Brief Definitive Report

B Lymphocytes Are Essential for the Initiation of T Cell–mediated Autoimmune Diabetes: Analysis of a New "Speed Congenic" Stock of NOD.*Igμ*^{null} Mice

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Summary

The T lymphocytes mediating autoimmune destruction of pancreatic β cells in the nonobese diabetic (NOD) mouse model of insulin-dependent diabetes mellitus (IDDM) may be generated due to functional defects in hematopoietically derived antigen-presenting cells (APC). However, it has not been clear which particular subpopulations of APC (B lymphocytes, macrophages, and dendritic cells) contribute to the development and activation of diabetogenic T cells in NOD mice. In the current study we utilized a functionally inactivated immunoglobulin (*Ig*)μ allele (*Igμ*^{null}) to generate a "speed congenic" stock of B lymphocyte–deficient NOD mice that are fixed for linkage markers delineating previously identified diabetes susceptibility (*Idd*) genes. These B lymphocyte NOD.*Igμ*^{null} mice had normal numbers of T cells but were free of overt IDDM and insulitis resistant, while the frequency of disease in the B lymphocyte intact segregants was equivalent to that of standard NOD mice in our colony. Thus, B lymphocytes play a heretofore unrecognized role that is essential for the initial development and/or activation of β cell autoreactive T cells in NOD mice.

Insulin-dependent diabetes mellitus (IDDM) in the nonobese diabetic (NOD) mouse model results from autoimmune destruction of pancreatic β cells mediated by both $CD4^+$ and $CD8^+$ T lymphocytes (1, 2). However, while T lymphocytes are clearly the final mediators of β cell destruction in NOD mice, there is evidence that these autoreactive effectors are generated as a consequence of functional defects in hematopoietically derived APC such as B lymphocytes, macrophages, and dendritic cells (2). Diabetes susceptibility (*Idd*) genes both inside and outside of the unusual *H2*^{g7} MHC haplotype appear to contribute to diabetogenic APC defects in NOD mice. However, the mechanisms whereby APC contribute to the development of autoimmune IDDM in NOD mice are unknown. Possibilities include a unique ability of NOD APC to process and immunologically present certain β cell proteins, and/or a reduced capacity to activate tolerogenic mechanisms that normally anergize or delete diabetogenic T lymphocytes. Another, not mutally exclusive, possibilty is that NOD APC preferentially induce β cell autoreactive $CD4^+$ T lymphocytes to secrete cytokines characteristic of a potentially pathogenic Th1 response, rather than those of a Th2 response that may dampen pathogenesis. However, it is not known if any of these potentially diabetogenic dysfunctions in NOD mice are exerted by all or particular subpopulations of APC.

It has been proposed that B lymphocytes may play a more critical role in the induction of immunological tolerance than other APC populations. This possibility is supported by reports that B lymphocytes have a greater capacity than other types of APC to induce T cell anergy and to preferentially activate Th2 rather than Th1 cytokine responses (3, 4). However, this conclusion was questioned by a recent report (5) demonstrating that peripheral T cell tolerance could be induced normally in mice made B lymphocyte deficient by congenic transfer of the previously described Igμ allele (6) that has been functionally disrupted by homologous recombination (originally abbreviated μMT^0, now formally designated *Igh6*^{tm1Cgn}, and here designated *Igμ*^{null} for clarity). Specific subpopulations of APC may also differentially contribute to IDDM susceptibility in NOD mice if they present varying mosaics of β cell autoantigens to T lymphocytes. Indeed, some proteins can only be antigenically processed and presented to T cells by B lymphocytes (7). This process may lead to the development of autoimmunity if B lymphocytes activate normally quiescent self-reactive T cells that had escaped tolerogenic mechanisms because the antigenic determinants they recognize are not

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normally processed and presented by other populations of APC (8). To understand the relative roles various APC subpopulations play in the development of autoimmune IDDM, we examined if pathogenesis was altered in a congenic stock of B lymphocyte–deficient NOD.$Ig\mu^{null}$ mice.

