Effect of MHC Transgene Expression on Spontaneous Insulin Autoantibody Class Switch in Nonobese Diabetic Mice

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A study of spontaneous anti-insulin autoantibodies in nonobese diabetic (NOD) mice revealed that when first detected, the antibodies are immunoglobulin M (IgM), but by age 10 weeks, immunoglobulin G (IgG) autoantibodies have appeared in many of these animals. When NOD strains, partially or completely protected from IDDM by the insertion of transgenes in the class II region, were compared, it was found that the switch to IgG autoantibodies was inhibited and the autoantibodies remained IgM indefinitely. We speculate that the switch to IgG may be a marker of events leading to IDDM in NOD mice and an indication that T-cell help has been generated for responses to β-cell antigens. Such help not only directs the development of IgG autoantibodies, but more importantly, allows the emergence of potentially pathogenic T-cell clones that are capable of infiltrating the pancreas and mediating β-cell damage.
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Immune responses to insulin have been detected in prediabetic humans and animal models of IDDM (1–4). Furthermore, manipulation of this response through tolerance induction to insulin has been shown to reduce the spontaneous incidence of IDDM in NOD mice (5–7). A variety of protocols have been used to induce tolerance, including insulin administered intravenously, intranasally, and orally (5,8,6) or subcutaneous injection of insulin or its B-chain in incomplete Freund's adjuvant (IFA; [8,9]). The mechanism(s) by which such tolerance protocols prevent IDDM remains to be clarified. Because there appears to be an association (but not necessarily a correlation) between the spontaneous development of antibody responses to insulin and the development of IDDM (1,10), we have compared the spontaneous response to insulin in NOD mice with that of transgenic NOD mice with either zero or reduced incidence of disease. The NOD transgenic mice that we have investigated express a transgene encoding either an I-Eα^d chain (NOD-E mice) (11) or a mutated I-Aβ^{g7} chain (NOD-A-Pro mice and NOD-A-Asp mice) (11,12). IDDM never develops in NOD-E mice, NOD-A-Pro mice, or NOD-A-Asp male mice and only develops at a 15% incidence in NOD-A-Asp female mice (12). We show that all three lines have markedly differing spontaneous antibody responses to insulin when compared with NOD mice. We have also examined spontaneous responses to insulin in normal inbred and outbred mice and in Biozzi mice, which do not develop IDDM but do share the NOD I-Aβ^{g7} gene (13).

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CFA, complete Freund's adjuvant; ELISA, enzyme-linked immunosorbent assays; IFA, incomplete Freund's adjuvant; Ig, immunoglobulin; MHC, major histocompatibility complex.

RESEARCH DESIGN AND METHODS

Mice and assessment of diabetes. NOD and NOD-A-Asp mice of both sexes, 4–24 weeks of age, were obtained from breeding colonies established in our own facilities at the Department of Pathology, while the NOD-A-Pro and the NOD4R mice were maintained at the Central Biological Services. Other normal mice were obtained from National Institute for Medical Research (Mill Hill, London) or from Harlan U.K. (Bicester, Oxon). Sera from Biozzi mice were from S. Amor and D. Wraith. The clinical onset of diabetes was judged by frequent testing for the presence of glucose in the urine and blood, using Glucostix and Diastix reagent strips (Miles Laboratories, Slough, U.K.) and a glucose meter (Ames, Slough, U.K.). Two consecutive readings >12 mmol/l coupled with a positive urine test were considered to be an indication of overt diabetes.
Transgenic and congenic mice. The generation of NOD-E, NOD-A-Pro, and NOD-A-Asp mice and their disease characteristics are fully described in our previous publications (11,12). NOD-E and NOD-A-Pro mice and NOD-A-Asp male mice never develop IDDM, while NOD-A-Asp female mice develop the disease with ~15% incidence at 30 weeks. Generation of the congenic NOD4R (NOD × B10A.4R) mice is described by Wicker et al. (14,15), and the breeding nucleus maintained at Cambridge was obtained from Dr. Linda Wicker, Merck Research Laboratories, New Jersey. These mice have the NOD-susceptibility genome but do not have I-A^{g7}. Some animals develop insulitis but they do not get diabetes.
Enzyme-linked immunosorbent assays (ELISAs). Anti-insulin antibodies were evaluated using standard ELISA methods (16), coating the plate overnight at 4°C with bovine insulin (or B-chain peptide 9-23 where specified in text) at 10 μg/ml. Our own unpublished observations have shown that anti-insulin antibodies, both spontaneous and induced, bind to bovine and sheep insulin with equal efficiency. A goat anti-mouse polyvalent immunoglobulin (IgM, IgG, and IgA), an anti-mouse IgM, or an anti-mouse IgG conjugated to alkaline-phosphatase (Sigma Immunochemicals) were used as developing antibodies. Where normal mouse serum was shown for comparison, there was a standard aliquot of pooled sera from outbred normal mice, and where required, standard sera of known concentration were included. The values shown are the mean optical density readings at 410 nm ± SE, and where relevant, these are converted to nanograms per milliliter of immunoglobulin. Sera were diluted to 1/50, and although assayed at four further doubling dilutions, only the values for 1/50 are shown for ease of comparison.
Priming for induced antibodies. 50 μg sheep insulin (obtained from Sigma) was administered in the base of the tail in a 1:1 emulsion with complete Freund's adjuvant (CFA; H37Ra, Difco, Detroit, MI) and repeated on day 7 in the footpad. The mice were bled retro-orbitally at 3 weeks to assess anti-insulin antibodies.
Statistical analysis. Student's *t* test was used where appropriate.

