Influence on Spontaneous Tissue Inflammation by the Major Histocompatibility Complex Region in the Nonobese Diabetic Mouse

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Abstract

We investigated the role of the major histocompatibility complex (MHC) region in the specificity of autoimmunity by analysing specifically the development of sialadenitis, but also insulitis, nephritis and autoantibody production in autoimmune-prone nonobese diabetic (NOD) mice where the MHC $H2^{g7}$ haplotype had been exchanged for the $H2^{q}$ (NOD.Q) or $H2^{p}$ (NOD.P) haplotype. The exchange of H2 haplotype did not affect the frequency of sialadenitis because the $H2^{q}$ and $H2^{p}$ congenic NOD strains developed sialadenitis with the same incidence as NOD. However, the severity of sialadenitis varied among the strains, as NOD.Q > NOD > NOD.P. At 11–13 weeks of age, the NOD.Q ($H2^{q}$) female mice developed more severe sialadenitis compared to NOD.P ($H2^{p}$) ($P = 0.038$). At 20 weeks, the NOD ($H2^{g7}$) female mice showed more severe sialadenitis than NOD.P ($P = 0.049$). This is in contrast to the development of insulitis in the present strains, because the incidence of insulitis was almost completely inhibited by the replacement of the $H2^{g7}$ haplotype of NOD. The incidence of insulitis in NOD.Q was 11–22%, compared to 75% in NOD, which correlated well with lower titres of anti-glutamic acid decarboxylase (anti-GAD) antibodies in NOD.Q compared to NOD ($P = 0.009$). However, the introduction of the $H2^{q}$ haplotype into the NOD strain instead directed the autoimmune response towards the production of lupus types of autoantibodies, because the incidence of antinuclear antibodies (ANA) in NOD.Q was 89% compared with 11% in NOD.P and 12% in NOD mice, which in turn correlated with a high incidence of nephritis in NOD.Q compared to NOD. Consequently, we show that different haplotypes of MHC are instrumental in directing the specificity of the spontaneous autoimmune inflammation.

Introduction

The major histocompatibility complex (MHC) gene region has been documented as an important player in susceptibility in several human autoimmune disorders as well as in animal models for such disorders.

Sjögren's syndrome (SS) is an autoimmune disease characterized by dry mouth (xerostomia) and dry eyes (keratoconjunctivitis sicca). The disease is characterized by lymphocytic infiltrates in the salivary and lacrimal glands [1]. This disorder is denoted as primary SS (pSS) when it exists alone (sicca syndrome) and as secondary SS (sSS) when combined with another autoimmune rheumatic disease. The aetiology of SS is unknown, but both genetic and environmental factors have been suggested to play a role in disease susceptibility [2]. Autoimmune diseases aggregate in families, and family and animal studies indicate that genetic susceptibility also confers a significant risk for pSS [3–6].

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The development of autoimmune diseases such as SS is complex and dependent on multiple genes and environmental factors. To date, the most consistent association for susceptibility to autoimmune disease has been with the MHC class II region complex [7–9]. This is also the case for SS, as certain MHC haplotypes have been associated with development of SS [10–13]. The MHC class II molecules function both by shaping the T-cell receptor (TCR) repertoire in the thymus and by selecting and presenting antigenic peptides to T cells in the periphery, but the exact mechanisms underlying these findings remain hypothetical.

Earlier studies have shown that the MHC region in the mouse (H2) is associated with autoimmune inflammatory diseases such as experimental autoimmune encephalomyelitis (EAE) [14], collagen-induced arthritis (CIA) [15] and insulin-dependent (type 1) diabetes mellitus (T1DM) [16]. For EAE, $H2^p$-expressing mouse strains developed a more severe disease than $H2^q$-expressing mouse strains, which correlated with a higher affinity of peptide binding to the $H2^p$ molecule as compared with the affinity for the $H2^q$ molecule [14]. In both EAE and CIA, the disease-associated gene in the MHC region has been identified as being the MHC class II Aβ gene, the DQ homologue in the mouse [14, 15]. Earlier studies of peptide-binding properties of the A^q and A^p molecules in CIA and EAE have shown that disease severity is associated with stronger binding by the disease-associated peptide to A^q and A^p, respectively [14, 17]. Susceptibility to diabetes is clearly controlled by the MHC region. [16]. Strong circumstantial evidence points towards a role of the A^{g7} molecule. However, the data are not conclusive because this molecule plays an immunosuppressive role, and transgenic overexpression of any A class II molecule tends to induce immunosuppression [18–20].

The nonobese diabetic (NOD) mouse (H2 I-A^{g7}) is used as a model for T1DM, thyroiditis and SS because of its spontaneous development of polyendocrine autoimmunity with chronic inflammation in several organ systems [21–27]. The most well-characterized manifestation in the NOD mouse is the diabetes where the first signs of inflammation in the islet of Langerhans (insulitis) are found at about 4 weeks of age. Most NOD mice develop insulitis, but a substantial number never proceed to the diabetic stage. In high-incidence colonies, 80–90% of the females and 40–50% of the males become diabetic after 3–7 months because of extensive β-cell losses in the pancreas.