Materials and Methods

Development of a "Speed Congenic" Stock of NOD.$Ig\mu^{null}$ Mice. The $Ig\mu^{null}$ allele functionally disrupted by insertion of a neomycin resistance gene (*neo*^{r}) was backcrossed from the original chimeric stock with a mixed 129 and C57BL/6 (B6) genome (6) onto the NOD/Lt inbred background. The heterozygous carriers of the $Ig\mu^{null}$ allele used as breeders at each backcross generation were identified by typing DNA isolated from PBL by PCR with the primer set 5′-GCTATTCGGCTATGACTGGG-3′ and 5′-GAAGGCGATAGAAGGCGATG-3′, which generates a 706-bp product from within the *neo*^{r} insert. Each of 35 amplification cycles on a PTC-100 thermal cycler (MJ Research, Watertown, MA) consisted of denaturation at 94°C for 30 s, primer annealing at 60°C for 30 s, and extension at 72°C for 30 s. Using previously described PCR methodologies (9, 10), segregants from the fourth and sixth backcross (N5 and N7, respectively) generation were genotyped for the microsatellite markers shown in Table 1 that are linked to the indicated *Idd* loci. At the N7 backcross generation, $Ig\mu^{null/+}$ heterozygotes shown by these PCR analyses to be fixed as homozygous for NOD alleles at the indicated linkage markers of *Idd* susceptibility loci were intercrossed. PBL from the resulting F1 progeny were typed by two-color flow cytometric analysis (FACScan®, Becton Dickinson and Co., San Jose, CA) for the presence or absence of B lymphocytes using an FITC-conjugated goat polyclonal antiserum specific for mouse IgM (Southern Biotechnology Associates, Birmingham, AL) and the B220 specific mAb RA3-6B2 conjugated to PE. Segregants lacking B lymphocytes were scored as NOD.$Ig\mu^{null}$ homozygotes, and have been maintained by brother–sister mating. B lymphocyte intact N7F1 progeny were scored as wild-type $Ig\mu^{+/+}$ homozygotes if the *neo*^{r} insert could not be detected by PCR. N7F1 progeny carrying the *neo*^{r} insert, but positive for B lymphocytes, were scored as $Ig\mu^{null/+}$ heterozygotes. All mice were maintained under specific pathogen–free (SPF) conditions and allowed free access to food (Agway diet NIH 31A; PMI Feeds Co., South Henley, MO) and acidified drinking water.

Assessment of Diabetes, Insulitis, and Leukocyte Subsets. B lymphocyte intact and deficient segregants were monitored weekly for the development of glycosuria with Ames Diastix® (kindly supplied by Miles Laboratories Inc., Elkhart, IN). Glycosuric values of ≥3 were considered diagnostic of diabetes onset. Pancreases from mice that remained normoglycemic through 20 wk of age were fixed in Bouin's solution, sectioned at three nonoverlapping levels, and stained with aldehyde fuchsin for histological analysis of insulitis development. Islets (at least 25/mouse) were individually scored as follows: 0, no lesions; 1, periinsular leukocytic aggregates, usually periductal infiltrates; 2, <25% islet destruction; 3, >25% islet destruction; 4, complete islet destruction. An insulitis score for each mouse was obtained by dividing the total score for each pancreas by the number of islets examined. Data are presented as mean insulitis score ± SEM for the indicated number of B lymphocyte intact or deficient segregants. Splenic leukocytes from mice that remained non diabetic through 20 wk of age were typed by FACS® for the presence of B lymphocytes with a goat polyclonal antiserum specific for mouse Ig (Southern Biotechnology Associates), and for macrophages (MØ), $CD4^+$, and $CD8^+$ T lymphocytes with the mAbs M1/70, GK1.5, and 53-6.72, respectively.

