Genetic Control of Autoimmunity: Protection from Diabetes, but Spontaneous Autoimmune Biliary Disease in a Nonobese Diabetic Congenic Strain


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Genetic Control of Autoimmunity: Protection from Diabetes, but Spontaneous Autoimmune Biliary Disease in a Nonobese Diabetic Congenic Strain


At least 20 insulin-dependent diabetes (Idd) loci modify the progression of autoimmune diabetes in the NOD mouse, an animal model of human type 1 diabetes. The NOD.c3c4 congenic mouse, which has multiple B6- and B10-derived Idd-resistant alleles on chromosomes 3 and 4, respectively, is completely protected from autoimmune diabetes. We demonstrate in this study, however, that NOD.c3c4 mice develop a novel spontaneous and fatal autoimmune polycystic biliary tract disease, with lymphocytic peribiliary infiltrates and autoantibodies. Strains having a subset of the Idd-resistant alleles present in the NOD.c3c4 strain share component phenotypes of the liver disease: NOD mice with B6 resistance alleles only on chromosome 3 have lymphocytic liver infiltration without autoantibody formation, while NOD mice with B10 resistance alleles only on chromosome 4 show autoantibody formation without liver infiltration. The liver disease is transferable to naive NOD.c3c4 recipients using splenocytes from affected NOD.c3c4 mice, demonstrating an autoimmune etiology. Thus, substitution of non-NOD genetic intervals into the NOD strain can prevent diabetes, but in turn cause an entirely different autoimmune syndrome, a finding consistent with a generalized failure of self-tolerance in the NOD genetic background. The complex clinical phenotypes in human autoimmune conditions may be similarly resolved into largely overlapping biochemical pathways that are then modified, potentially by alleles at a few key chromosomal regions, to produce specific autoimmune syndromes.

Materials and Methods

Mice

The development of the NOD.c3c4 strain from the parental strains, NOD.B10 Idd9.1/9.2/9.3 and NOD.B6 Idd3/17/10/18, was described previously (9). The c3c4 strain has ~80 cM genetic intervals on chromosomes 4 and 3 introgressed from B10 and B6, respectively (Fig. 1). The NOD.B10 Idd9.1/9.2/9.3R28 strain (9), called c4(a) and used as the parental chromosome 4 congenic strain in this study, has a smaller introgressed region (~60 cM) from the B10 strain as compared with the NOD.B10 Idd9.1/9.2/9.3 parental strain (which is no longer extant). The c3(a) strain was described previously (10) as the NOD.B6 Idd3/10 strain. Although the Idd17 resistance gene is not present in the c3(a) strain, diabetes resistance of this strain is identical with that of the NOD.B6 Idd3/10 parental strain (which is also no longer extant), which was called NOD.B6 (Idd3/Idd10)R1 (10). Please note that subsequent to the publication of Ref. 10, the Idd10 region was shown to consist of two Idd genes, Idd10 and Idd18 (11), and therefore the strain designations were updated for the descriptions used in the current study. The c3c4 and c3(a) strains were selectively bred to produce the novel c3(a)c4 strain. The c3c4, c3(a), c4(a), and c3(a)c4 strains are available from Taconic Farms (Germantown, NY) through the Emerging Models Program (lines 1112, 1538, 1104, and 1802, respectively).

Histology

Organs were fixed in 10% buffered formalin and processed for paraffin embedding. Tissue sections (5 μm) were stained with H&E and evaluated microscopically for leukocytic infiltration and cyst scores.

Antinuclear Ab (ANA) immunofluorescence

Prepared HEP-2 slides were purchased from Diasorin (Stillwater, MN). Mouse sera were diluted 1/20 in 10 mM PBS, pH 7.3. A total of 25 μl of the diluted sera was incubated on the slide for 30 min in a dark, moist chamber and washed in 10 mM PBS in a Coplin jar for 10 min. Fluorescein-conjugated goat Ab to mouse IgG, IgM, and IgA (Valeant Pharmaceuticals, Costa Mesa, CA) was diluted 1/100 in PBS. A total of 35 μl of the diluted conjugate was placed on each well, and the slide was incubated 30 min in a dark, moist chamber and washed in PBS for 10 min. Slides were viewed and scored at ×400 magnification with a dry lens of a Leitz fluorescence microscope equipped with a mercury vapor lamp (Leica Microsystems, Bannockburn, IL).