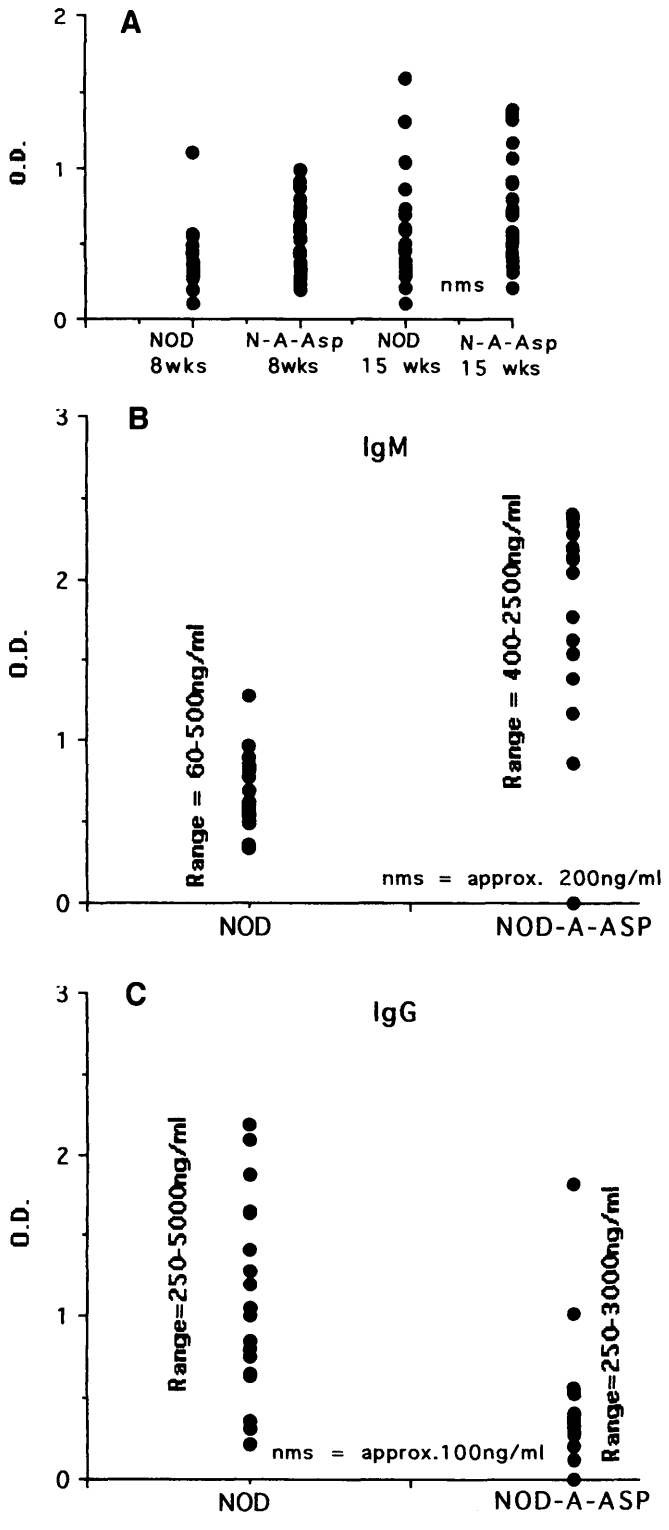

FIG. 1. *A*: cohorts of 25 female NOD and NOD-A-Asp mice were bled at intervals, and the levels of spontaneous anti-insulin autoantibodies in sera diluted to 1/50 were assessed in an ELISA developed with goat anti-mouse polyvalent immunoglobulin. The optical density (OD) was read at 410 nm. The data shown are from representative bleeds at 8 and 15 weeks. Sera with the highest titers of anti-insulin autoantibodies, selected from NOD (n = 20) and NOD-A-Asp (n = 16) mice between 8 and 22 weeks of age, were diluted to 1/50. Using standard sera, the class and concentration of antibody was determined by developing with goat anti-mouse IgM (*B*) or goat anti-mouse IgG (*C*). The means ± SE for IgM were NOD = 0.7 ± 0.05; NOD-A-ASP = 1.9 ± 0.13. The means for IgG were NOD = 1.1 ± 0.13; NOD-A-ASP = 0.5 ± 0.1. (P = 0.001 for both when comparing NOD with NOD-A-ASP.)

RESULTS

Spontaneous autoantibody responses to insulin in NOD and NOD transgenic mice. Autoantibodies to insulin have been shown to arise spontaneously in NOD mice (2,3). Such antibodies were detected in the sera of NOD and NOD-A-Asp mice of both sexes and can be found as early as 6 weeks in some animals. They are not detected in the sera of young NOD-E mice, although antibodies to insulin develop in some of these animals later at ~4 months of age.

The development of spontaneous anti-insulin antibodies was followed over a period of time in cohorts of 25 female NOD and NOD-A-Asp mice. Anti-insulin antibodies developed in some mice from both cohorts from 8 weeks onward. Figure 1*A* shows the incidence and range of these antibodies at two representative bleeds.