Table 1. *Linkage Markers Analyzed to Fix NOD-derived Idd Loci to Homozgosity in the NOD.$Ig\mu^{null}$ Congenic Stock*

Idd locus/chromosome	Linkage marker homozygous for NOD allele	Relative microsatellite size
Idd1=H2^{g7}/17	**D17Mit34=C4*	B6=129>NOD
Idd2/9	**D9Mit25*	NOD>B6>129
Idd3/3	*D3Mit206*	NOD>129>B6
	**D3Nds6=Il2*	B6>NOD=129
	D3Mit95	NOD>B6=129
Idd4/11	*D11Mit115*	NOD>B6=129
	**D11Nds16=Acrb*	NOD=129>B6
	D11Mit320	129>NOD>B6
Idd5/1	**D1Mit5*	NOD=129>B6
	D1Mit18	NOD>B6=129
	**D1Mit46*	NOD=129>B6
Idd6/6	*D6Mit52*	B6=129>NOD
	D6Mit339	B6>NOD>129
Idd7/7	**D7Mit20*	B6=129>NOD
Idd8, Idd12/14	**D14Mit11*	B6>NOD
	D14Mit110	129>NOD> B6
	D14Mit222	NOD>B6=129
Idd9, Idd11/4	**D4Mit59*	NOD>B6=129
Idd10/3	*D3Nds11*	B6=129>NOD
	**D3Nds8=Tshb*	NOD=129>B6
	D3Mit103	B6>NOD>129
Idd13/2	*D2Mit395*	129>B6>NOD
	**B2m*	NOD=129≠B6‡
	D2Mit17	NOD>129>B6
Idd14/13	**D13Mit61*	NOD>B6=129
Idd15/5	**D5Mit48=Pgyl*	B6>NOD
	D5Mit69	NOD>B6=129

Microsatellites markers with the indicated allelic size variants were typed in N5 backcross mice and in the N7 segregants used for the intercross.

*Markers typed as homozygous for allelic variants characteristic of NOD in the single N5 female serving as the progenitor for all mice in subsequent backcross generations.

‡β2m allelic variants were typed as previously described (9) by BglI digestion of the PCR product.

Results

Genetic Characterization of the NOD.$Ig\mu^{null}$ "Speed Congenic" Stock. Heterozygous carriers of the $Ig\mu^{null}$ allele from the N5 backcross generation were genotyped by PCR for the markers denoted by an asterisk in Table 1 that were previously shown to be closely linked to the indicated *Idd* loci.

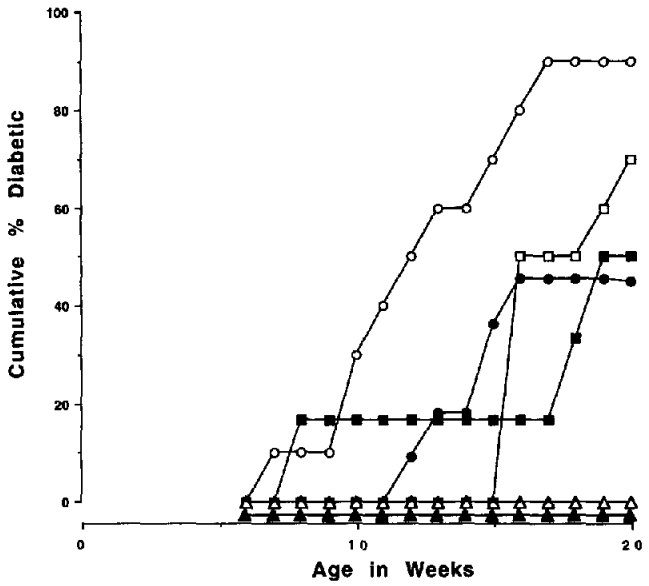

Figure 1. Diabetes development through 20 wk of age in NOD.$Ig\mu^{null}$, NOD.$Ig\mu^{null/+}$, and NOD.$Ig\mu^{+/+}$ segregants at the BC6 generation. Symbols: NOD.$Ig\mu^{null}$ females (n = 8, △) and males (n = 9, ▲); NOD.$Ig\mu^{null/+}$ females (n = 10, ○) and males (n = 11, ●); NOD.$Ig\mu^{+/+}$ females (n = 10, □) and males (n = 6, ■). Chi-squared analysis demonstrated that while significantly higher (P <0.01) than in B lymphocyte–deficient NOD.$Ig\mu^{null}$ male and females, the cumulative incidence of IDDM at 20 wk of age did not differ statistically (P >0.9) between sex-matched B lymphocyte–intact $Ig\mu^{null/+}$ or $Ig\mu^{+/+}$ segregants.