Protein immunoprecipitation (IP)

Protein IP assays were set up, as previously described (12). Briefly, rapidly growing, undifferentiated K562 cells were grown at 37°C and 5% CO2 in DMEM supplemented with 10% FBS. Cells were collected in 200-ml conical tubes, centrifuged at 800 rpm for 8 min, washed once with prewarmed methionine-free DMEM culture medium, and centrifuged again. The medium was removed, and for each sample the following were added: 5 ml of methionine-free DMEM supplemented with 3% FBS, 50 μCi of [35S]methionine (ExpreSS; New England Nuclear, Boston, MA), and 1 × 10^6 K562 cells. The cells were labeled at 37°C overnight (8–16 h), collected, washed, and suspended in 5–10 ml of IP buffer: 10 mM Tris-HCl, pH 8.0, 500 mM NaCl, and 0.1% Igepal. They were then sonicated, and the sonicate was centrifuged for 10 min at 4°C at 14,000 rpm to remove debris. A
gels were dried and autoradiographed. Gels were enhanced by soaking for 30 min in 0.5 M sodium salicylate. The standards run on the same gel.

The addition of Na₂EDTA (pH 8.0) to a solution in PBS was transferred i.v. into the irradiated recipients; irradiated controls received PBS alone. The livers of the donors were examined histologically. An approximately linear fashion over time (data not shown); all mice with histological evidence of disease over age 30 wk. The histological scores for cyst formation and lymphocytic infiltration increased in an approximately linear fashion over time (data not shown); all mice with histological evidence of cyst formation had coexistent peribiliary lymphocytic infiltration histologically.

Component immunophenotypes in donor strains: NOD mice congenic for resistance alleles on chromosome 3 mice develop lymphocytic liver infiltrates not seen in other NOD congenic mice

Notably, like the NOD strain, neither of the parental strains that had been bred together to develop the NOD.c3c4 strain, c3 (NOD.B6 Idd9.1/9.2/9.3) and c4 (NOD.B10 Idd9.1/9.2/9.3), showed any hepatomegaly after aging for over 1 year (n > 100 females for each strain). However, we hypothesized that subclinical abnormalities potentially related to the liver disease might be present in one or both of these strains. Because the c3 and c4
strains used to develop the NOD.c3c4 strain are not extant, the examination of chromosome 3 and 4 congenic strains for component immunophenotypes was performed with related congenic strains (see Materials and Methods for strain derivation). NOD.c3(a) and NOD.c4(a) (Fig. 1). Upon histological examination, four of four 33-wk-old female NOD.c3(a) mice demonstrated peribiliary lymphocytic aggregations without cyst formation (Fig. 4). This abnormality was not present in NOD mice nor in NOD.c4(a) mice.

ANA testing was performed on each strain. Female NOD.c3c4 mice began developing positive ANA at ~12 wk, with 45% (5 of 11) positive at 16 wk and 83% (58 of 70) ANA⁺ at 22 wk and older (Fig. 5). NOD.c4(a) mice also had a high incidence of ANA positivity, with 12 of 23 female mice ANA⁺ at 20 wk and older. Most (greater than 95%) NOD, B6, and NOD.c3(a) mice were ANA⁻ (Fig. 5). In c3c4 mice, serum antinuclear autoantibodies generally formed after the development of histological disease. However, the correlation was not absolute because occasionally c3c4 mice were ANA⁺ while showing no histological evidence of biliary disease.