The NOD mouse is also recognized as a relevant model for SS characterized by many of the features of the human disease [28]. NOD mice show focal lymphocytic infiltration in the salivary and lacrimal glands [25] and production of the autoantibodies such as anti-Ro/SSA and anti-La/SSB. Signs of sialadenitis appear by the age of 8–12 weeks in females and greater than 12 weeks in males and correlate with the appearance of lymphocytic infiltration of exocrine tissues [29]. The NOD mouse is the only strain described that, like patients with SS, shows a decrease in tear and saliva flow rate due to inflammation in lacrimal and salivary glands, respectively [26, 30].

In addition, even at 1 week of age, the NOD mouse may develop a mild thyroid inflammation, with an incidence ranging from a few per cent up to 80% [23]. Interestingly, the diabetes-resistant MHC congenic NOD strain, NOD.$H2^{h4}$ (I-A^k), shows extensive infiltration in the thyroid gland, resembling Hashimoto's thyroiditis [31, 32], suggesting an important role for the MHC region in the disease development.

Numerous studies have been conducted to search for genes influencing several autoimmune features in NOD [summarized in Ref. 33]. The NOD mouse offers a particular opportunity to study the relationship between the development of these different phenotypes and the genes that directs the autoimmunity to the different tissues.

The purpose of this study was to characterize the effect of different MHC (H2) haplotypes on the development of spontaneous tissue inflammation in the autoimmune-prone genetic environment of the NOD mouse. H2 congenic NOD mice, where the I-A^{g7} haplotype had been exchanged for I-A^q (NOD.Q) or I-A^p (NOD.P), were characterized regarding development and severity of inflammation in the salivary glands (sialadenitis), in the islet of Langerhans (insulitis) and kidney (nephritis) and development of autoantibodies.

Materials and methods

Congenic mice. Mice were bred and kept in conventional animal facilities under standard conditions at the animal unit, Section of Medical Inflammation Research, University of Lund, Sweden. The mice were kept in a climate-controlled environment with 12-h light/dark cycles and polystyrene cages containing wood shavings and fed with standard rodent chow and water *ad libitum* (as defined at http://www.inflam.lu.se). To create the NOD congenic mice, we crossed one group of mice from the NOD colony with the $H2^q$-positive strain C3H.Q (from D.C. Schreffler, St. Louis, MO, USA) and another group with the $H2^p$-positive strain C3H.NB (from Jackson Laboratories, Bar Harbor, ME, USA). The females from the F_1 generation of each cross were backcrossed to the males from the NOD ($H2^{g7}$) parental strain for 10 generations. The mice were then intercrossed three times to obtain $H2^q$ and $H2^p$ homozygous mice. Genomic DNA was extracted from tail biopsies of mice by standard procedures. MHC screening was performed for every generation of backcross and intercrossing breeding by using polymerase chain reaction (PCR) amplification with specific primers (Pharmacia, Biotech, Piscataway, NJ, USA) for the different MHC haplotypes: $H2^q$ forward primer 5′-GGGACGCGCATACGATC-3′

and reverse primer 5′-CCGCCGCAGGGAGGTGGG-GAC-3′ and $H2^p$ forward primer 5′-ATTTCGTGGCC-CAGTTGA-3′ and reverse primer 5′-CCGCAGGGA GGTGTGGGT-3′. The length of the congenic H2 fragment was detected using microsatellite markers polymorphic between the H2 haplotypes [39].

Histopathological techniques. For histopathological studies of sialadenitis, submandibular salivary glands were dissected, snap frozen in liquid nitrogen and stored at −70 °C until sectioning. Mice at two different ages were evaluated (11–13 and 20 weeks). Serial sections (6 μm) from submandibular glands were prepared in a cryostat and stained with hematoxylin and eosin and studied in a light microscope. The degree of inflammation was determined with a focus scoring (FS) based on the enumeration of inflammatory mononuclear cell (MNC) infiltrates (>50 MNC) per millimetre square of submandibular salivary gland, and greater than or equal to five different areas within focal infiltrates were counted. The total inflamed area relative to the submandibular salivary gland area was measured using a ratio index (RI) by the use of digitalized morphometric equipment (Videoplan, Kontron Bildanalyse, Eching, Germany).

Pancreatic tissue samples were taken from mice killed at 20 weeks of age, fixed in 4% paraformaldehyde for 24 h, dehydrated and embedded in paraffin. Serial sections (5 μm) were prepared and stained with hematoxylin and eosin. Insulitis was scored using a 5-grade scale as described previously [34]. Kidney was fixed in 4% paraformaldehyde for 24 h, dehydrated and embedded in paraffin. Serial sections (5 μm) were prepared and stained with hematoxylin and eosin. The degree of inflammation was determined with FS based on inflammatory MNC infiltrates (>50 MNC) per millimetre square.