Sera with high anti-insulin titers were selected from NOD and NOD-A-Asp female mice aged between 8 and 22 weeks and tested to determine the class of antibody. Such antibodies in NOD mice were frequently IgG, whereas those in NOD-A-Asp mice were predominantly IgM (Figs. 1*B* and *C*; P = 0.001 when comparing NOD and NOD-A-Asp for both IgM and IgG). It is possible, therefore, that the presence of high titers of spontaneous IgG anti-insulin antibodies might correlate with the predisposition to develop IDDM in NOD mice. This was further supported when examination of sera from NOD-A-Pro mice and older NOD-E mice (which do not develop IDDM) showed that some mice in both transgenic groups also have spontaneous anti-insulin antibodies; however, like the NOD-A-Asp, these antibodies appear to be predominantly IgM (Fig. 2). Nontransgenic littermates of NOD-A-Pro mice developed IgG autoantibodies (data not shown).

The presence of anti-insulin antibodies per se does not predict disease (10), but the switch to IgG in NOD mice may be a significant marker in the progress toward IDDM. By following the development of these antibodies from 6 weeks of age, it was apparent that in NOD mice the gradual switch to IgG occurs around 8 weeks. In the majority of NOD-A-Asp mice, the switch is delayed indefinitely (Fig. 3), and animals as old as 22 weeks still have high titers of IgM anti-insulin antibodies (data not shown).

Does the appearance of IgG anti-insulin antibodies relate to subsequent development of IDDM? Because some NOD male mice develop IgG anti-insulin antibodies but have a much lower incidence of disease, the switch to IgG cannot inevitably lead to diabetes; other factors, including sex, must have a role in determining the onset of disease. A cohort of nine female NOD mice, 7 weeks old, were bled serially and monitored for onset of IDDM over 14 weeks to see if titers of IgG had any bearing on subsequent development of disease. The three mice that had the highest titer of IgG were the three mice that developed IDDM during the period of observation (Fig. 4).