Autoantibody production was further investigated using IP. NOD.c3c4 mice demonstrated anti-Sm autoantibodies on IP, which are considered highly specific for systemic lupus erythematosus (SLE) in humans (Fig. 6). At 16 wk, 5 of 8 NOD.c3c4 mice tested were positive for anti-Sm Abs by IP. By 35 wk, 17 of 29 (58%) NOD.c3c4 mice were anti-Sm⁺. The specificity for Sm Ag was confirmed by RNA IP (data not shown). NOD, B6, and NOD.c3(a) mice were negative for anti-Sm Abs. NOD.c4(a) mice, however, formed anti-Sm Abs at a similar percentage to NOD.c3c4 mice, with 13 of 25 anti-Sm⁺ at 20 wk or older (Fig. 6).

**FIGURE 3.** Kinetics of liver and pancreatic histopathology in NOD.c3c4 mice. A–C, Progressive biliary cyst formation in 8 (A), 16 (B), and 40 (C)-wk-old NOD.c3c4 mice. D–F, Minimal pancreatic islet inflammation in the same individual mice. G–I, Progressive peribiliary tract lymphocytic infiltration in the same mice. Arrows point into biliary cyst cavities. Lymphocytes infiltrate the pericystic space.

**FIGURE 4.** Component immunophenotypes of donor strains: lymphocytic infiltrates were found in NOD.c3(a) (left), but not NOD (right) nor NOD.c4(a) strains.

Component immunophenotypes in donor strains: NOD.c4(a) mice develop antinuclear and anti-Smith autoantibodies not seen in other NOD congenic mice
Overall, the ~50% penetrance of anti-Sm Abs in the NOD.c3c4 and NOD.c4(a) mice is similar to that observed in the autoimmune MRL/Mp-lpr/lpr mouse (16).

**Anti-DNA autoantibodies in NOD.c3c4 mice**

We further characterized autoantibody production in the NOD, NOD.c3(a), NOD.c4(a), B6, and NOD.c3c4 strains by analyzing anti-ssDNA and anti-dsDNA Ab levels. The IgM dsDNA titer was significantly elevated in NOD.c3c4 mice aged less than 6 mo, but in none of the other strains (Fig. 7A). As the mice aged past 8 mo, only NOD.c3c4 mice demonstrated significant elevations of IgG dsDNA autoantibody compared with the other genetically related strains (Fig. 7A). The increase in titers was statistically equivalent to anti-DNA titers in NZB mice (Fig. 7B). NOD.c3c4 mice older than 8 mo also demonstrated increased IgM dsDNA and ssDNA, and IgG ssDNA autoantibody titers (Fig. 7C, and data not shown). Older female NOD.c3c4 mice demonstrated significant increases in titers of IgM dsDNA, IgG ssDNA, and IgG dsDNA compared with male mice (Fig. 7C), suggesting a more severe autoantibody response in female mice.

**Splenocytes from diseased NOD.c3c4 mice can transfer liver disease to naive irradiated NOD.c3c4 recipients**

One fundamental test of autoimmune etiology in experimental models is transfer of autoimmunity by lymphocytes from affected to unaffected mice (14). An autoimmune etiology for NOD diabetes was first suggested by the transfer of diabetes with splenocytes from diabetic mice (14, 15). We used the NOD splenic transfer protocol to test whether the liver disease could be transferred with immunocytes or whether the liver inflammation was secondary to a degenerative process intrinsic to biliary/hepatic cells. A total of $25 \times 10^6$ splenocytes, or PBS alone, was transferred from diseased older mice to young, irradiated NOD.c3c4 recipients. Five of seven splenocyte recipients developed histological evidence of liver disease, compared with one of four PBS recipients (Fig. 8 and Table I). Consistent with results in NOD, irradiation was protective of disease in absence of cotransfer of pathogenic splenocytes (14).

**Infectious agents are not cofactors for liver disease in NOD.c3c4 mice**

To exclude any contribution by an infectious agent, NOD.c3c4 mice were rederived by embryo transfer at Taconic Farms. The liver disease emerged with equal penetrance following rederivation, excluding a pathogenic bacterial/viral/parasitic etiology for our observations.