Immunohistological technique. The primary antibodies used in the immunohistological analyses are as follows: for CD4 (T cells), H129.19, a rat IgG2a, κ (PharMingen, San Diego, CA, USA); for CD8 α chain (T cells), 53.6.7, a rat IgG2a, κ (PharMingen); and for CD11b (macrophages), M1/70.15, a rat IgG2b (Seralab, Leicestershire, UK). Rabbit anti-rat immunoglobulin G (IgG) (Vector Laboratories, Burlingame, CA, USA) was used as the secondary antibody. Frozen sections (6 μm) from submandibular gland tissue of mice at two different ages (11–13 and 20 weeks) were prepared in a cryostat. The sections were stained by using the avidin–biotin peroxidase complex (ABC technique; Dako A/S, Glostrup, Denmark) [35]. Briefly, the endogenous peroxidase activity in the sections was blocked with 0.3% H_2O_2 for 5 min, after fixing the sections in cold acetone. To reduce background staining in glandular tissue, we undertook pretreatment with Avidin solution (Vector Laboratories) for 15 min, followed by a 15-min wash with Tris-buffered saline (TBS), and finally, the slides were covered with Biotin solution (Vector Laboratories) for 15 min. After washing in TBS, the slides were incubated with blocking protein (normal rabbit serum, 1:20 in TBS) for 15 min. After the blocking procedure, the sections were incubated with the primary antibody for 30 min, followed by a brief wash in TBS before incubation with the secondary (biotinylated) antibody for 1 h. Binding of biotin-labelled antibodies was detected following stepwise incubation with avidin–biotin complexes (ABC) for 1 h and substrate consisting of H_2O_2 and 3-amino-9-ethyl-carbazol (AEC)-containing buffer for 15 min. Between each step, sections were washed with TBS for 5 min. Sections were counterstained with 50% Mayer's hematoxylin. As a negative control, 1% bovine serum albumin (BSA)/TBS instead of the primary antibody was used.

Counting of the numbers of $CD4^+$, $CD8^+$ and macrophage-positive cells infiltrating submandibular salivary glands was performed using a light microscope and 40× ocular fitted with a 10 × 10 graticule. For each group and each cell marker, approximately 1500 cells of the MNC population were counted. The total number of cells in corresponding areas did not vary significantly between the different mouse and age groups. Consequently, we used percentage figures to illustrate our findings. The average number of positive cells per field within each mouse was calculated, and the results are given as percentage of stained cells/total number of MNC.

Terminal-deoxynucleotidyl-transferase mediated dUTP–digoxigenin nick end labelling analysis. Frozen sections (6 μm-thick) of salivary glands were prepared in a cryostat and stored at −70 °C until used. The terminal-deoxynucleotidyl-transferase (TdT)–mediated dUTP–digoxigenin nick end labelling (TUNEL) method [36, 37] was used. Frozen sections were dried for 10 min at room temperature, fixed for 30 min in 10% paraformaldehyde (room temperature) and washed in TBS for 5 min. To neutralize endogenous peroxidase, we incubated sections with 0.1% H_2O_2 for 2 × 15 min at room temperature and subsequently washed them for 5 min in TBS. The sections were covered with TdT reaction buffer [0.5 M cacodylate, pH 6.8, 1 mM $CoCl_2$, 0.5 mM dithiothreitol (DTT), 0.05% BSA and 0.15 M NaCl] for 2 × 5 min at 37 °C. To label the apoptotic cells, we covered the sections with 75 μl of TdT reaction buffer containing 4 units of TdT and 2 μl of digoxigenin-conjugated dUTP (Boehringer-Mannheim, Mannheim, Germany) and incubated them at 37 °C for 1 h. After washing [once with TBS and once with TBS supplied with 2% fetal calf serum (FCS)], the sections were incubated with sheep anti-digoxigenin (5 μg/ml) diluted 1:200 in 2% FCS/TBS for 1 h. The sections were subsequently washed once in TBS and once in 2% FCS/TBS. The sections were then incubated with horseradish peroxidase (HRP)-conjugated anti-sheep IgG (5 μg/ml) (Dako) diluted 1:100 in 10% pooled human serum (PHS)/2% FCS/TBS for 1 h at room temperature. Next, the sections were washed once in TBS, and the bound HRP was detected by incubation

with 0.1 mg/ml of AEC in 0.17 M NaAc and 0.01% H_2O_2 at room temperature for 15 min. The sections were washed once again in TBS, counterstained in 50% Mayer's hematoxylin for 10 s, washed in running water and monitored under low-power light microscopy. Four mice from each group were evaluated at two different ages (11–13 and 20 weeks). Negative controls included tissue sections incubated as described above, but omitting TdT. Lymph nodes from MRL/*lpr* mice served as a positive control for apoptosis and were included in every run.

