Investigation of the Role of B-Cells in Type 1 Diabetes in the NOD Mouse

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B-cells are important in the development of type 1 diabetes, but their role is not completely defined. Although B-cells produce autoantibodies, these are not thought to be pathogenic; however, their antigen-presenting function is postulated to be critical. To examine the relative importance of these functions of B-cells, we have generated nonobese diabetic (NOD) B-cell–deficient mice that express a transgene encoding a mutant heavy chain immunoglobulin transgene on the cell surface but cannot secrete immunoglobulins (mIgs). This allowed us to dissect the importance of the relative roles of antigen presentation, dissociated from antibody production. We found that the expression of the mgl transgene increased insulitis and the incidence of diabetes compared with transgene-negative NOD B-cell–deficient mice, indicating that the ability to produce antibodies is not necessary for B-cells to have some effect on the development of diabetes. However, diabetes was not restored to the level seen in normal NOD mice. This may relate to reduced ability to activate an islet-specific T-cell repertoire, presumably due to the reduced islet-specific B-cell repertoire. Our results implicate a specific antigen-presenting function for B-cells. Diabetes 53:2581–2587, 2004

Autoimmune diseases such as type 1 diabetes involve the interaction of different subsets of lymphocytes and antigen-presenting cells (APCs), as in immune responses against pathogens. These responses involve both CD4 and CD8 T-cells responding to antigens presented by B-cells, macrophages, and dendritic cells. Much work has been done to investigate the role of T-cells in type 1 diabetes. The actual part that B-cells play in the development of diabetes, however, is not precisely known. Many studies have shown that autoantibodies are present in pre-diabetic and newly diagnosed patients with diabetes. These include antibodies to proteins such as insulin (1), the 64K protein/GAD (2,3), and the tyrosine phosphatase IA2 (insulinoma-associated protein 2)/ICA 512 (islet cell antibody 512) (4–6). Combinations of autoantibodies are known to be highly predictive of future development of disease in relatives of patients with type 1 diabetes, especially when susceptibility genes to the disease are present as well (7). However, in spite of this, a question remains as to whether autoantibodies are pathogenic in this disease.

To study the role of B-cells in the nonobese diabetic (NOD) mouse model of diabetes, NOD mice have been crossed to mice deficient in B-cells by gene targeting (μMT<sup>−/−</sup>) such that the mice have arrested development of B-cells and cannot express or secrete IgM (8). These mice have a very low incidence of diabetes (9,10) and have a delay and reduction in the severity of insulitis (11,12). Similarly, mice treated with anti-IgM antibody show no insulitis or diabetes compared with mice treated with a control antibody (13). However, B-cells are not absolutely required for diabetes to occur because NOD,μMT<sup>−/−</sup> mice can in rare instances develop diabetes (14,15). In addition, when T-cells from NOD,μMT<sup>−/−</sup> mice develop in lymphocyte-deficient NOD.SCID mice, these T-cells can expand rapidly, and an increased incidence of diabetes is observed (14).

It is clear that B-cells are important APCs. Although B-cells do not present antigens as efficiently as dendritic cells, they bind antigen specifically via cell surface immunoglobulin and thus can present soluble proteins much more effectively than B-cells that do not bear specific receptors, and the specificity of the immunoglobulin directs processing of the protein (16). Antigen-specific B-cells may therefore be very important in diversifying the immune response, presenting sequestered antigens to CD4 T-cells (17). Antigen-specific T-cells are able to activate antigen-specific B-cells, and these can then stimulate T-cells to break self-tolerance (18,19). These antigen-specific B-cells may particularly downregulate presentation of immunodominant epitopes and increase presentation of cryptic epitopes as shown by experiments using GAD-specific B-cell lines (20). It has been postulated (21,22) that B-cells in the NOD mouse may have a preferential ability to present β-cell autoantigens such as GAD. Further, it has also been suggested that I-A<sup>β7</sup>–mediated antigen presentation on B-cells is important (23). In these experiments using bone marrow chimeras, I-A<sup>β7</sup> was deficient in the B-cell compartment, but not in other APCs. However, there was also a change in the major histocompatibility complex class I region in the I-A<sup>β7</sup>–deficient

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†Dr. Charles A. Janeway, Jr., sadly passed away before the submission of this manuscript. He fully endorsed the work and contents of this study.

