd10/18 strains, there was a delayed appearance of IAAs, and although the frequency of IAA positivity appears to be reduced at 13–15 and 21–34 weeks, the decrease did not reach statistical significance.

At all three time points examined, NOD mice exhibited a high prevalence of IAAs. In contrast, whereas almost all NOD mice had insulitis at 8–10 weeks of age (16 of 20 females), the severity was significantly less at 8–10 weeks than at 13–15 weeks (P < 0.0001) and 21–34 weeks of age (P = 0.0001). One diabetes-protected strain, Idd3/5, had a highly significant reduction of IAAs compared with that of NOD mice at all three time points (P < 0.0001 for each). The Idd3/10/18 and Idd5 strains were protected from insulitis at 13–15 and 21–34 weeks, times by which many of the NOD mice develop severe insulitis (P values for these four comparisons ranged from <0.0001 to 0.015). The congenic strains Idd3 and Idd10/18 had significant protection from insulitis only at 13–15 weeks (P < 0.0001 and 0.006, respectively). Thus, in the strains of mice having diabetes-resistant genes that protect against insulitis most completely, Idd3/5 and Idd3/10/18, IAAs are rarely detected. When mice have fewer insulitis and diabetes protective genes, such as the Idd3, Idd5, and Idd10/18 strains, IAAs are more frequently observed, albeit with different patterns and prevalence than those in the NOD parental strain.

Idd9 mice have a delayed IAA response that is significantly lower than the IAA prevalence of NOD mice (n = 10, 20 vs. 70% in NOD mice, P = 0.05), but it becomes essentially equivalent to the prevalence of NOD at 13–15 weeks (n = 10, 80%, P = NS) and is similar at 21–34 weeks.

**FIG. 2.** IAAs and insulitis in diabetes-resistant strains having genes that protect from both insulitis and diabetes. A: Percent positive for IAAs among NOD and NOD congenic mice at 8–10, 13–15, and 21–34 weeks. B: The mean histology score and the standard error is plotted at the three ages tested.
Expression of IAAs correlates with diabetes frequency in NOD congenic strains with genetic resistance to insulitis. To further evaluate the importance of IAAs as an immunologic predictor of type 1 diabetes, we have examined the correlation between IAA prevalence and diabetes frequency in NOD mice and the subset of NOD congenic strains having genes that protect from insulitis in addition to diabetes. The correlation of diabetes incidence (determined at 7 months) with IAA positivity in these mice was statistically significant at 8–10 weeks ($r = 0.91, P < 0.05$) and at 13–15 weeks ($r = 0.87, P < 0.05$) (data not shown). Because the genetic protection in Idd9 mice appears to occur at a stage subsequent to the development of insulitis and IAAs, these mice were excluded from the correlation analysis.

DISCUSSION

Type 1 diabetes is an immune-mediated disease in which the expression of IAAs are believed to be predictive of the risk of diabetes progression and age at onset of disease (3,4). The finding of anti-IAAs in patients who do not progress to type 1 diabetes, “nonprogressors,” may relate to the presence of genetic loci providing protection at a stage subsequent to the development of insulin (similar to the Idd9 mice). In these patients, anti-IAAs may be poor immunologic predictors of diabetes. Idd9 mice have a high incidence and titer of IAAs in association with a high degree of insulitis but are genetically resistant to diabetes (12). The insulitis in these mice is associated with a cytokine profile that is skewed toward a Th2 “protective” phenotype (i.e., interleukin-4 dominant). We evaluated the IgG subclass of the IAAs in the Idd9 mice to determine whether it was consistent with a Th2-dominant response and found no difference compared with control NOD mice, which predominantly have IgG1 IAAs. Of note, we have found that this resistance to diabetes is not reversed with cyclophosphamide treatment (data not shown). It remains to be determined what genetic mechanism underlies this potent resistance to diabetes.

Despite significant effects on IAA prevalence, the protective loci studied here did not appear to have a significant effect on the IAA index of “IAA positive” mice. Analyzing only IAA positive sera, the range of IAA titers was similar among the NOD and NOD congenic mice, and there was no statistically significant difference between the IAA titers. It may be that the timing of IAA appearance is more important than the peak levels for predicting diabetes risk. This may relate to the extremely high risk for children developing IAAs before age 3 (4). An alternative but not mutually exclusive hypothesis is that IAA positivity in the Idd9 mouse and in the “nonprogressors” is associated with a specific yet very slow β-cell destructive process, such that diabetes may occur only after a long follow-up period. In this case, the very slow loss of β-cells may not reveal itself in the form of overt diabetes until years later and thus death from an unrelated cause may occur before the development of diabetes. The data shown here supports the concept that the prevalence of IAAs in NOD mice is related to the prevalence of insulitis in the pancreas, and analysis of congenic-inbred mice indicates that IAAs can be dissociated from diabetes (e.g., Idd9).

As it is important to advance our understanding of the immunopathogenesis of type 1 diabetes and to enhance prediction in individuals with genetic risk, it is equally important to predict which autoantibody-positive individuals will not progress to diabetes. Thus, more analyses of the genetic regulation of IAA expression and the genetic determinants of diabetes progression need to be performed, and the information gained may be useful when deciding which individuals should be targeted to prevent trials.

ACKNOWLEDGMENTS

This study was supported by grants DK-32083 and DK-32493 and a grant from the Children’s Diabetes Foundation at Denver. L.S.W. is supported by a joint grant from the Juvenile Diabetes Research Foundation (JDRF) and the Wellcome Trust. The availability of NOD congenic mice through the Taconic Farms Emerging Models Program has been supported by grants from the Merck Genome Research Institute, the National Institute of Allergy and Infectious Diseases, and the JDRF.

We thank Dongmei Miao, MD, for expert technical assistance and Lorena Jaramillo for assistance with the data analysis.

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