Serology. Antinuclear antibodies (ANA) in sera were measured by indirect immunofluorescence using Hep-2-cell–coated slides (BIOCHIP slides, Euroimmun, Lübeck, Germany) and a fluorescein-conjugated IgG Fc-specific anti-mouse (goat)-specific secondary antibody (Euroimmun). Samples were screened at a dilution of 1:10. Sera from MRL/*lpr* mice were used as positive control at a dilution of 1:100. Titres of anti-histone/dsDNA and anti-glutamic acid decarboxylase (anti-GAD) were measured individually in triplicates in sera collected from mice of age 11–13 and 20 weeks. Anti-histone/dsDNA antibodies were measured by enzyme-linked immunoadsorbent assay (ELISA) plates (Costar Corporation, Cambridge, MA, USA), coated 2 h in 37 °C with histone in phosphate-buffered saline (PBS) at 10 μg/ml (Sigma, St. Louis, MO, USA) and, after washing with PBS with Tween, coated with dsDNA in PBS at 50 μg/ml (Sigma) for 1 h at 37 °C. The plates were blocked with 2% FCS in PBS at 4 °C overnight, and sera were assayed at a dilution of 1:100. Peroxidase-conjugated anti-mouse IgG antibody was diluted in PBS containing 0.05% Tween. Results were calculated in milligrams per millilitre from a standard curve generated using positive sera from MRL/*lpr* mice. The levels of anti-GAD antibodies were measured by coating ELISA plates with recombinant human GAD65 protein (produced in baculovirus) (10 μg/ml in 0.1 mol/l $NaHCO_3$, pH 8.5) at 4 °C overnight, as described elsewhere [38].

Statistical evaluation. Incidence data, given in percentage of total number of affected animals, were evaluated using contingency summary table and χ^2 tests. The Mann–Whitney *U* nonparametric test was used to test differences in severity of tissue inflammation. Fisher's protected least significance difference (PLSD) test was used to evaluate antibody titres. A *P* value less than 0.05 was considered significant. Unless indicated, mean values and standard errors are shown.

Results

Incidence and severity of sialadenitis in the congenic and NOD strains

In this study, we investigated the autoimmune response in NOD mice compared with NOD mice where the $H2^{g7}$ haplotype of the NOD had been exchanged for the $H2^{q}$ and the $H2^{p}$ haplotypes by congenic breeding. In the NOD.Q ($H2^{q}$) mice, the $H2^{q}$ fragment had been previously shown to be 4.5 cM by using microsatellite marker screening of chromosome 17 including the MHC gene region [39]. The outer borders of the congenic fragment were defined by the markers D17Mit175 and D17Mit11 [39]. The $H2^{p}$ fragment of the NOD.P congenic mice was of similar size (data not shown).

We investigated the incidence and severity of sialadenitis in mice of two different ages, 11–13 as well as 20 weeks. Generally, male mice developed sialadenitis with lower incidence compared to female mice (Fig. 1A,B). However, within sexes, no significant difference in incidence was observed between the different H2 congenic NOD strains and NOD in the different age groups. In males, the

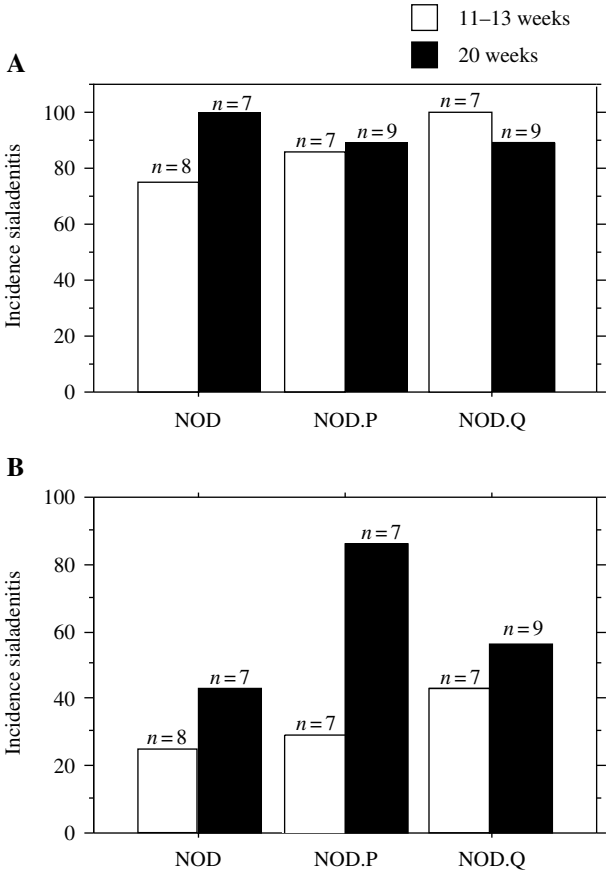

Figure 1 No difference in the incidence of sialadenitis in nonobese diabetic (NOD) and H2 congenic NOD mice. Incidence (%) of sialadenitis was determined by the presence of inflammatory mononuclear cell (MNC) infiltrates in frozen sections of submandibular salivary glands. Tissues were taken at 11–13 or 20 weeks of age. Serial sections (6 μm) from submandibular glands were prepared in a cryostat and stained with hematoxylin and eosin and scored in a light microscope. No difference in the incidence of sialadenitis in the H2 congenic NOD mice and that in either (A) female NOD or (B) male NOD mice was observed. In all three strains, the incidence of sialadenitis was increased by age. *n*, number of mice.

