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*J Immunol* 2010; 184:859-868; Prepublished online 9 December 2009;
doi: 10.4049/jimmunol.0903149
http://www.jimmunol.org/content/184/2/859

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An NZW-Derived Interval on Chromosome 7 Moderates Sialadenitis, But Not Insulitis in Congenic Nonobese Diabetic Mice

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Autoimmune lymphocytic infiltration of the salivary glands, termed sialadenitis, is a pathologic feature of Sjögren’s syndrome (SjS) that is also prominent in nonobese diabetic (NOD) mice. Genetic factors regulate sialadenitis, and a previous (NOD × NZW)F2 study detected linkage to murine chromosome (Chr) 7. The locus, subsequently annotated as Ssial3, maps to the distal end of Chr7 and overlaps a region associated with type 1 diabetes susceptibility in NOD mice. To examine whether Ssial3 could contribute to both diseases, or was specific for SjS, we generated a congenic mouse strain that harbored an NZW-derived Chr7 interval on the NOD genetic background. This congenic strain exhibited reduced sialadenitis compared with NOD mice and confirmed Ssial3. This reduction, however, did not ameliorate saliva abnormalities associated with SjS-like disease in NOD mice, nor were congenic mice protected against insulitis (lymphocytic infiltration of the pancreatic islets) or diabetes onset. Thus, the Ssial3 locus appears to have a tissue-specific effect for which the NZW allele is unable to prevent other autoimmune traits in the NOD mouse. Anomalous increases for antinuclear Ab production and frequency of marginal-zone B cells were also identified in congenic mice, indicating that the NZW-derived Chr7 interval has a complex effect on the NOD immune system. The Journal of Immunology, 2010, 184: 859–868.

The nonobese diabetic (NOD) mouse strain spontaneously develops autoimmunity with prominent leukocytic infiltration of various tissues, including the pancreas and salivary glands (1). Because of this pathology, NOD mice have become a widely used animal model for type 1 diabetes (T1D) and Sjögren’s syndrome (SjS). In the case of T1D, infiltration of the pancreatic islets (insulitis) can be first detected in NOD mice by 4–5 wk of age, typically leading to a chronic level of β cell destruction resulting in hypoinsulinemia and hyperglycemia (2). In contrast, infiltration of the salivary glands (sialadenitis) is not detectable until ≥8 wk of age, and intrinsic abnormalities within the NOD salivary gland have been implicated in precipitating this infiltration (3–6). Although other mouse models exist for SjS, NOD mice appear to reflect more closely the human condition because of their age-related loss of saliva production concomitant to sialadenitis (7). It has also become apparent that the similar, yet distinguishable, autoimmune pathologies for T1D and SjS are dependent on genetic factors inherited within this unique autoimmune-prone mouse strain (8). One important challenge is to determine which of these factors contribute to dysregulation of the immune system and which contribute to tissue specificity of the autoimmune attack.

Linkage analyses have identified a number of loci contributing to T1D and SjS in various NOD outcross studies. Confirmation of these loci has been achieved predominantly using congenic mouse strains. To date, congenic NOD mice have confirmed T1D loci (termed Idd loci) on chromosomes (Chrs) 1, 2, 3, 4, 6, 7, 11, 13, 17, and 18 (2, 9, 10). Besides the MHC region on Chr17, at least five of these loci have been dissected into smaller intervals through analysis of subcongenic NOD mice, and strong evidence has pinpointed B2m, Ii2, Cila4, Nram1, and Tprv1 as likely genes for which NOD mice harbor TID susceptibility alleles (11–15). Preliminary evidence also suggests that Arnl2 and Stat5b are potential TID susceptibility genes (16, 17). In contrast to T1D, fewer linkage and congenic mouse studies have been performed to identify SjS susceptibility loci. Boulard et al. (18) detected linkage of sialadenitis to Chr1 and Chr3, termed Aec1 and Aec2, which overlapped two TID susceptibility loci, termed Idd5 and Idd3 respectively. This genetic overlap had been identified by an earlier study in which SjS-related phenotypes were reduced in NOD mice harboring congenic intervals for Idd5 and Idd3 (19). Conversely, placing the combined Idd5 and Idd3 NOD congenic intervals onto the C57BL/6 genetic background was sufficient to induce SjS-related phenotypes (20). A different NOD outcross study by Johansson et al. detected significant linkage to a locus on Chr4, but this locus does not appear to overlap the T1D susceptibility loci on...
designated an Idd7 (Fig. 1), suggesting that this locus may contribute to both T1D and SjS-like disease in NOD mice.