NOD-A-Asp mice can be induced to develop IgG anti-insulin antibodies. To determine whether there was an underlying defect in the development of IgG antibodies to insulin in NOD-A-Asp mice, female mice, 2–3 months old, were immunized with sheep insulin in CFA. Figure 5 shows that these mice made excellent IgG responses to exogenous insulin and that 3 weeks after immunization, the induced anti-insulin response was wholly IgG with no detectable IgM. (Any spontaneous anti-insulin antibodies would not be detectable at the titers used to assay induced responses.)

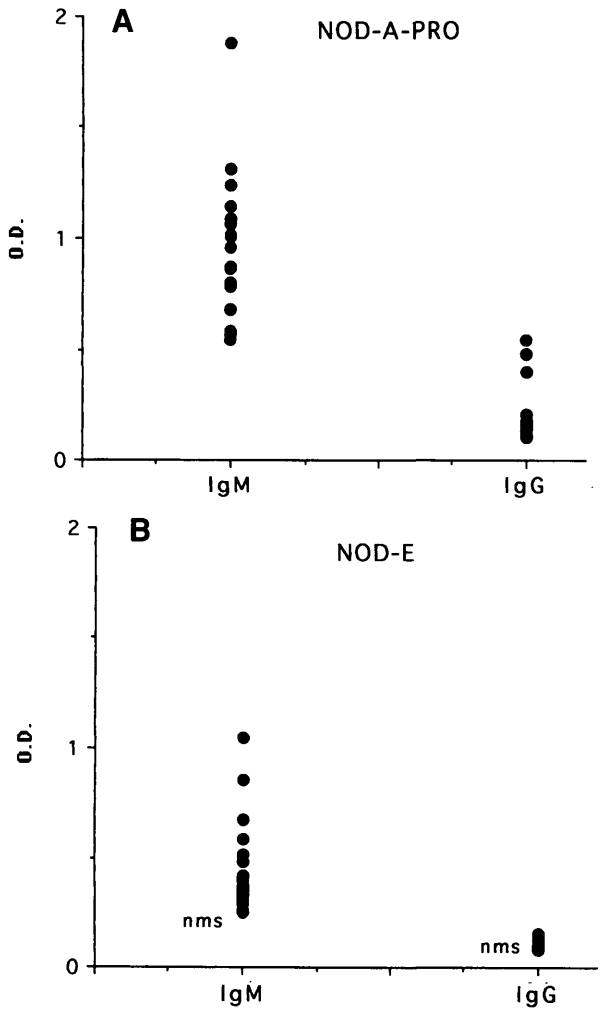

FIG. 2. Sera from NOD-A-Pro male and female mice (n = 19) and NOD-E female mice (n = 20) were diluted to 1/50 and tested in an ELISA developed with anti-mouse IgM or anti-mouse IgG to determine class of spontaneous autoantibody. The means ± SE for IgM were 0.9 ± 0.07 and 0.5 ± 0.05 for NOD-A-Pro and NOD-E, respectively, and for IgG were 0.2 ± 0.02 and 0.1 ± 0.01.

Isotype and specificity of the autoantibodies. Because the development of IgG2a antibodies is an indication of Th1 responses, it might be expected that anti-insulin antibodies of this isotype would correlate with the progression toward IDDM. We examined the isotypes in sera from prediabetic NOD mice of different ages and failed to detect any IgG2a. As in insulin immunized mice, only IgG1 and IgG2b were present at detectable levels (data not shown). IgG2a in spontaneous antibody was <400 ng/ml, whereas some sera reached levels in excess of 5,000 ng/ml of IgG1. Some other researchers have similarly found the dominant spontaneous isotype to be IgG2b (17). Those IgG antibodies, which were present occasionally or present at low titers in NOD-A-Asp mice (see Fig. 1), had the same isotype profile as NOD mice. Autoantibodies to insulin from NOD and NOD-A-ASP mice included specificities for both A-chain and B-chain and, interestingly, the B9-23 B-chain peptide (data not shown).