This analysis identified a single N5 female that was homozygous for allelic variants characteristic of NOD mice at all of these *Idd* linkage markers. This female served as the progenitor for all mice in subsequent backcross generations. We also genotyped the NOD, 129, and B6 parental strains for a series of additional microsatellites marking chromosomal regions carrying *Idd1-15*. This enabled us to identify the series of linkage markers shown in Table 1 that delineate NOD genomic elements from those derived from either the 129 or B6 strains. Typing of these markers confirmed the homozygous presence of NOD derived genome at all previously identified *Idd* loci in the N7 progenitors used for the intercross (Table 1).

NOD.$Ig\mu^{null}$ Mice Are IDDM and Insulitis-resistant. Currently, IDDM develops in 90% of standard NOD/Lt female and 63% of male mice at The Jackson Laboratory by 1 year of age. This susceptibility has been fully reconstituted in the speed congenic stock of NOD.$Ig\mu^{null}$ mice as evidenced by the IDDM incidence in the B lymphocyte positive segregants from the N7 backcross generation. By 20 wk of age, IDDM developed in 70% (7/10) of female and 50% (3/6) of male N7F1 NOD.$Ig\mu^{+/+}$ segregants. Similarly, IDDM also developed in 90% (9/10) of female and 45.5% (5/11) of male N7F1 NOD.$Ig\mu^{null/+}$ segregants. Mean insulitis scores of the B lymphocyte intact segregants that remained free of overt IDDM through 20 wk were 3.89 ± 0.10 (n = 4) in females and 3.28 ± 0.32 (n = 9) in males. The extensive insulitis and high frequency of IDDM in these two groups of B lymphocyte intact N7F1 segregants is equivalent to that observed in our standard NOD mice at the same age. This provided a functional confirmation of the linkage marker analyses (Table 1) demonstrating that the NOD-derived *Idd* loci necessary for disease development had been fixed in the N7 segregants. In marked contrast, IDDM failed to develop in any B lymphocyte–deficient female (0/8) or male (0/9) N7 NOD.$Ig\mu^{null}$ mice (Fig. 1). These NOD.$Ig\mu^{null}$ mice were also virtually free of insulitis as evidenced by mean insulitis scores at 20 wk of age of 0.31 ± 0.06 (n = 5) in females and 0.23 ± 0.02 (n = 5) in males. These scores reflected, at most, the presence of perivascular/periductal leukocytic aggregates, but no intraislet infiltrates. This virtual absence of insulitis in NOD.$Ig\mu^{null}$ mice indicates that IDDM development is not merely delayed, but that β cell–autoreactive T cell responses are not initiated in this stock. The $Ig\mu$ structural gene is located on distal chromosome 12 in a region where genome-wide scans have failed to detect any *Idd* loci. Collectively, these results indicate that elimination of B lym-

Table 2. *Splenic Leukocyte Profiles of NOD.$Ig\mu^{null}$, NOD.$Igm\mu^{null}1$, and NOD.$Ig\mu^{+/+}$ Segregants*

Genotype	Percent B cells	No. B cells (× 10^6)	Percent CD4 T cells	No. CD4 T cells (× 10^6)	Percent CD8 T cells	No. CD8 T cells (× 10^6)	Percent MØ	No. MØ (× 10^6)
$Ig\mu^{null}$	0.12	0.05	58.1	22.6	21.8	8.0	8.6	2.9
(n = 3)	± 0.05	± 0.03	± 3.6	± 8.2	± 0.7	± 2.6	± 1.4	± 0.8
$Ig\mu^{null/+}$	35.8	34.3	32.4	30.7	8.5	8.1	5.9	5.5
(n = 4)	± 1.2	± 3.0	± 1.1	± 2.4	± 1.2	± 1.4	± 0.6	± 0.5
$Ig\mu^{+/+}$	39.9	40.6	28.8	29.4	9.1	9.3	5.7	5.6
(n = 6)	± 1.2	± 4.1	± 1.0	± 3.1	± 0.7	± 1.1	± 0.5	± 0.4