It was notable that the (NOD × NZW)F2 study only detected linkage to Chr7 and Chr1 (18). In both cases, the NZW mouse strain contributed a resistant allele to F2 mice with the Chr7 allele appearing to have a greater effect in males. The limited number of detected loci within this specific NOD outcross was perhaps not surprising, because NZW mice maintain a latent autoimmune predisposition (30, 31). As linkage to Chr1 had been confirmed by previous congenic mouse studies (19, 20), we generated a congenic mouse strain in which an NZW-derived Chr7 interval was fixed to homozygosity on the NOD genetic background. We report in this study the characterization of this congenic NOD strain for sialadenitis, T1D incidence, antinuclear Ab (ANA) production and marginal-zone B cells (MZBs).

Materials and Methods

Mice

NOD/Lt (NOD) and NZW mice were bred and maintained at the Walter and Eliza Hall Institute. NOD.NZW-Chr7−D7Mit23−D7Mit370 (NOD.NZW-Chr7) congenic mice were generated by backcrossing (NOD × NZW)F1 females to NOD males. Serial backcrosses were then performed using NOD males or females and appropriate backcross progeny based on genotyping results. An NZW-derived Chr7 interval was fixed to homozygosity on the NOD background by brother-sister matings using N10 generation mice. Genotyping was performed as previously described (32). Mice were housed in a conventional animal facility for the duration of the experiments. All animal experiments described within this text were performed in Australia and comply with the current laws of Australia regarding such experiments.

Histology

Pancreata and submandibular salivary glands were harvested from euthanized mice at 150 (±10) d old, immersed in Bouin’s fixative, and embedded in paraffin. Five consecutive sections were cut from each sample, ~100 μm apart and ~2 μm thick, and stained with H&E. Sialadenitis was evaluated as described by Cha et al. (20). Briefly, focus scores were calculated as the mean number of foci (aggregations of >50 mononuclear cells) per 4-mm² area of tissue after scoring whole tissue sections for total foci number. Five tissue sections per mouse were scored, compared with three tissue sections scored by Boulard et al. (18). Although we observed heterogeneity for the number of foci between tissue sections, foci having >50 mononuclear cells were readily detectable. Insulitis was evaluated in pancreatic sections by scoring each individual islet as described by Charlton et al. (33).

Measurement of stimulated saliva flow rate

Mice were fasted for 6 h with water ad libitum and injected i.p. with a secretagogue comprising pilocarpine (0.05 mg per 100 g body weight; Sigma-Aldrich, St. Louis, MO) and isoproterenol (0.2 mg per 100 g body weight; Sigma-Aldrich) dissolved in PBS. After 1 min, the mouse was restrained in the hand of the operator, and saliva was collected from the animal’s mouth using a micropipette for a period of 10 min. The total

![FIGURE 1.](image)

Schematic diagram of Chr7 and congenic NOD mouse strains. Marker positions (Mb, left column; cM, right column) are based on National Center for Biotechnology Information Build 37 and the Mouse Genome Informatics database. The diabetes incidence and markers for the depicted congenic intervals for NOD.CBALs-Tyr+/Lt (CBA-derived Chr7 interval), NOD.Lc7 (C57L/J-derived Chr7 interval), and NOD.B6-Gpi1b+/Lt (C57BL/6-derived Chr7 interval) mouse strains were previously described (28, 29). These congenic strains are depicted for comparison purposes described in the introduction and Discussion. The Idd7 and Ssial3 intervals are based on peak linkage scores as previously described (18, 25–27). The Idd7 interval, which has not yet been designated an Idd number, is based on diabetes incidence results for NOD.CBALs-Tyr+/Lt, NOD.Lc7, and NOD.B6-Gpi1b+/Lt mouse strains (28, 29). Diabetes incidence and sialadenitis are described relative to NOD mice as reported in this study or previously. <NOD, no statistical difference between congenic and NOD mice; <NOD, congenic mice had a significant decrease for T1D incidence; nd, not determined.
volume of saliva collected from each mouse was measured and normalized by weight. Samples were stored at −20°C.

Measurement of salivary protein concentration

Protein concentration in salivary samples was determined using the Bradford method per reagent instructions (Bio-Rad, Hercules, CA). Samples were diluted 1:8 in PBS, and 5 μl of each was added to a 96-well assay plate. BSA diluted in H2O was used as standards at concentrations of 0, 0.125, 0.25, 0.5, 0.75, and 1 μg/l; 200 μl Bradford reagent (Bio-Rad) was added to each standard and sample well. The plate was incubated for 15 min at room temperature. Absorbance readings were taken at 595 nm using a GENios microplate multidetection reader (Tecan, Manndorf, Switzerland).