APC, antigen-presenting cell; KLH, keyhole limpet hemocyanin; LPS, lipopolysaccharide.

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B-cells, and, therefore, it cannot be concluded that reduced diabetes is solely due to lack of I-A^d on B-cells. Removal of maternally produced antibodies prevents NOD mice from developing diabetes (24). It is possible that soluble islet-specific antibodies could indirectly promote disease either by enhancing antigen presentation by dendritic cells or by causing tissue damage that releases islet cell antigens. Furthermore, B-cells could play roles other than as APCs, for example, by promoting normal lymphoid architecture and follicular dendritic cell formation or cytokine secretion (25,26). Although the studies thus far do, on balance, suggest that the role of B-cells relates to their function as APCs rather than in the production of autoantibodies, this has not been directly tested.

To do this we generated NOD transgenic mice that express an IgM heavy chain transgene that could only be expressed on the surface of B-cells but unable to secrete expressed on the surface of B-cells but unable to secrete antibody (mIg). This allowed us to investigate restoration of B-cells in B-cell–deficient mice and to try to dissect the importance of antigen presentation compared with the autoantibody-producing function of B-cells. The hypothesis was that if the mIg transgenic mice developed diabetes to a greater extent than the transgene-negative NOD,µMT^−/− mice, then the ability to secrete antibody is not necessary for the production of diabetes.

RESEARCH DESIGN AND METHODS

NOD/Caj mice were used for all backcrosses. µMT^−/− mice lacking B-cells (8) were originally obtained from Klaus Rajewsky and backcrossed for 14 generations onto NOD/Caj mice and are designated NOD,µMT^−/− mice. Breeders were typed for markers at NOD susceptibility loci as described (9), and all mice observed in these studies were homozygous for these markers from the ninth backcross generation. The mice were all housed in specific pathogen-free conditions. All of the animal studies were performed under protocols approved by the Yale University Animal Care and Use Committee. Transgenic mouse generation. The mlg construct has been previously described (27). This consists of a rearranged variable-diversity-joining (VDJ) segment containing VH182.2, reactive to the hapten (4-hydroxy-3-nitrophenyl) acetyl ligated to an IgM constant region from which the secreted exon and polyadenylation site had been excised, resulting in production of only membrane-bound but not secreted Ig. A control construct (28) was identical except that the secreted exon was intact (m+slg). Both constructs were injected directly into NOD ova (Yale Diabetes and Endocrinology Research Center transgenic injection facility). Two founder lines each of both mlg and m+slg were obtained, and because similar results were obtained, data were pooled. NOD mice express IgM^a, which facilitated screening by the use of fluorescein isothiocyanate–conjugated anti-IgM^a antibody (PharMingen, San Diego, CA). These mice were then crossed to the NOD,µMT^−/− mice and further intercrossed to make the µMT mutation homozygous so that only the B-cells expressed were transgenic B-cells. All mice used in the experiments were tested for the presence of the IgM^a alleotype to confirm that the transgenes were expressed on a homozygous NOD,µMT^−/− background. Thus all mice used were homozygous NOD,µMT^−/− and mlg or m+slg transgene-positive or -negative littermates.

Antibodies. Anti-CD4, anti-CD8, anti-CD45R (B220), anti-IgM^a, and anti-IgM^b antibodies used for flow cytometry were purchased from PharMingen. 3H-thymidine incorporation proliferation assays. Purified T-cells from spleen (nonimmunized mice) or popliteal lymph node (immunized mice) were resuspended in RPMI 1640 medium (Invitrogen, Carlsbad, CA) with 5% FCS. The cells were incubated in a 96-well plate at a concentration of 2.5 × 10^5 cells/well, together with 2 × 10^5 irradiated (3,000 rad) T-cell–depleted APCs. The antigens were used at different concentrations in a final volume of 200 µl. After 72 h, the plates were pulsed with 0.5 µCi 3H-thymidine for a further 14 h.