Are spontaneous anti-insulin antibodies linked with the I-A^{g7} haplotype? Different mouse strains were examined for the presence of serum anti-insulin antibodies to establish whether the presence of anti-insulin antibodies related to a genetic background predisposing to IDDM. We found that among Biozzi mice and the NOD4R male and female mice, there were a number of animals with above normal titers of anti-insulin antibodies (Figs. 6*A* and *B*). In contrast, when sera from several different normal strains of mice ranging in age from 8 to 17 weeks were examined, only very low titers of spontaneous anti-insulin antibodies were found (Fig 6*C*; $P <$ 0.0001, when comparing normal strains with either NOD or NOD-A-Asp mice, whereas NOD4R mice were not significantly different from NOD-A-Asp mice).

Biozzi mice share the I-A^{g7} haplotype with the NOD mice but do not spontaneously develop diabetes or any other autoimmune disease. The NOD4R mouse is a congenic strain that expresses I-A^{k} but is I-E negative and was generated by crosses of NOD with B10A.4R (NOD.H-2^{h4}). Only ~20% of the mice develop insulitis, and none develop diabetes. NOD mice and Biozzi mice share I-A^{g7}, but they also share certain other putative IDDM susceptibility loci (13). Taken together, these data suggest that it is not the I-A^{g7} haplotype that controls the development of spontaneous anti-insulin antibodies, but probably other genes in the NOD repertoire are important.

DISCUSSION

The data presented in this report show that the development of IgG anti-insulin autoantibodies is associated with a predisposition to develop IDDM. The development of IgM anti-insulin antibodies is common to strains of mice sharing certain NOD alleles predisposing to IDDM; however, class switching to IgG occurs only in strains where IDDM follows.

We have shown that although NOD mice in common with their transgenic lines have anti-insulin antibodies of the IgM class at 6 weeks, most NOD mice switch to predominantly IgG anti-insulin antibodies at ~8 weeks of age. In the majority of individuals from the less susceptible transgenic strains, this class switch does not occur, although IgM antibodies may remain high. It is tempting to speculate that since this switch coincides with the onset of islet infiltration in NOD mice, there may be an as yet unidentified event that leads to both destructive islet infiltration and the class switch to IgG autoantibodies.

At present, there is no direct evidence implicating IgG anti-insulin antibodies in the pathogenesis of IDDM. Because not all animals that develop IgG antibodies go on to get diabetes, we must conclude that the IgG antibodies themselves are not responsible for disease. However, because antibody class switch is T-cell–dependent (18), the development of IgG autoantibodies may be a marker for the emergence of helper T-cells recognizing islet antigens. The onset of destructive intra-islet infiltration may result in the release of cytokines that facilitate the switch to IgG. Our data shown in Fig. 4 confirm that a rise in IgG anti-insulin antibodies and the subsequent onset of IDDM is not unrelated.

Recent studies have turned the spotlight back on insulin as an important β-cell antigen. Wegmann et al. (4,19) have shown conclusively that the insulin B-chain peptide 9-23 is the dominant epitope recognized by diabetogenic clones isolated from the infiltrated pancreas of NOD mice. Moreover, it has also been shown by Daniel and Wegmann (8) and ourselves (P.H., A.C., unpublished observations) to be a very effective tolerizing agent when administered subcutaneously in IFA. We have observed that a significant proportion of the