Measurement of saliva amylase activity

Saliva amylase activity was determined using the Infinity Amylase Liquid Stable Reagent (Thermo Electron, Noble Park, Victoria, Australia) in which pNP-G7 is the substrate. Briefly, 7 μl of a 1:250 dilution of saliva was mixed with 280 μl Infinity Amylase Stable Reagent and incubated for 60 s at 37°C. Two absorbance readings with a 2-min interval were taken at 405 nm using a GENios microplate multidetection reader. Activity was calculated in units of activity (U) per liter according to the manufacturer’s instructions using the formula: amylase activity (U/L) = Δ Abs/min × 5140 × dilution factor.

Detection of proteolysis of parotid secretory protein

Aberrant proteolytic activity of an enzyme specific for parotid secretory protein (PSP) was detected using an adaptation of the method developed by Cha et al. (34). Synthetic peptide (EA VPQNLNLDVELLQQ, Mimotopes, Clayton, Australia) was dissolved at 1 mg/ml in 10 mM Tris-HCl pH 8.0; 8 μl saliva sample was mixed with 42 μl peptide and incubated at 42°C for a specified time period before addition of 50 μl of 50 mM Tris-HCl pH 8.0. All samples were filtered through a 10-kDa cut-off regenerated cellulose filter unit (Millipore, Bedford, MA) at 10,000 rpm using a Heraeus Biofuge pico centrifuge (Kendro Laboratory Products, Lane Cove, New South Wales, Australia) for 5 min then 13,000 rpm for 15 min at room temperature. Samples were automatically loaded onto a Jupiter 300-0.5 μm C18 column (250 × 4.6 mm internal diameter; Phenomenex, Torrence, CA) and chromatographed using an Agilent HP1100 system (Agilent, Palo Alto, CA). The chromatographic conditions were: 5 min loading and washing with 100% buffer A (0.1% trifluoracetic acid [Pierce, Rockford, IL] in Milli-Q water), followed by a 40-min linear gradient from 100% buffer A to 100% buffer B (80% acetonitrile [Mallinckrodt, St. Louis, MO], 0.088% trifluoracetic acid, in Milli-Q water) at a flow rate of 1 ml/min. A final 5-min equilibration to 100% buffer A was achieved before injection of the next sample by the autosampler. The signal was monitored at 214 nm. Control samples (peptide alone, buffer alone, saliva alone, filtered and unfiltered saliva, and peptide) were used to identify each of the peaks in the chromatograms.

Monitoring of spontaneous diabetes

Cohorts of female mice were maintained in a conventional animal facility and tested weekly from 50 d of age for elevated urinary glucose (>110 mmol/l) using Distastr reagent strips (Bayer, Pymble, Australia) over a 300-d period. Following three consecutive elevated urinary glucose readings, diabetes onset was confirmed with a tail-bleed using an Accu-Check Advantage blood glucose meter (Roche, Basel, Switzerland). Mice that did not become diabetic were euthanized at 300 d. Pancreata and submandibular glands were harvested from each mouse and assessed for lymphocytic infiltration as described above.

ANA detection

ANAs were detected by indirect immunofluorescence. Serum samples were diluted 1:100 in PBS; 20 μl diluted sample was placed onto each well of an HEP-2 coated slide (Immuno Concepts, Logan, UT) and incubated for 30 min in a humidified container. Each well was washed with PBS before addition of sheep anti-mouse IgG-FITC Ab (Silenus Labs, Boronia, Australia) and humid incubation for an additional 30 min. Slides were washed and cover slips mounted using Fluorescent Mounting Medium (Dako, Glostrup, Denmark). Slides were viewed by fluorescent microscopy and scored as described by Hesselstrand et al. (35). Each sample was assigned a score of 0, 1, 2, or 3, with 0 being no nuclear fluorescence, and 3 being high intensity staining. Double blind scoring was performed for all samples.