T-cell purification. Splenocytes or lymphocytes from popliteal lymph nodes were incubated with anti-Fc receptor antibody (2.4G2) and anti-I-A^d^ for 30 min, washed, and then incubated on ice with BioMag anti-mouse IgG, anti-mouse IgM, and anti-rat IgG (PerSeptive Biosystems, Framingham, MA). The cells were then incubated for 45 min. The beads were then removed using a magnet and the cells washed and resuspended in culture medium. The T-cell preparations were >90% pure.

Antigen-specific antibody measurement. Plates (96 well) were coated with insulin or affinity-purified GAD at a concentration of 50 µg/ml or insulin overnight at 4°C. After washing with PBS + 0.1% Tween-20, the plates were blocked for at least 1 h with PBS + 1% BSA. Serum (50 µl) was incubated at concentrations of 1 in 50. The plates were further washed with PBS + 0.1% Tween-20 and then incubated with 50 µl anti-IgM conjugated to alkaline phosphatase and streptavidin, followed by development with disodium paranitrophenyl phosphate substrate (Sigma) and read at OD_405 (optical density at 405-nm wavelength).

Immunization. mlg transgenic and transgene-negative NOD,µMT^−/− littermates or NOD mice were immunized with 50 µg antigen (GAD, insulin, or KLH) with complete Freund’s adjuvant in the hind footpads. Three weeks later, they were boosted with the same antigen emulsified with incomplete Freund’s adjuvant. One week later, the mice were killed, and the popliteal lymph nodes and spleen were removed for assay. Peripheral blood was taken and serum separated for analysis.

Histology. Pancreatic tissue was fixed in formalin, paraffin embedded, and stained with hematoxylin and eosin. The sections were examined microscopically, and insulitis of individual islets was assessed by two independent observers according to the scale of 0–4 as follows: 0, no insulitis; 1, peri-insulitis; 2, peri-insulitis with less than one-half of the islet infiltrated; 3, more than one-half of the islet infiltrated; and 4, complete islet destruction. Additionally, a proportion of pancreata was fixed in periodate-lysine-parafomaldehyde, sucrose infused, and then frozen in Tissue-Tek OCT (Bayer, Elkhart, IN). Sections (7-µm thick) were stained with biotinylated YTL4.3 antibody recognizing CD4, TIB 105 antibody recognizing CD8, and B220 antibody, which stains the majority of B-cells. The color was developed using diaminobenzidine tetrahydrochloride and nickel ammonium sulfate. The sections were then counterstained with hematoxylin. Diabetes screening. Animals were tested weekly for glycosuria using Diastix (Bayer, and if present, diabetes was confirmed by a blood glucose measurement of >250 mg/dl (13.9 mmol/l) using OneTouch test strips (LifeScan, Milpitas, CA) (30).

RESULTS

The presence of the transgene restores expression of IgM^a-positive B-cells. The transgene was identified using antibodies against IgM^a. It can be seen from Fig. 1 that the transgenes restored B220^+ (CD45R) B-cells in both types of transgenic mice. However, although the levels of B-cells in the mlg transgenic mice was normal, the number of B-cells in both lines of m+slg transgenic mice was reduced. The ratio of CD4 to CD8 in all of these mice was normal (data not shown).

mlg transgenic mice develop mild insulitis and increased incidence of diabetes compared with NOD,µMT^−/− mice. Our previous experience had shown that NOD,µMT^−/− mice develop mild insulitis (10) in accordance with an earlier report (11) and have a low incidence of diabetes. Insulitis in these NOD,µMT^−/− mice can be seen from the age of 8 weeks onwards, and an example of the insulitis at 12 weeks of age is shown here; although, in general, NOD,µMT^−/− mice have milder insulitis.
litis compared with NOD mice. A fraction of the transgenic mice showed an increased amount of insulitis compared with NOD $\mu$MT$^{-/-}$ mice (Fig. 2B). Expression of the transgenes significantly altered the severity of insulitis overall ($P < 0.001$), especially in the male transgenic animals (Fig. 2C).