Splenic leukocytes from BC6 segregants remaining nondiabetic through 20 wk of age were typed by FACS® as described in Materials and Methods for the presence of B lymphocytes, CD4 and CD8 T lymphocytes, and MØ. Data represent percentage or absolute number ± SEM of each leukocyte population.

phocytes by congenic transfer of the $Ig\mu^{null}$ mutation is sufficient to block IDDM development in an otherwise genetically susceptible NOD mouse stock.

Normal T Cell Lymphocyte Development in B Lymphocyte–deficient NOD.$Ig\mu^{null}$ Mice. It was important to determine if the absence of IDDM and insulitis in NOD.$Ig\mu^{null}$ mice resulted solely from the elimination of B lymphocytes, or whether this mutation also rendered mice T lymphopenic. Thus, we used FACS® analysis to compare the percentages and absolute numbers of B lymphocytes, $CD4^+$ and $CD8^+$ T lymphocytes, and MØ in the spleens of 20-wk-old NOD.$Ig\mu^{null}$, NOD.$Ig\mu^{null/+}$, and NOD.$Ig\mu^{+/+}$ segregants. As shown in Table 2, B lymphocytes were present and did not differ in proportion or absolute number in spleens of $Ig\mu^{null/+}$ and $Ig\mu^{+/+}$ segregants. As expected, B lymphocytes were absent in spleens of $Ig\mu^{null}$ segregants. The proportion of $CD4^+$ and $CD8^+$ T lymphocytes in spleens of $Ig\mu^{null}$ segregants was two to three times higher than in $Ig\mu^{null/+}$ and $Ig\mu^{+/+}$ segregants. However, this was due solely to the absence of B lymphocytes in mice homozygous for the $Ig\mu^{null}$ allele, since the absolute numbers of splenic $CD4^+$ and $CD8^+$ T lymphocytes were similar in all three segregation classes. Similarly, the proportion, but not the absolute number of MØ was also increased in spleens of $Ig\mu^{null}$ segregants compared with either class of B lymphocyte–intact segregants. When compared at 6 wk of age, total numbers of splenic T cells and MØ also did not differ between NOD.$Ig\mu^{null}$ and standard NOD mice. Thus, the genetic elimination of B lymphocytes does not appear to quantitatively effect the development of other leukocyte populations in NOD mice.

Discussion

It has been previously demonstrated that T lymphocytes from diabetic NOD donors can efficiently transfer disease to neonatal recipients depleted of B lymphocytes by treatment with a μ-specific mAb (11). Thus, after diabetogenic T lymphocytes have been generated they can clearly mediate destruction of pancreatic β cells in the absence of B lymphocytes. However, our current study demonstrates that neither IDDM nor significant levels of insulitis develop in B lymphocyte–deficient NOD.$Ig\mu^{null}$ mice that have fixed the necessary NOD-derived *Idd* susceptibility loci to permit disease development in the presence of B lymphocytes.