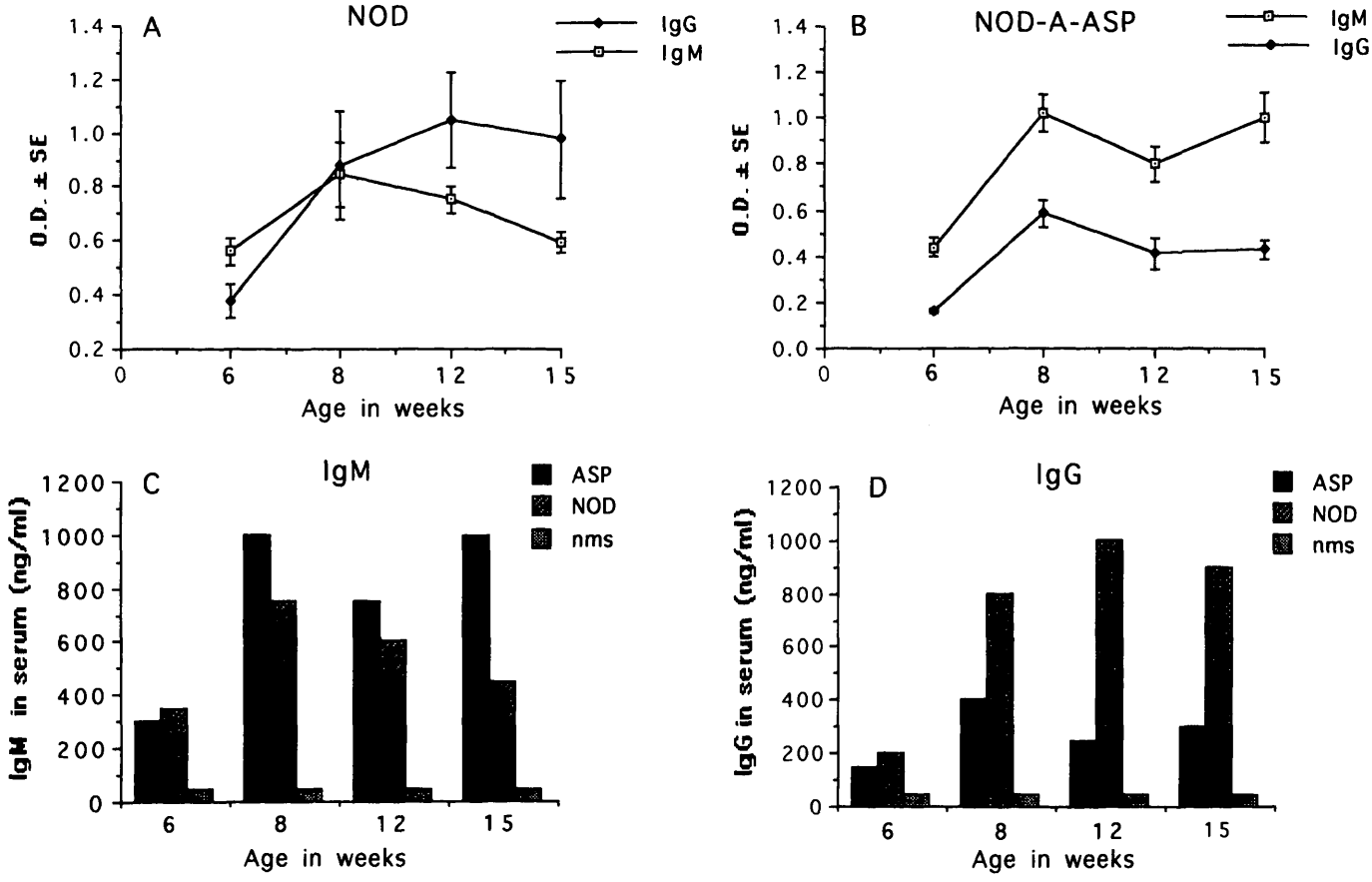

FIG. 3. Randomly chosen sera from NOD and NOD-A-Asp female mice aged 6–15 weeks were diluted to 1/50 and assayed for IgM and IgG spontaneous anti-insulin autoantibodies. Between 10 and 20 mice were tested for each time point. NOD IgG antibody differed significantly from NOD-A-ASP IgG at 12 and 15 weeks (P = 0.005 and 0.028).

spontaneous anti–B-chain serum antibodies bind to B9-23 in an ELISA, which supports the notion that this peptide must have a significant role in the events that lead either to protection from IDDM or, conversely, to the development of β-cell destruction.