incidence increased with age (Fig. 1B), whereas in the females, the high incidence was already established at 11–13 weeks (Fig. 1A). However, the severity of sialadenitis was influenced by the H2 haplotypes. The severity was investigated by morphometric analyses of submandibular glands (Table 1) and measured by FS as well as RI. Generally, male mice developed milder disease (Table 1) compared to females. In females at the age of 11–13 weeks, NOD.Q had more severe disease as compared with NOD.P ($P = 0.038$). Although not statistically significant, a trend was noted towards more severe sialadenitis of NOD.Q and a less severe sialadenitis in the NOD.P compared to NOD.

At the age of 20 weeks, a similar trend was noted where female NOD mice had developed more severe disease than female NOD.P, when FS was compared ($P = 0.049$). The same relation was seen in male mice at the older ages (20 weeks), where the NOD.P developed a more severe disease than NOD ($P = 0.004$ for FS and $P = 0.006$ for RI) (Table 1). In summary, analyses of the severity at these two time points revealed a trend towards more severe sialadenitis in the NOD.Q strain compared to NOD and less severe sialadenitis in the NOD.P strain compared to NOD.

Immunohistochemical characterization of sialadenitis

To investigate whether the cell composition of sialadenitis in the H2 congenic NOD strains was different from that in NOD, we performed immunohistochemical analyses of different subsets of MNCs in the focal infiltrates. No differences of the percentages of $CD4^+$ T cells, $CD8^+$ T cells and macrophages infiltrating submandibular glands of NOD.Q, NOD.P and NOD strains could be observed (data not shown). A CD4 : CD8 ratio of less than 2 was noted in almost all 11- to 13-week-old strains. Twenty-week-old mice had increased average levels of $CD4^+$ T cells compared with levels in the younger mice. The $CD4^+$ cells and $CD8^+$ cells were evenly distributed in the infiltrates, whereas macrophages were mostly present in the periphery of the inflammatory foci.

Normal levels of apoptosis in salivary glands

Altered apoptosis has been put forward as a possible mechanism for pathogenesis in sialadenitis. Therefore, using TUNEL technique on frozen sections of salivary glands, we investigated the level of functional apoptosis in the NOD.Q and NOD.P mice compared to NOD. No apoptotic cells were detected in acini or ducts. Less than 0.1% of apoptotic cells were seen in the focal infiltrates, which indicates normal levels of apoptosis in all strains (data not shown).

Significant reduction of insulitis in $H2^q$ congenic NOD

The first sign of diabetes in the NOD mice is the inflammation in the islet of Langerhans (insulitis), normally found at about 4 weeks of age. We therefore investigated the influence of changing the H2 haplotype on insulitis in the NOD mice. Both the incidence and severity of insulitis were reduced in the NOD.Q mice as compared with those in the NOD mice (Fig. 2A). The titres of antibodies against the islet autoantigen GAD were reduced in the NOD.Q mice as compared with NOD mice (Fig. 2B), a finding that correlates with the reduction of insulitis in the NOD.Q mice.

Production of ANA and development of nephritis are more pronounced in $H2^q$ congenic NOD mice

Most aged NOD mice (>200 days old) have been shown to develop ANA and haemolytic anaemia [22]. The introduction of the $H2^q$ or the $H2^p$ haplotype increased the

Table 1 Severity of sialadenitis in nonobese diabetic (NOD) and major histocompatibility complex (MHC) congenic NOD mice

		n		FS $\times 10^{-1} \pm$ SEM		RI $\times 10^{-3} \pm$ SEM	
Strain	Sex	11–13 weeks	20 weeks	11–13 weeks	20 weeks	11–13 weeks	20 weeks
NOD	Female	8	7	2.5 ± 0.7	1.5 ± 0.3*	5.5 ± 1.9	4.8 ± 1.7
NOD.P	Female	7	9	1.2 ± 0.4†	0.8 ± 0.3*	3.1 ± 0.9‡	2.8 ± 1.4
NOD.Q	Female	7	9	2.7 ± 0.5†	1.6 ± 0.4	6.6 ± 1.1‡	11.0 ± 6.0
NOD	Male	8	7	0.2 ± 0.1	0.0 ± 0.03§	0.3 ± 0.2	0.1 ± 0.1¶
NOD.P	Male	7	7	0.2 ± 0.1	0.7 ± 0.2§	0.4 ± 0.4	4.2 ± 1.8¶
NOD.Q	Male	7	9	0.2 ± 0.1	0.3 ± 0.1	0.6 ± 0.3	0.7 ± 0.3

The degree of inflammation of the salivary glands was determined by focus scores (FS) and ratio index (RI) by counting infiltrating cells in greater than or equal to five different areas within focal infiltrates of frozen section. FS is the mean number of foci of mononuclear cell (MNC) infiltrates (>50 MNC) per millimetre square of submandibular salivary gland tissue. RI is the mean total focus (infiltration) area per total submandibular salivary gland area. The total inflamed area relative to the submandibular salivary gland area was measured using an RI. SEM, standard error of the mean.