Verifying that all *Idd* loci normally necessary for IDDM development are fixed in an NOD congenic stock is an essential prerequisite when determining whether the genetic elimination of a particular immunological component affects pathogenesis. This is illustrated by two consecutive studies from the same group, which analyzed the development of β cell autoimmunity in NOD mice congenic for a segment of chromosome 14 derived from the 129 strain that contained a functionally inactivated TCR-α locus (12, 13). It was originally reported that in a heterozygous state, the inactivated TCR-α locus could block the development of β cell autoimmunity in NOD mice (12). However, as portions of the congenic segment recombined to NOD type with further backcrossing, it was subsequently found that the originally reported resistance was not due to the inactivated TCR-α locus, but rather to the presence of linked 129-derived resistance alleles at the previously identified *Idd8* and/or *Idd12* loci on chromosome 14 (13). Our genotypic analysis, coupled with the normal rate of disease development in the B lymphocyte–intact segregants, indicates that complete IDDM resistance in the NOD.$Ig\mu^{null}$ stock cannot be ascribed to the presence of 129 or B6 derived resistance alleles at any previously identified *Idd* loci. To date, when any single non-MHC *Idd* resistance locus has been congenically fixed to homozygosity on the NOD background, it has not resulted in complete IDDM and insulitis resistance in females as observed in our NOD.$Ig\mu^{null}$ stock. Thus, it is highly unlikely that the absence of IDDM and insulitis in our NOD.$Ig\mu^{null}$ stock is due to a previously unidentified recessively acting 129 derived resistance allele linked to the functionally inactivated $Ig\mu$ gene. This conclusion is further supported by the fact that while numerous *Idd* susceptibility and resistance loci with major to very minor effects have mapped in outcross studies of NOD mice with a number of different diabetes resistant strains, none of these have mapped to chromosome 12 where $Ig\mu$ is located (1, 14). Hence, these past studies coupled with our current findings clearly indicate that B lymphocytes play a heretofore unrecognized role that is essential for the initial development and/or activation of β cell autoreactive T cells in NOD mice.

The mechanism(s) by which B lymphocytes contribute to the initial development of diabetogenic T lymphocytes in NOD mice remains to be elucidated. It has been previously proposed that in very young NOD mice, autoantibodies secreted by B lymphocytes bind to pancreatic β cells, and that this triggers an autoreactive T cell cascade through an Ab-dependent cell-mediated cytotoxicity (ADCC) response (15). However, if such an ADCC mechanism is essential to diabetogenesis in NOD mice, it cannot be mediated by maternally transmitted autoantibodies, since equivalent degrees of disease resistance were observed in NOD.$Ig\mu^{null}$ segregants derived from either B lymphocyte intact ($Ig\mu^{null/+}$) or deficient ($Ig\mu^{null}$) dams. Future studies will examine the possibility that diabetogenic T lymphocytes are initially triggered by an ADCC response by determining if disease resistance is abrogated in NOD.$Ig\mu^{null}$ mice injected with Ig isolated from standard NOD donors with high islet cell autoantibody titers. Another, not mutually exclusive, possibility is that B lymphocytes contribute to the development of autoimmune IDDM in NOD mice through their role as APC. It has been reported that B lymphocytes may have a greater capacity than other APC to activate immunoregulatory functions that may block the development of IDDM, such as rendering autoreactive T cells anergic or skewing the pattern of cytokines they produce from a Th1 to a Th2 profile (3, 4). However, our finding that autoimmune IDDM is completely inhibited rather than enhanced in NOD.$Ig\mu^{null}$ mice indicates that the development and/or activation of β cell–autoreactive T cells is not normally blocked by such B lymphocyte–controlled immunoregulatory mechanisms.

In this way our results are in agreement with a recent report that B lymphocytes are not required for the induction of peripheral T cell tolerance (5). On the other hand, our data do not exclude the possibility that the diabetogenic function of B lymphocytes in NOD mice is as APC with a unique ability to process and present certain β cell autoantigens to autoreactive T cells. This possibility is currently being tested by determining if the B lymphocyte–deficient NOD.$lg\mu^{null}$ stock differs from standard NOD mice in ability to process and present certain candidate β cell autoantigens such as glutamic acid decarboxylase and insulin to T cells (16–18).

Most previous analyses of B lymphocyte function in the NOD mouse have focused on the spontaneous development of autoantibodies against putative islet cell antigens. Autoantibody production in NOD mice has been assumed to be a secondary consequence of β cell destruction, and hence, B lymphocytes have been regarded as accessories rather than primary instigators of pathogenesis. Our results clearly delineate a previously unidentified primary pathogenic role for B lymphocytes in IDDM initiation in the NOD mouse. The mechanism(s) by which B lymphocytes contribute to the initiation of autoimmune IDDM in NOD mice remain to be elucidated. However, knowledge of these mechanisms may provide new avenues for predicting and/or preventing the development of autoimmune IDDM in humans.

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