Flow cytometric analysis

Spleens were harvested from nondiabetic mice at ~70 (±5) or 150 (±10) d old. Cell suspensions were made by gently grinding each organ between the frosted ends of glass microscope slides (Menzel Glaser, Braunschweig, Germany) into PBA buffer (PBS containing 0.1% BSA and 0.1% Na3cit). RBCs were depleted using red cell lysis buffer (10 mM potassium bicarbonate, 150 mM ammonium chloride, 0.1 mM EDTA, 5% heat-activated FCS, in water). Cells were washed with PBA before labeling for flow cytometric analysis. Cell suspensions were labeled with the following fluorochrome-conjugated Abs: anti-B220 (RA3-6B2), anti-CD21, anti-CD1d, and anti-CD23 (B3B4; BD Biosciences, San Jose, CA). Secondary Abs were used as required. Calibration beads (BD Biosciences) were added to each sample prior to analysis for estimation of cell counts. All samples were run on a FACSCalibur (BD Biosciences) flow cytometer and analyzed using FlowJo software (Tree Star, Ashland, OR).

Statistical analysis

Pair-wise comparison of diabetes incidence curves was performed using a log-rank test. Comparison of ANA scores from different groups was performed using a Mann-Whitney U test. All other results were evaluated using unpaired Student t tests.

Results

The region containing Ssial3 is located on mouse Chr7 between D7Mit213 and D7Mit242, with the peak linkage occurring at D7Mit53 (Fig. 1) (18). To begin defining this locus, we generated a congenic NOD mouse strain that contained an NZW-derived interval for Chr7 on the NOD genetic background. Although the distal portion of the NZW interval was irrevocably lost during the generation of the NOD.NZW-Chr7 mouse strain, the NZW-derived interval still encompassed D7Mit53 (Fig. 1) and these congenic mice were characterized for Sis- and T1D-related phenotypes.

Severity of sialadenitis, but not insulinitis, is reduced in NOD.NZW-Chr7 mice

Sialadenitis appears as periductal lymphocytic foci within the glandular architecture of the NZW submandibular salivary gland. It has been previously noted that NOD females exhibit a higher sialadenitis incidence than NOD males with detection of Chr7 linkage most evident in (NOD × NZW)F2 males (36, 37). To assess this sex bias, female and male nondiabetic cohorts of NOD and NOD.NZW-Chr7 mice were examined for sialadenitis at 150 (±10) d old, the time at which linkage was initially detected (18). As expected, NOD females demonstrated a significantly higher inflammatory focus score than NOD males (p < 0.001; Fig. 2A). A similar trend was observed between congenic females and males, but congenic mice of both sexes demonstrated a significant reduction in lymphocytic infiltration of the salivary gland compared with NOD mice, but not as low as observed in NZW mice (Fig. 2A). This result provided confirmation and localization of the previous linkage to Chr7 (18), but suggests one or more of the other Ssial loci contributes to sialadenitis in the NOD.NZW-Chr7 congenic mouse. The reduction in lymphocytic infiltration was tissue-specific, because the NZW-derived interval did not result in a significant reduction in insulinitis between these same NOD and congenic mouse cohorts (Fig. 2B).

Saliva flow rate and composition are unaltered in NOD.NZW-Chr7 mice

The complications associated with SjS, such as dental caries and increased oral bacterial infections, are attributed in part to secretory hypofunction (38). Previous studies have shown that NOD mice older than 140 d, subsequent to sialadenitis, have a significant reduction in their capacity to produce saliva on stimulation (39–41). To determine whether the reduction of sialadenitis can ameliorate salivary gland dysfunction observed in NOD mice, we compared stimulated saliva flow rate between NOD, NOD.NZW-Chr7, and NZW mice. Stimulated saliva flow rate was measured in female and male cohorts (NOD and congenic mice were nondiabetic) after administration of a secretagogue at 150 (±10) d old. NOD...
and congeneric mice exhibited similar mean normalized flow rates, which were within the range of normalized rates observed for similarly aged NOD mice in other studies (Fig. 3) (42, 43). NZW mice exhibited higher mean saliva flow rates than NOD and congeneric mice, but because of large variation in male mice, only the female difference was statistically significant (Fig. 3). In either case, reduced sialedenitis did not result in improved saliva production in NOD.NZW-Chr7 mice compared with NOD mice.

In addition to sialedenitis and reduced saliva flow rate, the biochemical properties of saliva are also known to alter as NOD mice age and develop SjS-like disease. Elevated protein concentration and decreased amylase activity are often used as SjS markers in NOD mice (3, 39, 44). Saliva samples were further assessed for these other SjS-related phenotypes. As observed for saliva flow rate, the levels of saliva protein concentration and amylase activity were not significantly different (p < 0.05) between NOD and congeneric mice, except for a relatively small difference (p = 0.04) between NOD and congeneric females (Table I). Curiously, NZW mice exhibited the highest saliva protein concentration of the three strains tested despite the relative absence of sialedenitis (Table I).