*$P = 0.049$ NOD versus NOD.P.

†$P = 0.038$ NOD.Q versus NOD.P.

‡$P = 0.038$ NOD.Q versus NOD.P.

§$P = 0.004$ NOD versus NOD.P.

¶$P = 0.006$ NOD versus NOD.P.

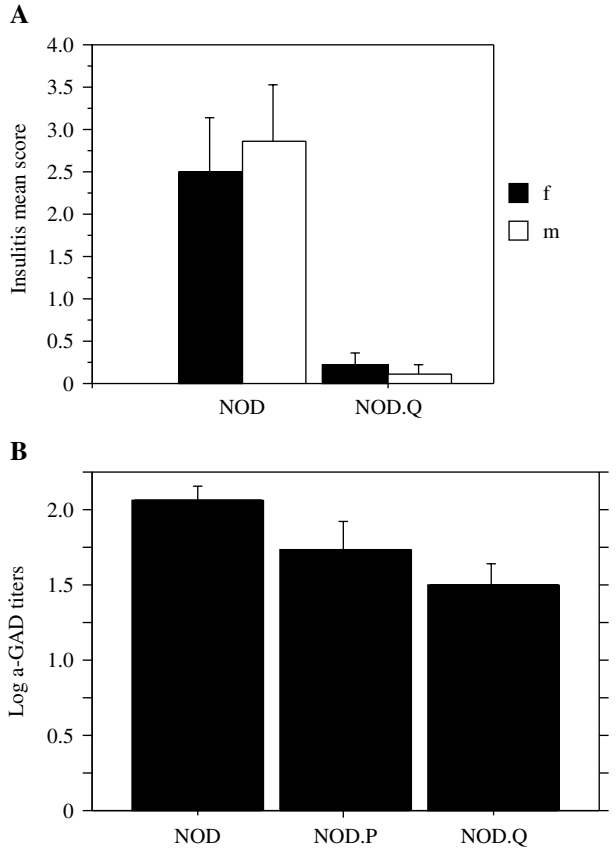

Figure 2 Exchanging the $H2^{g7}$ haplotype for $H2^{q}$ haplotype mediates reduced incidence of insulitis and production of anti-glutamic acid decarboxylase (anti-GAD) antibodies. (A) Pancreatic tissue samples were taken at 20 weeks of age. Tissues were fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. Serial sections (5 μm) were prepared and stained with hematoxylin and eosin. Insulitis was scored using a 5-grade scale as described previously [34]. The incidence of insulitis in the nonobese diabetic (NOD) mice was 75% in both females and males compared to the incidence of 22% in NOD.Q females (2/9) and 11% in NOD.Q males (1/9). The severity, expressed as mean score, was significantly lower in NOD.Q compared to NOD ($P_{female} = 0.009$, $P_{male} = 0.005$). The incidence of insulitis in NOD.P was not determined. (B) Titres of anti-GAD autoantibodies were assayed in sera from animals 20 weeks old. Anti-GAD antibodies were measured by enzyme-linked immunoadsorbent assay (ELISA) using recombinant human GAD65 protein (produced in baculovirus) as described elsewhere [38]. NOD versus NOD.Q $P = 0.0094$, NOD versus NOD.P $P = 0.061$ and NOD.P versus NOD.Q $P = 0.26$ [Fisher's protected least significance difference (PLSD)].

incidence of ANA production (Table 2). ANA production is a hallmark of human systemic lupus erythematosus (SLE) as well as of the lupus in murine models. The NOD.Q mice also developed nephritis, with a higher incidence than that in the NOD mice (Fig. 3), a finding that correlates with the higher incidence of ANA production in the NOD.Q mice compared to NOD mice.

In addition to the ANA production, we investigated the influence of the different H2 haplotypes on the development

Table 2 Incidence of antinuclear antibody (ANA) response in nonobese diabetic (NOD) and major histocompatibility complex (MHC) congenic mice

		ANA response at	
Strain	Sex	13 weeks (%)	20 weeks (%)
NOD	Female	1/8 (13)	0/8 (0)
NOD.P	Female	2/10 (20)	0/9 (0)
NOD.Q	Female	7/7 (100)	8/9 (89)
B10.Q	Female	ND	0/5 (0)
NOD	Male	0/8 (0)	0/8 (0)
NOD.P	Male	1/7 (14)	1/7 (14)
NOD.Q	Male	5/7 (71)	4/9 (44)
B10.Q	Male	ND	0/5 (0)

ANA were measured in sera from mice 13 and 20 weeks old by indirect immunofluorescence using Hep-2-cell–coated slides and a fluorescein-conjugated immunoglobulin G (IgG) Fc-specific anti-mouse (goat) specific secondary antibody. ND, not determined.

of anti-histone/dsDNA autoantibodies also associated with autoimmune response, in particular with lupus. The NOD.P strain developed lower levels of anti-histone/dsDNA antibodies compared to the NOD and NOD.Q stains ($P = 0.002$ and $P = 0.022$, respectively) (Fig. 4).