A number of mice expressing the mlg transgene developed diabetes (Table 1). This was significant ($P < 0.05$) in...
TABLE 1
Diabetes incidence in transgenic and nontransgenic NOD,µMT−/− mice at 50 weeks compared with diabetes incidence in the NOD colony at 30 weeks

<table>
<thead>
<tr>
<th>Percentage with diabetes</th>
<th>NOD</th>
<th>mlg</th>
<th>Transgene negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>90</td>
<td>10.64*</td>
<td>1.56*</td>
</tr>
<tr>
<td>n</td>
<td>20</td>
<td>47</td>
<td>64</td>
</tr>
<tr>
<td>Male</td>
<td>70</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>n</td>
<td>20</td>
<td>35</td>
<td>56</td>
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*Fisher’s exact test was performed, and the difference between the incidence of diabetes in the mlg female mice compared with the NOD,µMT−/− transgene-negative mice is significant (P < 0.05). No diabetes occurred in m+slg transgenic mice (n = 56 males and 50 females).

female mice. Diabetes was not, however, restored to the level seen in NOD mice, and no diabetes was seen in the m+slg control transgenic mice.

NOD,µMT−/− mice can generate low proliferative responses to islets. In keeping with the fact that the NOD,µMT−/− mice develop insulitis, they can, in the absence of B-cells, also generate spontaneous T-cell proliferative responses to islets, as shown in Fig. 3A. NOD,µMT−/− mice have low spontaneous T-cell responses to GAD and insulin, and the introduction of the mlg transgene did not lead to increased responses to these antigens (data not shown). In order to check that the B-cells from the mlg transgenic mice were able to function normally, T-depleted spleen cells (predominantly B-cells) from NOD and mlg mice were stimulated with lipopolysaccharide (LPS) as a nontpecific stimulus. Results shown in Fig. 3B indicate that the T-depleted spleen cells respond normally to this stimulus.

T-depleted spleen cells from the mlg transgenic mice were used as APCs to stimulate islet responses from NOD T-cells. The APCs were able to present exogenous antigens derived from islets to NOD T-cells (Fig. 3C). When this experiment was performed with purified B-cells, it can be seen that there is no difference in the ability of the mlg transgenic B-cells to present islet antigen to NOD T-cells compared with wild-type NOD B-cells (Fig. 3D).

Transgenic mice are able to generate T-cell proliferative immune responses after immunization. mlg transgenic mice were immunized with KLH, insulin, or affinity-purified GAD in complete Freund’s adjuvant. The T-cell proliferative responses to the KLH after immunization were similar in the transgenic, compared with the nontransgenic, B-cell–deficient and NOD mice shown in Fig. 4. However, immunized T-cell responses to GAD did not increase in the presence of the transgene compared with transgene-negative mice, and responses to insulin were uniformly low.

Transgenic mice generate low spontaneous antibody responses to islet lysate and are not able to generate antibodies in response to immunization. Islet lysate was coated onto enzyme-linked immunosorbent assay plates to represent multiple islet antigens. Nonimmunized NOD mice have a greater spontaneous antibody response to these islet antigens than m+slg transgenic mice (P < 0.05). As expected, mlg transgenic mice as well as transgene-negative mice (B-cell deficient) do not secrete any detectable antibody (Fig. 5). Serum was also taken from the mice that were immunized with KLH and insulin and tested against these antigens coated onto enzyme-linked immunosorbent assay plates. Antibody responses to both of these antigens were low in all mice compared with NOD mice (data not shown).

DISCUSSION
In order to separate the role of the B-cells in antigen presentation from antibody production in the development of diabetes, we generated NOD transgenic mice expressing B-cells (mlg) that were only able to present antigen but not secrete antibody due to a mutation in the secreted exon. Although the transgenic construct had a fixed heavy chain, the potential for selection of a diversity of light chains was maintained. Therefore, in theory, the cells bearing the transgenic construct should, at albeit reduced