Another biochemical marker of aberrant saliva in aged NOD mice is the increased presence of a serine protease that is capable of cleaving PSP (34). This proteolytic enzyme is yet to be identified, but its activity in saliva can be detected by quantifying cleaved PSP products using reverse-phase HPLC. Synthetic peptide containing the NLNL cleavage sequence from PSP was incubated with saliva samples obtained from the flow rate experiment. A preliminary experiment was performed using samples from two NOD males and two NOD females to quantify peptide cleavage at various incubation time points (0 min, 5 min, 30 min, 2 h, 6 h, 16 h; data not shown). Although not reported in previous studies (34, 45, 46), we observed a sex bias for peptide cleavage such that male saliva cleaved peptide more quickly than female saliva, presumably owing to male saliva containing a higher concentration of the NLNL cleavage sequence from PSP (Fig. 4B, 4C). After incubation for 16 h, however, both sexes of NOD, NOD.NZW-Chr7, and NZW mice demonstrated pronounced serine protease activity (mean peptide cleavage >30%) based on PSP peptide cleavage (Fig. 4C), suggesting that the NZW-derived interval for Chr7 does not significantly affect this SjS-associated phenotype in congeneric NOD mice. The combined saliva composition data also suggest that NZW mice, for which these traits have not been previously reported, may develop certain saliva abnormalities (PSP proteolytic cleavage, protein concentration), but they are not correlated with sialedenitis or saliva flow rate as observed in NOD mice.

Table 1. Saliva protein concentration and amylase activity

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Mean Protein Concentration ± SEM (µg/µl)</th>
<th>Mean Amylase Activity ± SEM (U/l × 10³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOD female (n = 14)</td>
<td>6.50 ± 0.87</td>
<td>1.86 ± 0.32</td>
</tr>
<tr>
<td>NOD.NZW-Chr7 female (n = 14)</td>
<td>4.27 ± 0.53*</td>
<td>2.55 ± 0.45*</td>
</tr>
<tr>
<td>NZW female (n = 20)</td>
<td>9.26 ± 0.95**</td>
<td>3.36 ± 0.42*</td>
</tr>
<tr>
<td>NOD male (n = 14)</td>
<td>6.31 ± 0.98</td>
<td>2.44 ± 0.34</td>
</tr>
<tr>
<td>NOD.NZW-Chr7 male (n = 17)</td>
<td>8.57 ± 0.88</td>
<td>2.50 ± 0.29</td>
</tr>
<tr>
<td>NZW male (n = 19)</td>
<td>9.81 ± 1.61</td>
<td>2.98 ± 0.33</td>
</tr>
</tbody>
</table>

*p < 0.05, in regard to NOD mice for given sex; **p < 0.05, in regard to NOD, NZW-Chr7 mice for given sex.
FIGURE 4. Detection of PSP proteolytic activity in saliva of female and male cohorts of NOD, NOD.NZW-Chr7 (Cg), and NZW mice. Saliva was collected from individual mice at 150 (± 10) d old and incubated with a synthetic peptide containing the PSP NLNL cleavage site for activated proteases. Saliva and peptide were incubated for 5 min and 16 h, respectively. A, Representative profiles of elution fractions from reverse-phase HPLC analysis. Chromatographic conditions are described in Materials and Methods. The intact synthetic peptide elutes at ~22.9 min (A, arrow) and the two cleavage products elute at ~17.5 and ~21.8 min (B, arrows). B, Proteolytic activity was measured as the percentage of the intact peptide peak area remaining after incubation. After 5 min of incubation, saliva from male mice exhibited greater PSP proteolytic activity compared with saliva from female mice. No difference was observed between female NOD and female congenic mice, but NOD males exhibited greater proteolytic activity than congenic males. C, After 16 h of incubation, saliva from all mice exhibited peptide cleavage. Unlike NOD and congenic females, all NOD and congenic males exhibited essentially complete digestion as little to no intact peptide was detected after incubation (B and C, bars indicate mean ± SE).
NOD.NZW-Chr7 mice develop T1D and have an increase in ANA production

Although no difference for insulitis was observed between NOD and NOD.NZW-Chr7 mice, diabetes onset was also assessed because previous congenic studies indicated that T1D could be delayed or prevented despite the presence of insulitis (22, 23, 28, 29). Female and male cohorts of NOD and NOD.NZW-Chr7 mice were monitored for diabetes onset for 300 d (Fig. 5). Similar to sialadenitis, females demonstrated a higher diabetes incidence, and these results reflect the values typically observed in the Walter and Eliza Hall Institute conventional animal facility for female and male mice (47). Whereas there was a noticeable difference between the diabetes incidence curves for NOD and congenic mice, the observed decrease was not statistically significant in either female or male comparisons between NOD and congenic mice ($p > 0.10$), as ascertained by a log-rank test. If there is a T1D susceptibility locus within this congenic interval, then NZW mice harbor an allele that has a relatively small effect on T1D pathogenesis when placed on the NOD genetic background.