**Diabetes, not liver disease, is dominant in (NOD.c3c4 × NOD)F$_1$ mice**

To begin to dissect the relationship of the genetic control of T1D and the autoimmune liver disease, we tested for liver disease and diabetes in two F$_1$ crosses. Of 20 (NOD.c3c4 × NOD)F$_1$ mice aged to over 1 year, 9 developed diabetes and none developed liver disease. Thus, one dose of the chromosome 3 and 4 liver disease alleles is insufficient to induce liver disease in the context of the NOD background. (NOD.c3c4 × B6.H2$^{c7}$)$F_1$ mice showed neither diabetes nor liver disease. This result demonstrated that the B6/B10 alleles, which are homozygous in this F$_1$ (assuming B6 and B10 mice share disease alleles at both chromosomes), must interact with additional NOD genes present in a homozygous state on the NOD genetic background to generate the NOD.c3c4 liver disease phenotype. These results also demonstrate that a spontaneous dominant mutation is not the cause of liver disease in the c3c4 mice.

Autoantibody studies were performed in the F$_1$ mice. Seven of 10 (NOD.c3c4 × NOD)F$_1$ mice were ANA$^+$, and 4 of 5 tested by IP were anti-Sm$^+$. In contrast, 6 of 17 (NOD.c3c4 × B6.H2$^{c7}$)$F_1$ mice were ANA$^+$, and 0 of 17 were anti-Sm$^+$. This result demonstrates that c3c4 loci are dominant for autoantibody production when present on a homozygous NOD background. In contrast, anti-Sm autoantibodies cannot arise when the B6/B10 c3c4 alleles are homozygous on a heterozygous NOD/B6 background (see Table II).

**The presence of B6 Idd17 is not required for the development of liver disease**

To determine whether the same genes that contribute protection from T1D confer liver disease susceptibility or, alternatively, that the colocalization is due to linked loci, we have initiated the development of congenic strains that will continue to be susceptible to liver disease if the Idd genes themselves are also the liver disease genes. Because the NOD.c3(a) and the no longer extant NOD.c3 strains had the same degree of protections from diabetes (see Materials and Methods) and the NOD.c3(a) mice showed the
Component immunophenotype of liver infiltration (Fig. 4), we reasoned that the presence of a B6 allele at Idd17 may not be necessary to develop liver disease. We therefore developed the NOD.c3(a)c4 strain (Fig. 1), which contains resistant alleles from B6 at Idd3, Idd10, and Idd18, but is NOD at Idd17. We crossed the NOD.c3(a) mouse to the NOD.c3c4 mouse and by selective intercrossing developed the NOD.c3(a)c4 strain, which has significantly less B6 genetic material on chromosome 3 as compared with the NOD.c3c4 strain (Fig. 1). Preliminary results show that this new strain develops the NOD.c3c4 liver disease phenotype. Ten of 21 mice aged over 39 wk developed liver disease, excluding the B6 Idd17 allele as an essential genetic component for the development of liver disease, and significantly narrowing the chromosome 3 intervals implicated in liver pathogenesis.

Discussion
These results demonstrate that an autoimmune biliary tract disease arises when genetic intervals on chromosomes 3 and 4 from diabetes-resistant strains are introgressed onto the NOD autoimmune background. There is no autoimmune diabetes in NOD.c3c4 congenic mice, and conversely, there is no evidence of inflammatory liver disease in NOD mice. NOD strains congenic for diabetes-resistant regions on chromosome 3 or 4 alone show component immunophenotypes of the complete clinical syndrome. NOD.c3(a) mice demonstrate liver lymphocytic infiltrations, but neither cysts nor the autoantibodies found in the NOD.c3c4 and NOD.c3(a)c4 mice (Fig. 4), while NOD.c4(a) mice demonstrate some autoantibody formation, most notably anti-Sm Abs (Fig. 6), but no liver lymphocytic infiltrates (see Table II for summary). We hypothesize that the introgressed regions from chromosomes 3 and 4 individually contribute susceptibility to liver disease, but only when they interact with the NOD genetic background does a progressive, destructive liver disease occur.