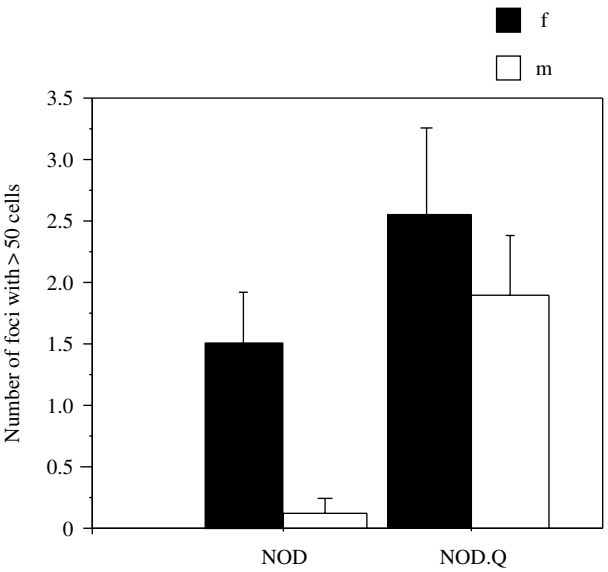

Figure 3 Introduction of the $H2^{q}$ haplotype into nonobese diabetic (NOD) mice mediates increased incidence and severity of nephritis. The severity of nephritis was assayed as the degree of inflammation determined by focus score (FS) based on inflammatory mononuclear cell (MNC) infiltrates (>50 MNC) per millimetre square. Kidney was taken from 20-week-old animals, fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. Serial sections (5 μm) were stained with hematoxylin and eosin. Incidence of nephritis in NOD females was 75% and in males 13%. In both female and male NOD.Q, incidence was 78%. The severity of kidney inflammation was significantly increased in the NOD.Q male mice compared to NOD males (P_{male} NOD versus NOD.Q = 0.01). The incidence of nephritis in NOD.P was not determined.

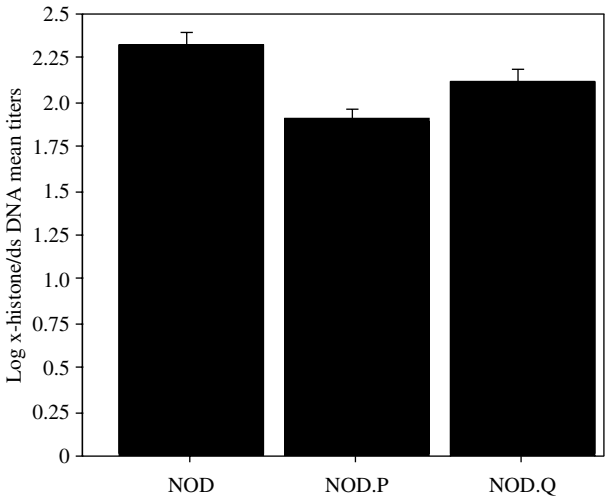

Figure 4 The $H2^p$ haplotype reduces anti-histone/dsDNA autoantibody production in nonobese diabetic (NOD) mice. Levels of anti-histone/dsDNA autoantibodies, expressed as $\log_{10}$ of titres in milligrams per millilitre, were assayed in sera from 20-week-old animals by enzyme-linked immunoadsorbent assay (ELISA). Sera from MRL/*lpr* mice were used as positive control. NOD.P mice produced lower levels of anti-histone/dsDNA autoantibodies compared to NOD and NOD.Q [$P_{\text{NOD–NOD.P}} = 0.002$ and $P_{\text{NOD.P–NOD.Q}} = 0.02$; Fisher's protected least significance difference (PLSD)].

Discussion

In this study, we investigated the influence of different MHC haplotypes on the development of autoimmune response in the NOD mouse, in particular the incidence and severity of sialadenitis. The MHC region is associated with susceptibility to several autoimmune diseases, and the $H2^{g7}$ haplotype of the NOD strain has been shown to be fundamental to the development of diabetes in the strain [16]. Our results show that the $H2^{g7}$ was in fact not critical to the development of sialadenitis in the NOD background, a finding that is supported by others [29]. The NOD.Q ($H2^q$) and NOD.P ($H2^p$) congenic strains showed levels of sialadenitis incidence that were similar to that in the NOD strain. The severity of sialadenitis was, however, influenced by the MHC haplotype, in that increasing severity was observed when exchanging $H2^{g7}$ for $H2^q$ and decreasing severity was observed when $H2^{g7}$ was exchanged for $H2^p$. We conclude that the cell composition of sialadenitis, including $CD4^+$ and $CD8^+$ cells and macrophages present in the inflammatory foci, in the H2 congenic NOD strains was not different from that in the NOD strain.