The presence of serum ANAs is a diagnostic criterion in human SjS (38, 48), and also a systemic autoimmune phenotype in the NOD mouse strain, although the level of ANA production appears to vary between NOD mouse colonies (38, 43, 44, 48, 49). Serum was obtained from female and male cohorts of nondiabetic NOD, NZW, and NOD.NZW-Chr7 mice at 150 (±10) d old and assessed for the presence of ANAs. Different levels of ANAs were observed based on the presence of Ab to nuclear components of HEp-2 cells, with NZW mice having the lowest median score for the three mouse strains (Fig. 6). This low score for NZW mice was similar to previous reports (30, 31, 50, 51) and suggested that there is little to no ANA production in this mouse strain. Congenic females, on the other hand, had the highest median ANA score, which was significantly higher than that of NOD females, a difference not observed between congenic and NOD males (Fig. 6). This result indicates that the NZW-derived interval is able to exacerbate this autoimmune phenotype in a sex-specific fashion when placed on the NOD genetic background, and suggests an interaction between genes in this interval and the NOD background such that the congenic ANA production exceeds either of the parental strains.

NOD.NZW-Chr7 mice exhibit an increase in the frequency of splenic MZBs

Recent studies have highlighted the role of B cells in the development of SjS-like disease in mice (52). In particular, an increase in MZBs has been correlated with SjS-like disease in C57BL/6 congenic mice, harboring NOD-derived intervals for Idd3 and Idd5, and in C57BL/6 BAFF transgenic mice (46, 53). Although sialadenitis was reduced, we hypothesized that an increased level of MZBs may be correlated with saliva abnormalities (e.g., flow rate, PSP proteolytic activity) observed in congenic mice. Splenic lymphocytes isolated from female and male cohorts of nondiabetic NOD, NZW and NOD.NZW-Chr7 mice were assessed at ∼70 and ∼150 d old by flow cytometry to determine whether there was a difference for the MZB population at a time when sialadenitis was present in NOD mice. There was a detectable increase in MZB cell frequency from 70–150 d old for all mouse strains, but congenic female mice exhibited the highest number and frequency of MZB cells at 150 d old (Fig. 7). Congenic male mice also demonstrated an increased frequency in MZB cells compared with NOD and NZW males at 150 d old, but no difference was observed for total number (Fig. 7E, 7F). The reason for the difference in total number of MZBs between congenic female and male mice is not clear, but congenic females did exhibit more splenic lymphocytes compared with all congenic males, as well as NOD and NZW mice of both sexes at 150 d old (Fig. 7G). These differences also reflect the sex bias observed for sialadenitis (Fig. 2A) and ANA production (Fig. 6), specifically that females have more severe sialadenitis and higher ANA production compared with males.

Discussion

The initial step in testing the role of Ssial3 in SjS and T1D was to generate a congenic mouse strain for the previously linked region on Chr7 (18). In this study, we replaced the NOD Ssial3 Chr7 interval with the NZW-derived interval, which was predicted to be protective. The resulting congenic mouse strain, NOD.NZW-Chr7, demonstrated a significant reduction for sialadenitis compared with NOD mice. This result provided confirmation of the previous linkage of sialadenitis to Chr7. However, NZW homozygosity for the Ssial3 locus does not entirely eliminate salivary gland infiltration, because no female and few male NOD.NZW-Chr7 mice were completely free from sialadenitis as defined by the presence of lymphocytic foci. Boulard et al. (18) observed that NZW mice exhibit minor infiltration of the salivary glands by 300 d of age, despite having a protective allele for this locus. Thus, it is not unexpected that the NZW-derived Chr7 interval did not completely eliminate sialadenitis in congenic NOD mice. Other loci on Chr1 (Ssial1/Aecc2), Chr3 (Ssial2/Aecc1), and Chr4 (Nss1) have been shown to contribute to sialadenitis susceptibility, and protective alleles at these combined loci may be necessary to provide complete protection against SjS-like disease in NOD mice (19, 21). It was also noted that linkage to the distal end of Chr7 was strongest in males (18). Nevertheless, our results indicate that the NZW allele for this locus reduces sialadenitis in male and female congenic mice.