The combination of the B10 c4 interval and the B6 c3 genetic intervals in NOD.c3c4 mice resulted in anti-Sm autoantibodies and anti-DNA Abs. The NOD and NOD.c3(a) strains, however, showed no autoantibodies, while the NOD.c4(a) mice produced antinuclear and anti-Sm Abs, but no dsDNA Abs. The capacity of the B10 Idd9 interval to generate autoantibody production on the NOD background is interesting in light of the overlap of this interval with a chromosome 4 region (Sle2) previously shown to predispose to B cell hyperactivity in New Zealand mixed lupus-prone mice (17, 18). The present studies suggest the possibility that the B10 Idd9 region may, in combination with NOD background genes, drive autoantibody production via a similar biochemical pathway as Sle1, 2, and 3 in the murine lupus model (18).

Anti-Sm Abs target proteins in the U small nuclear ribonucleoprotein (snRNP) family, which perform pre-RNA splicing in the nucleus. The Sm proteins are shared by many of the different U snRNPs; autoantibodies to specific U snRNPs are specific for different autoimmune syndromes (such as anti-U1 snRNPs, which are found for polymyositis, and anti-U2 snRNPs, which are seen in autoimmune overlap syndromes) (19). The emergence of anti-Sm autoantibodies was somewhat unexpected, as these are considered specific for SLE in human SLE and murine lupus models (19). Some groups, however, have reported ANA in unmanipulated NOD mice, but detected antinuclear and anti-Sm Abs after treatment with bacillus Calmette-Guérin (Mycobacterium bovis) (22). The same group subsequently showed that bacillus Calmette-Guérin acted via an adjuvant-like effect on APCs (23). Subsequently, this group demonstrated that M. bovis injection of NOD mice produced anti-Sm and anti-dsDNA reactivities of an IgG2a subtype, suggesting against immune deviation to Th2 as an etiology (24). Linkage analysis of the effect of M. bovis treatment on NOD mice was performed in a backcross to BALB/c mice, and identified loci on chromosomes 1 and 17 (25). Cha et al. (26) found that NOD Idd3/5 intervals introgressed onto the B6 background allowed detection of ANA, as well, suggesting that Idd3 and 5 intervals could contribute to the development of ANA production. Although these loci do not all overlap with the genetic intervals identified in our model, the general picture emerging from our studies combined with these previous studies is that either environmental (M. bovis, and possibly different mouse-room flora in different colonies) or genetic perturbations of the NOD immune system can drive it toward an SLE-like autoantibody response. As in many autoimmune syndromes, the pathological contribution of the autoantibodies is open to question; we will test the pathogenicity of the autoantibodies in future studies using a transfer system.

The outcross studies that we performed provided insights into the genetic control of liver disease in NOD.c3c4 mice. Because nearly 50% of the (NOD.c3c4 × NOD)F1 mice developed diabetes, but none developed liver disease, it is possible to conclude that
the two disease processes, T1D and the biliary autoimmune process, cannot occur simultaneously, as if the autoimmune response focuses on one organ system. However, the observed result of diabetes in the F1 mice could be explained by a recessive mode of inheritance for the liver disease as opposed to a dominant mode for T1D. Regardless, it is intriguing to speculate that the underlying genetic pathway controlling these two different organ-specific autoimmune syndromes may overlap substantially (require the NOD genetic background), but that the choice of target organ is influenced by other loci. This genetic result has implications for understanding the pathogenesis of autoimmune syndromes in humans. The occurrence of a diverse clinical set of autoimmune syndromes in a human kindred could be explained by a shared autoimmune-prone genetic background modified by additional genetic loci that then focus the clinical manifestations of the breakdown of tolerance. Similarly, one can hypothesize that autoimmune syndromes/diseases that appear radically different from a clinical perspective may share genetic/biochemical pathways that differ only at a few points.