Notably, in humans, no clear association between SS and MHC class II has been documented, in contrast to the association observed, e.g., between multiple sclerosis (MS) and DR2. Rather, the MHC class II alleles are associated with the presence of autoantibodies in SS, such as anti-Ro/SSA and anti-La/SSB, which in turn are associated with leucopenia and lymphopenia and with more severe glandular disease [2, 40].

The insulitis in the NOD strain precedes the development of sialadenitis. However, the sialadenitis process seems not to be secondary to the diabetes development. Wicker *et al.* [16] have previously shown that NOD mice in which the $H2^{g7}$ segment is replaced with the $H2^b$ haplotype of C57BL/6 mice, called NOD.B10.$H2^b$, have loss of insulitis and diabetes susceptibility. However, this congenic mouse strain retained the focal lymphocytic infiltrates of the submandibular glands, reminiscent of SS-like disease. Further, biochemical and physiologic characterization of this congenic strain has shown that these mice exhibit many of the pathologic hallmarks of the parental NOD mouse, such as loss of secretory function and increased expression of apoptotic cysteine proteases [29]. We have shown that the NOD.Q strain, carrying the $H2^q$ haplotype, has a clear reduction in insulitis development, a finding that is in agreement with what was shown by Wicker *et al.* The NOD.Q strain has also been shown to be protected from developing diabetes [39].

However, after introducing the $H2^q$ haplotype into the NOD strain, we found that the autoimmune response shifted towards a more lupus type of response, with increased incidence of nephritis and higher production of ANA. Also, the pattern of production of other types of autoantibodies, in this study measured by anti-histone/dsDNA autoantibodies, was shifted by the change of H2 haplotype in the NOD background.

Results of previous studies are in keeping with our results, in that the $H2^{g7}$ haplotype is not essential for the development of sialadenitis in the NOD mouse. We have extended these studies by investigating the effect of the $H2^q$ and $H2^p$ haplotype on the development and severity of sialadenitis.

In the $H2^q$ haplotype, the E-genes are not expressed and the Aα-chain is identical with the Aα-chain in the $H2^p$ haplotype. The only difference is found in the Aβ-chains, which differ only at four amino acid positions in the peptide-binding groove [41], and the Aβ-chains have been shown to be crucial for the development of CIA [15]. Therefore, the difference we observed in the severity of sialadenitis is most likely related to the differences in self-peptide binding by the MHC class II haplotypes. It has been suggested that defects in the regulation of apoptosis may lead to autoimmune diseases such as SS [42–46]. It has been hypothesized that defective apoptosis of lymphocytes could lead to lymphocytic accumulation in exocrine glands [45], and increased apoptosis of epithelial cells has been suggested to explain the loss of secreting epithelium [47]. Furthermore, apoptotic cells may function as a source of autoantigen, leading to autoantibody formation [48–50]. However, we detected only very few apoptotic cells in submandibular salivary glands of these strains, indicating that increased levels of apoptosis are not fundamental to the pathogenesis of sialadenitis in the NOD mouse. This is in accordance with apoptotic activity of other murine models for SS such as MRL/*lpr* and $MRL^{+/+}$

[51]. However, we have not ruled out the possibility that the infiltrating lymphocytes may be blocked in the ability to undergo apoptosis.

These results show that MHC genes controlled sialadenitis severity, mainly in female mice. However, the effect was relatively weak, suggesting that the main contribution to the development of sialadenitis in the NOD strains is from non-MHC NOD genes. This is supported by the observation that C57BL/10.Q mice, harbouring the same $H2^q$ haplotype as NOD.Q, do not develop sialadenitis [39]. Recently, two separate genome-wide gene segregation experiments have been performed in crosses with the NOD strain to identify non-MHC genes involved in sialadenitis development [52, 53]. In the study by Johansson *et al.*, both parental strains carried the $H2^q$ haplotype, thereby equalizing the effect of the MHC allowing for the identification of non-MHC loci. A major locus, *Nss1* on chromosome 4, was shown to contribute to the development of sialadenitis in this cross. Interestingly, the *Nss1* locus does not overlap with any previously identified diabetes loci in NOD, suggesting a unique locus for sialadenitis development [52, 53]. Notably, an additional sialadenitis locus, the *Asm2*, has been located to chromosome 4 in a cross between MRL/*lpr* and C3H/*lpr* mice [54]. In the second genetic analysis of sialadenitis in NOD, two loci, located on chromosomes 1 and 3, were identified [52, 53]. These loci overlap the positions of the *Idd5* and *Idd3* loci, previously suggested to be involved in sialadenitis phenotypes of the NOD [55]. This has been recently confirmed in a double congenic strain for the *Idd5* and *Idd3* loci [56].

In conclusion, our studies of the NOD congenic strains confirm previous findings that the incidence of both diabetes and insulitis is dependent on the unique NOD MHC class II genes ($H\text{-}2^{g7}$), whereas the genetic contribution to susceptibility of sialadenitis in the NOD mouse has most probably a non-MHC II origin. An effect on the severity of sialadenitis and also on the development of ANA was, however, apparent, consistent with MHC class II association in human SS with autoantibody production rather than with prevalence.

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