Although monoclonal IgM autoantibodies have been isolated from normal mice (20), we were only able to detect significant levels of anti-insulin autoantibodies in Biozzi, NOD, and NOD4R mice. It was particularly intriguing to find that many Biozzi mice also have IgM insulin autoantibodies because this is the only other strain examined that expresses I-A^{g7}. NOD4R expresses I-A^{k}, which suggests that I-A^{g7} itself is not the predisposing feature in the development of anti-insulin antibodies but rather that some other background genes of NOD mice are involved. In support of this, it is known that

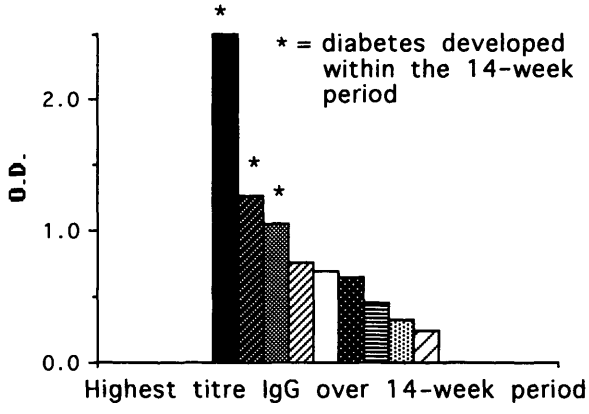

FIG. 4. A cohort of 9 female, 7-week-old NOD mice were bled serially over a 14-week period and monitored for the onset of diabetes. The sera were assayed for IgG anti-insulin autoantibodies at the end of this period during which time 3 mice had become diabetic. The IgG titers are shown for each mouse in order of the highest titer obtained.

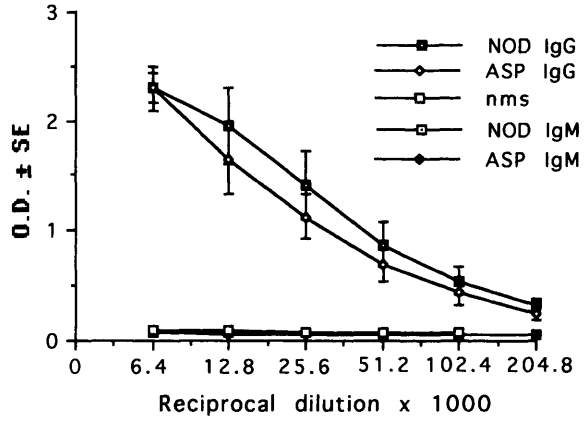

FIG. 5. Female NOD and NOD-A-Asp mice were primed with sheep insulin in CFA in the base of the tail and boosted 7 days later in the footpad. IgM and IgG antibodies to insulin were assessed 3 weeks after priming.