The discovery of a spontaneous, autoimmune biliary tract disease in NOD.c3c4 mice raises the question of analogy to any similar human disease process. PBC has been described at a greater incidence in relatives of patients with T1D (3, 4). PBC is a human autoimmune disease characterized by lymphocytic infiltration into

![Figure 7](http://www.jimmunol.org/)

**FIGURE 7.** Anti-DNA Ab production by NOD.c3c4 and related NOD congenic mice. A, IgM anti-dsDNA Abs in male and female mice younger than 6 mo; at this age, there was no sex difference in Ab levels. NOD.c3c4 mice (n = 19) had a significantly increased IgM dsDNA level compared with B6 (n = 16), NOD (n = 4), and NOD.c3(a) (n = 7) (p = 0.003, 0.02, and 0.01); the p value compared with NOD.c4(a) mice (n = 7) was 0.09. B, IgG anti-dsDNA in female congenic mice older than 6 mo. NOD.c3c4 mice (n = 11) had a significantly increased level of IgG dsDNA compared with B6 (n = 4) NOD.c3(a) (n = 10), NOD.c4(a) (n = 11), NOD (n = 4), and

![Figure 8](http://www.jimmunol.org/)

**FIGURE 8.** Splenocytes transfer liver disease to naive recipients. PBS (left) or 25 × 10⁶ whole splenocytes (right) from 40-wk-old NOD.c3c4 mice were transferred to irradiated 7- to 13-wk-old NOD.c3c4 recipients. Mice were sacrificed after 3 mo, and livers were examined for pathology. Three experiments were performed. See Table I for complete results.

New Zealand White (n = 6) mice (p = 0.04, 0.002, 0.001, 0.04, and 0.02, respectively), but equivalent levels to NZB (n = 6) (p = 0.95). C, Male (n = 16) vs female (n = 11) NOD.c3c4 anti-DNA responses in mice aged older than 8 mo. Female mice had significantly higher IgM dsDNA, IgG ssDNA, and IgG dsDNA titers than male NOD.c3c4 mice (p = 0.003, 0.01, and 0.0006, respectively).

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the biliary epithelium; progressive biliary epithelial damage resulting in fibrosis, biliary tract obstruction, and ultimately liver failure; and a specific immune response to mitochondrial enzymes (the pyruvate dehydrogenase complex (PDC)) (27). A series of studies demonstrated that the anti-PDC Ab is predominantly directed to the E2 enzyme of the PDC complex (28–31). A second series of studies demonstrated T cell epitopes in the E2 complex, and support the hypothesis that PBC is a T cell-mediated disease driven by PDC-reactive autoimmune T cells with a Th1 phenotype (32–36). As we demonstrate in this study, NOD.c3c4 mice develop a spontaneous biliary tract disease characterized by lymphocytic portal tract infiltrations, biliary obstruction, and eventual mortality due to obstructive liver failure, and the disease is transferable by splenocytes, implicating likely T cell involvement. The major difference between NOD.c3c4 mice and murine PBC is that NOD.c3c4 mice developed biliary epithelial hyperplasia (resulting in cysts) rather than the biliary epithelial damage with fibrosis seen in humans. Nonetheless, it is possible that a different pathological response of murine biliary epithelia to infiltrating lymphocytes could be due to genetically determined differences in tissue injury response. To pursue this issue, future studies will determine B and T cell responses of NOD.c3c4 mice to murine pyruvate dehydrogenase.

The results from the current study, that a resistance region for one disease (TID) contains genetic elements that confer increased susceptibility to another lethal syndrome (biliary disease), complement the recent demonstration that a single NOD Idd susceptibility allele can contribute to the pathogenesis of two different autoimmune diseases, TID and experimental autoimmune encephalomyelitis (37). The concept of the NOD genetic background providing a pan-autoimmune diathesis that is focused by specific modification is consistent with the observation that the B7-2 knockout placed on the NOD background develops an autoimmune demyelinating disease, and that HLA-DQ8 placed on the NOD background produces autoimmune myocarditis (38, 39). The development of biliary tract disease, Sjogren’s syndrome (40), thyroiditis (41), and autoimmune diabetes in a set of genetically similar NOD and NOD congenic strains serves as a model for human pedigrees containing multiple autoimmune diseases, including diabetes and primary biliary cirrhosis. Our results interpreted in the context of these other studies demonstrate that a genetically determined autoimmune pathway emerges from the NOD background genes, which can be changed to a completely different autoimmune phenotype by the introduction of different chromosomal intervals.

### References