Despite significant reduction of sialadenitis in NOD.NZW-Chr7 mice, the NZW-derived interval did not ameliorate saliva abnormalities associated with SjS-like disease in NOD mice. Congenic mice exhibited similar saliva flow rates and little to no difference for saliva biochemical properties (e.g., amylase activity, protein concentration, PSP protease activity) compared with NOD mice.
A predominant model suggests that genetically influenced morphologic disruption of the salivary glands occurs via delayed organogenesis and anomalous acinar apoptosis. This morphologic disruption precipitates inflammation with subsequent lymphocytic foci initially consisting of T cells, followed by B cell recruitment. The onset of disease, defined principally by hyposalivation, is then mediated by the combination of acinar destruction, Abs, and proinflammatory cytokines, likely produced by invading lymphocytes (3, 6, 34, 41, 43). Conversely, it has been proposed that reduced salivary secretion occurs independently of sialedenitis, but inflammation, once established, exacerbates salivary gland dysfunction (42). The reduced sialedenitis observed in NOD.NZW-Chr7 mice, without the concomitant changes in saliva flow rate and composition, would seem to support this second model. However, NOD.scid mice lack development of sialedenitis and do not exhibit a loss of salivary gland secretory function (3). It is therefore likely that different levels of lymphocytic infiltration and destructive activity is required for overt SjS-like disease to develop in NOD mice. The relatively low level of sialedenitis in NOD.NZW-Chr7 mice is apparently enough to alter saliva flow rate and composition.

In contrast to reduced sialedenitis, female NOD.NZW-Chr7 mice exhibited an increase in ANA production, and both sexes exhibited increased splenic MZB frequency at 150 d old. These phenotypes were unexpected, because NZW mice demonstrated little to no ANA production and had a lower MZB frequency than congenic mice. The combination of the NZW-derived Chr7 interval on the NOD background appears to allow an epistatic interaction, leading to greater phenotypic expression not observed in either of the parental NOD or NZW mice. A similar increase in ANA production was observed in another congenic NOD strain, which harbored a C57BL/10 interval for Idd9.5 on Chr4, whereas C57BL/10 mice do not exhibit ANA production (54). Interestingly, the NZW-derived interval in the NOD.NZW-Chr7 strain overlaps the defined Sle3 locus, which is linked to ANA development in the NZW/B model of systemic lupus erythematosus (55). Subsequent congenic analyses dissected the Sle3 locus and demonstrated that an NZW-derived allele could increase ANA production when placed on the C57BL/6 background (56). Notably, increased ANA production in these three congenic mouse examples is the result of epistatic interactions. This might be expected as severe disease, including nephritis and high levels of ANA production, is not apparent in NZW or NZB, but develops in their F1 hybrid (57). Further studies of NOD.NZW-Chr7 mice are required to determine whether they develop other lupus-related phenotypes.

The role of B cells is critical for the development of overt SjS in NOD mice, because NOD.Igκnull mice maintain normal saliva secretion despite extensive leukocytic infiltration of the salivary gland (58). Alternatively, ANAs do not appear to have a direct role in SjS-like disease in NOD mice (43); however, our observed increase in ANA production suggests that the B cell compartment of NOD.NZW-Chr7 female mice may be adversely affected by the congenic interval. A potential reservoir of autoreactive B cells is the MZB population (59), which has been implicated in mouse models of SjS-like disease (46, 53). MZBs are also increased in NOD mice compared with non-autoimmune-prone mouse strains (60, 61). The larger increase in splenic MZB frequency for NOD.NZW-Chr7 mice as they age may contribute to a more pathogenic infiltrate, despite a decrease in the number of lymphocytic foci within the salivary glands of these mice. It will, therefore, be of interest to determine whether subsequent subcongenic mouse strains, which dissect the Chr7 interval, segregate these various phenotypes that differ between NOD and NOD.NZW-Chr7 mice.