FIG. 3. Proliferative responses of nonimmunized mice. A: Spleens from four different NOD,µMT−/− mice (○) and NOD mice (□) were cultured with purified hand-picked islets from young pre-diabetic NOD mice (25 islets per well) for 72 h. The mice were all aged 6–10 weeks. Results are shown as Δcpm (proliferation — background [766–4,936 cpm]). B: Proliferation of 105 splenic B-cells purified from mlg and NOD mice and cultured with 10 μg/ml LPS for 24 h. Results are shown as mean and range of Δcpm — (proliferation — background [2,485–15,783 cpm]). C: Proliferation of purified NOD T-cells cultured with NOD islets (15 per well) and purified T-cell–depleted spleen cells from mlg and NOD mice. Results are shown as mean and range of Δcpm — (proliferation — background [6,426–18,805 cpm]). D: Proliferation of purified NOD T-cells from two separate mice (circles and triangles) cultured with NOD islets (15 per well) and purified B-cells from two separate mlg mice (open symbols) and two separate NOD mice (closed symbols). Thus to compare responses of the same T-cells to islets using different APCs, the open circles (○) should be compared with the closed circles (●) and the open triangles (△) with the closed triangles (▲).
frequency, be able to present the autoantigens important in diabetes to the autoreactive T-cells. The expression of the mIg transgene significantly increased insulitis in the male animals. There was also a sevenfold and statistically significant increase in the number of female mIg mice developing diabetes (5 of 47 [10.6%]) compared with the female nontransgenic NOD.\textsubscript{mt/H11002} littermate (1 of 64 [1.5%]) mice ($P < 0.05$). As the mIg transgenic mice do not secrete antibody, this increase in diabetes is clearly antibody independent and not related to the antibody-secreting function of B-cells. Thus we establish, using a genetic approach, that B-cells do, in fact, have an antibody-independent role in promoting diabetes.

Although the nonsecreting \textit{Ig} transgenic B-cells increase the incidence of diabetes, it is not restored to the level of wild-type NOD mice. This could be due to lack of a diverse repertoire, lack of antibody, or both. While repertoire almost certainly plays a role (see below), it is less clear whether antibody also has a role. The m+\textit{slg} transgenic mice did not develop diabetes, although they had insulitis comparable with the mIg mice (data not shown). This does not necessarily imply that the ability of these mice to produce antibody has an adverse effect on the development of diabetes. Rather, as the restoration of B-cells in both lines of m+\textit{slg} mice did not occur to the same extent as in the mIg transgenic mice, it is quite possible that the low total number of B-cells present in the m+\textit{slg} transgenic mice contributed to the lack of development of diabetes. For this reason, no major comparisons have been made with the m+\textit{slg} transgenic mice; however, the m+\textit{slg} mice were useful in assessing antibody responses to islet and other antigens.

In keeping with the fact that the NOD.\textit{mt/H11002} mice developed insulitis in the absence of B-cells, NOD.\textit{mt/H11002} mice had some spontaneous proliferative responses to islets, although responses specifically to both insulin and GAD were low. This indicated that the ability to generate anti-islet T-cell responses was not entirely dependent on B-cells to prime these responses. This agrees with a previous investigation (14) showing the development of diabetes in NOD.scid mice transplanted with NOD.m\textit{mt/H11002} fetal thymus. The presence of the mIg transgene did not increase the response to the specific islet autoantigens tested in transgenic mice. Although the B-cells from the transgenic mice were normally responsive to the nonspecific stimulus LPS and presented antigen normally to NOD T-cells, they were not able to allow the diversification to increase GAD and insulin responses. We further investigated the ability of T-cells from transgenic mice to respond after immunization with a nonislet-specific, foreign immunogen K\textit{LH} as well as with diabetes autoantigens, insulin, and GAD. The nontransgenic B-cell–deficient mice were able to respond to K\textit{LH} immunization as equally well as the transgenic NOD.\textit{mt/H11002} mice. However, none of the mice responded well to immunization with either GAD or insulin, whereas nontransgenic
NOD mice can respond to GAD but poorly to insulin after immunization. Thus, it is likely that the fixed heavy chain did not facilitate sufficient diversity for the recognition of these self-antigens.