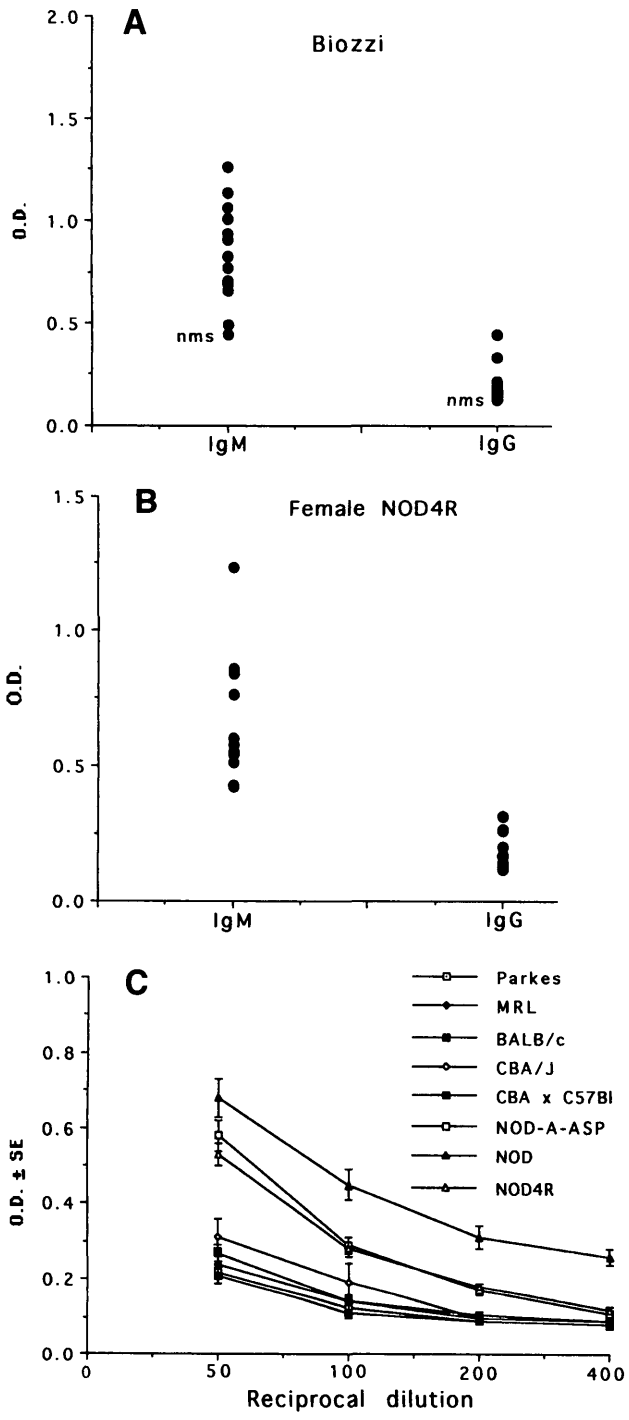

FIG. 6. IgM and IgG spontaneous anti-insulin autoantibodies were determined by ELISA in sera from Biozzi mice 10–12 weeks old (4 male and 8 female) (*A*) and 11 female NOD4R mice 4–8 months old (*B*). All sera diluted to 1/50. *C*: sera from 10-week male Parkes mice (n = 10), 6-week female MRL (n = 6), 16-week female BALB/c (n = 8), 12-week male CBA/J (n = 4), 10-week male CBA × C57BL/10 (n = 8), 4- to 8-month female NOD4R (n = 11), 13-week female NOD (n = 12), and 13- to 15-week female NOD-A-Asp (n = 12) were assayed by ELISA for spontaneous anti-insulin autoantibodies. P = 0.0001 when comparing normal strains with NOD or NOD-A-Asp at 1/50 dilution but NOD4R were not significantly different from NOD-A-Asp.

Biozzi mice share some of the same candidate IDDM susceptibility loci with NOD mice. For example, the FcγRI is truncated in both NOD and Biozzi mice (21), resulting in a failure to internalize antigen/antibody complexes adequately. This might result in a sequestered pool of antigen remaining on the cell surface and prolonging the period for which antigen is available for presentation.

Development of IgM anti-insulin antibodies may indicate the presence of IDDM susceptibility loci, which are nevertheless insufficient to cause disease. Following T-cell activation events associated with the subsequent development of IDDM (at present unknown and restricted mainly to NOD mice), the requisite T-cell help is provided to permit class switching of insulin autoantibodies and, coincidentally, the emergence of new T-cell clones with destructive potential. Our data therefore support the thesis that the presence of IgG insulin autoantibodies is a marker for the development of islet cell infiltration and, ultimately, β-cell destruction.

NOD-A-Asp mice differ from NOD mice by a single amino acid at position 57 on the β-chain of I-A^{g7}. This small change appears to affect not only the spontaneous incidence of IDDM (which is reduced from 70 to 15% in female mice) but also the ability of NOD-A-Asp to present and respond to islet antigens (12). The mutated I-A^{g7} has the potential to form a salt bridge between the aspartate at position 57 and the arginine at position 80. This alteration in the binding groove might permit higher affinity interactions between peptide, major histocompatibility complex (MHC), and T-cell receptors, leading to much more effective central tolerance. Additionally, as NOD-A-Asp antigen presenting cells bear both I-A^{g7} and I-A^{g7asp} on their surface, there may be competition for binding of diabetogenic peptides, or it may be that peptide presented on I-A^{g7asp} is either unable to activate diabetogenic T-cells or delivers a tolerogenic or anergic signal instead. In any event, we have shown that the presence of our MHC-encoding transgenes somehow prevents the switch to IgG anti-insulin autoantibodies, as well as preventing the chronologically related events that lead to β-cell destruction.

Spontaneous anti-insulin autoantibodies are known to be present in prediabetic humans and to correlate to some extent with the rate of progress toward overt diabetes (22). It would be of great interest to establish whether class switching to IgG occurs in human patients as in NOD mice and whether it is a marker for greater susceptibility to IDDM.

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