Genetic studies indicate that there are overlapping and distinct loci that contribute to SjS and T1D in the NOD mouse. Chr1 and Chr3, in particular, have shown to harbor overlapping loci (Idd5/Aec1 and Idd9/Aec2) that can affect pathogenesis for both diseases (19, 20, 45). Sialadenitis, however, is not a direct result of diabetes onset because NOD mice that are congenic for a different MHC locus are protected from diabetes, but still develop sialedenitis and salivary gland dysfunction (44). Furthermore, congenic mouse strains for a number of non-MHC loci were also not protected from SjS-like disease (19). With this in mind, we monitored NOD.NZW-Chr7 mice for insulitis and T1D onset because these mice harbored a congenic interval that overlapped two different regions on Chr7 implicated in T1D pathogenesis (Fig. 1). Despite conferring reduced sialedenitis, this NZW-derived congenic interval did not have a significant effect on insulitis when placed onto the NOD genetic background. This result suggests that the NZW allele for Ssial3 has a more pronounced effect on sialedenitis compared with insulitis. At best, this congenic interval resulted in a small decrease in T1D incidence for which the size of our mouse cohorts was statistically underpowered to detect, whereas the increased frequency of splenic MZB, which has been implicated in T1D pathogenesis (61), was not associated with exacerbation of T1D onset in NOD.NZW-Chr7 mice.

The contribution of Chr7 to autoimmunity in NOD mice is complex. At least two loci on this Chr are implicated in T1D pathogenesis (18, 25–29). The congenic interval in NOD.NZW-Chr7 mice is relatively large (∼97 Mb) and partially overlaps both T1D loci (Fig. 1). Previous linkage studies indicate that non–autoimmune-prone mouse strains (NON, C57BL/6, C57BL10) harbor a susceptibility allele for a proximal T1D locus, termed Idd7 (25–27). The NZW-derived interval, however, did not exacerbate T1D in NOD.NZW-Chr7 mice, suggesting that either the Idd7 locus is proximal to the congenic interval or the NZW allele is different from that harbored by NON, C57BL/6, and C57BL10 mice. Interestingly, a C57BL/6-derived interval, which is encompassed by our NZW-derived interval (Fig. 1), was also unable to exacerbate T1D in congenic NOD mice (29). This result indicated that Idd7 mapped proximal to this C57BL/6-derived interval, but a subsequent study demonstrated that this interval could affect TCR expression levels and thymic deletion of diabetogenic T cells (62). It may be that there are actually two T1D loci on the proximal end of Chr7 as proposed by Gonzalez et al. (27) in their linkage study, in which case C57BL/6 alleles may be required at
both of these loci on the NOD genetic background to exacerbate T1D. The distal end of Chr7 was also confirmed to harbor a T1D locus by two independent congenic mouse strains (28, 29). These congenic strains harbor different but overlapping intervals derived from CBA/LsLt and C57L/J mice (Fig. 1). And whereas they may represent different loci, both strains demonstrated significant

**FIGURE 7.** Flow cytometric analysis of splenic MZB in NOD and NOD.NZW-Chr7 mice. Single cell suspensions of splenocytes were prepared from mice at ∼70 (B, C, D) or ∼150 (E–G) d old. Splenic lymphocytes were stained for B220, CD23, CD21, and CD1d. A, Representative FACS profiles of CD21 and CD1d Ab staining on B220CD21+ gated splenocytes. B–D, Proportion and number of splenic MZB cells and total splenic lymphocytes at 70 d old. Congenic mice exhibited relatively higher levels of MZB cells compared with NOD and NZW mice at this time point. E–G, Proportion and number of splenic MZB cells and total splenic lymphocytes at 150 d old. All strains exhibited an increase in MZB cell frequency at 150 d old, but congenic mice, in particular females, exhibited the largest increase from 70 d old and had the highest frequency and number of MZB cells compared with NOD and NZW mice at 150 d old. Bars indicate mean ± SE.
decreases in TID incidence (28, 29). Alternatively, NOD.NZW-Chr7 mice were not significantly protected, suggesting that a TID locus maps distal to the NZW-derived interval or that the NZW allele is different than that harbored by CBA/LtJ and C57L/J mice. SsJ and TID represent polygenomic autoimmune diseases with complex aetiologies. The congenic study presented here has confirmed Ssial3 on Chr7 as the fourth locus related to SsJ-like disease in NOD mice. Although the NZW-derived allele can moderate sialadenitis, it does not prevent the diagnostic symptoms. The congenic study presented here has a major superiority region on chromosome 1. J. Immunol. 186: 4192–4201.


References


Acknowledgments

We thank Melissa Smith, Gabriela Panoschi, Ketti Stoev, Andrea Morcom, Sarah Jones, and Alex Delbridge for technical assistance; Dr. David M. Tarlinton for providing Abs and technical advice; and Dr. Pablo Silveira and Dr. Colleen Elso for helpful discussions.

Disclosures

The authors have no financial conflicts of interest.