The ability of the transgenes to recognize and diversify the immune response was further tested by investigating the antibody responses to islets. To test responses to antigens other than GAD and insulin, we used islet lysate. For this experiment, we investigated the antibody production from the m+slg control transgenic mice because this could not be examined in the mIg transgenic mice. NOD mice generated antibody responses to islet lysate that were greater than those from the m+slg transgenic mice. This was further evidence that antibody repertoire was reduced, and the failure to develop diabetes may, in part, be due to the fact that the mice could not sufficiently diversify the response to stimulate the T-cell repertoire necessary for diabetes to occur.

In the natural history of autoimmune diabetes, at the time of development of overt disease, there is a T-cell response to a diverse range of autoantigens that include GAD, insulin, HSP60 (heat shock protein 60) (31) and likely other autoantigens that have yet to be fully defined. In addition, autoantibodies are also produced, although there is not the same diversity as that seen for T-cells (32). Insulin autoantibodies are readily detected (33), but anti-GAD antibodies are only found in some NOD mice. One of the important aspects of the function of B-cells as APCs is the ability to concentrate antigen manyfold by virtue of the antigen specificity of the B-cell receptor (16,34–36). This would allow antigen-specific T-cells to be expanded if appropriately stimulated by relevant B-cell populations. Therefore, the ability to diversify the repertoire of B-cells that recognize different autoantigens might be important. Our study suggests that the reduced Ig repertoire resulting from the fixed heavy chain transgenes used here was not sufficient to produce islet-specific antigen receptors to allow appropriate diversity. This complements the other studies showing that B-cells expressing insulin-specific antibody receptors develop accelerated diabetes (37) and, the converse, that expression of a fixed antibody receptor to hen egg lysozyme, which does not allow any B-cell diversity to develop, retards the onset of diabetes (38).

Despite a number of contributory studies, the exact role of B-cells in diabetes development has still not been completely elucidated. The question of whether antigen presentation or production of autoantibodies is important in this process has been much debated but, as yet, has not been conclusively answered. Transfer of total serum from NOD mice does not precipitate disease (22). However, this experiment has not been done with antibodies tested to be specific for diabetes autoantigens. Certainly, autoantibodies are good predictive markers for the development of disease both in humans (7) and in NOD mice (33). In addition, the presence of specific autoantibodies may aid in the presentation of autoantigens (39). Recently, evidence has been provided that maternally transmitted antibodies are important in development of diabetes in NOD mice (24) as shown by experiments where NOD,μMT−/− females bred with NOD males had offspring that developed a lower incidence of diabetes than offspring from NOD females mated with NOD,μMT−/− males. Further, when NOD embryos were transferred to superovulated DBA/2 females or control NOD females, a lower incidence of diabetes was found in the mice of the DBA/2 surrogate mothers. While these experiments are compelling evidence that maternally transmitted antibodies from NOD mice can influence the development of disease, it does not directly show that it is the anti-islet antibodies that are specifically involved in the disease process. Analysis of the natural autoantibody repertoire of NOD mice has indicated that the repertoire has broad specificity for multiple antigens and is low avidity (40). In addition, experiments analyzing the autoantibody repertoire in NOD mice indicated that IgM may be important early in the process and that the role of IgG antibodies may increase after 14 weeks as pancreatic β-cells are destroyed (40).

Although there has been speculation over an antigen-presenting role for B-cells in diabetes, this is the first report that directly and experimentally separates the antigen-presenting role of B-cells from the ability to secrete antibody in the development of autoimmune diabetes. The data clearly show that antibody secretion is not essential for some of the diabetes-promoting role of B-cells. Our experiments also indicate, not surprisingly, that the ability of B-cells to recognize a diversity of islet autoantigens is likely to be important in the development of diabetes. Taken together, these results strongly implicate an antigen-specific antigen-presenting function for B-cells. On the other hand, these experiments do not address the role that antibody may play in the development of diabetes. This issue will require separate approaches. Our results are highly relevant to potential human studies to treat or prevent early diabetes via depletion of B-cells with anti-CD20 treatment; such therapy has a much greater effect on B-cells and presumably antigen presentation by B-cells than it does on antibody levels. Thus, our mechanistic data provide some experimental basis for the consideration of such a trial.

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