

Experimental autoimmune myasthenia gravis in naive non-obese diabetic (NOD/LtJ) mice: susceptibility associated with natural IgG antibodies to the acetylcholine receptor

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Abstract

Naive non-obese diabetic (NOD/LtJ) mice spontaneously produce natural IgG autoantibodies against self-antigens associated with the experimental autoimmune diseases to which they are susceptible: insulin-dependant diabetes mellitus, systemic lupus erythematosus and experimental autoimmune encephalomyelitis. We discovered recently that NOD/LtJ mice also spontaneously produce IgG antibodies to the acetylcholine receptor (AChR), an antigen that can induce experimental autoimmune myasthenia gravis (EAMG) in susceptible rodents. However, there are no reports indicating that NOD/LtJ mice are susceptible to EAMG. To test whether the presence of spontaneous IgG autoantibodies can predict susceptibility to an autoimmune disease, we challenged NOD/LtJ mice using a standard protocol to induce EAMG. We now report that NOD/LtJ mice developed EAMG, although to a somewhat lesser degree than did C57BL/6 mice, a strain regarded as highly susceptible to the disease. Both strains produced comparable levels of immune antibodies to AChR of the complement-fixing isotypes IgG2a and IgG2b; however, NOD/LtJ mice produced significantly more IgG1. An antigen-specific T cell proliferative response to AChR of the same magnitude was detected in both strains, together with the secretion of similar amounts of IFN- γ . Thus, NOD/LtJ mice are susceptible to EAMG and disease induction is accompanied by immune responses comparable to those seen in the susceptible strain C57BL/6. These results support the association between specific, natural IgG autoantibodies and susceptibility to the induction of a particular autoimmune disease.

Introduction

In a previous work, we analyzed the repertoire of natural autoantibodies in naive non-obese diabetic (NOD/LtJ) and C57BL/6 mice (1). Surprisingly, the natural IgG autoantibodies detected in both strains correlated with the autoimmune disease susceptibilities known to be associated with each strain. This suggested that specific natural IgG autoantibodies might indicate susceptibility to the induction of a particular autoimmune disease. Table 1 shows the natural IgG reactivities detected when pooled sera diluted 1:100 were tested against a panel of 54 self-antigens. Among the several reactivities found, both NOD/LtJ and C57BL/6 mice displayed IgG antibodies to the self-acetylcholine receptor (AChR) (1). Autoantibodies to AChR characterize both human myasthenia gravis and experimental autoimmune myasthenia gravis (EAMG) (2–4). However, only the C57BL/6 strain was known

to be susceptible to EAMG; there were no reports describing the induction of EAMG in the NOD/LtJ strain. Thus EAMG in NOD/LtJ mice could be viewed as a test of the relationship between specific IgG natural autoantibodies in naive animals and their susceptibility to induction of a particular experimental autoimmune disease. Therefore, we challenged NOD/LtJ mice using a standard protocol of immunization known to be effective in inducing EAMG in the susceptible C57BL/6 strain.

Methods

Mice and antigens

Eight-week-old female C57BL/6 and NOD/LtJ mice were bred in the animal facility of The Weizmann Institute of Science. The

Table 1. Natural IgG autoantibodies in naive NOD/LtJ and C57BL/6 mice

Strain	Antigen	IgG levels (OD _{405 nm}) ^a	Associated immunopathology	Reference
NOD/LtJ	double-stranded DNA	0.63	SLE	(49)
	single-stranded DNA	1.29	SLE	(49)
	glutamic acid decarboxylase	1.64	IDDM	(8)
	60-kDa heat shock protein	0.64	IDDM	(8)
	myelin basic protein	0.69	EAE	(48)
	myelin oligodendroglial glycoprotein	1.14	EAE	(48)
C57BL/6	AchR	1.58	EAMG	this paper
	myelin oligodendroglial glycoprotein	0.40	EAE	(51)
	AchR	0.36	EAMG	(2)

^aNatural autoantibodies at a dilution of 1:100 (1).

mice were maintained under pathogen-free conditions, and experimental protocols were approved by the Institutional Animal Care and Use Committee.

Torpedo californica AchR was generously provided by Professor Sara Fuchs (The Weizmann Institute of Science). BSA was purchased from Sigma (Rehovot, Israel).

Induction of EAMG

Mice were immunized intradermally in the hind footpads with 10 µg of *T. californica* AchR emulsified in complete Freund's adjuvant (CFA; Difco, Detroit, MI) enriched to 10 mg/ml of *Mycobacterium tuberculosis* H36 Ra (Difco) on day 0 and boosted with the same dose on day 30. Mice were observed for signs of muscle weakness, bled for the determination of serum antibody levels and sacrificed for the study of the T cell responses of their spleen cells.

Evaluation of clinical EAMG

The mice were examined every other day for signs of muscle weakness after exercise, in which each mouse was gently pulled backwards 10 times over a cage top, allowing the animal to grip the cage wires. The scale used to grade disease was as follows: 0, no weakness; 1, muscle weakness after exercise; 2, weakness at rest before exercise; 3, severe weakness, paralysis, dehydration and a moribund state (5). The results are presented as the percentage (incidence) of sick animals and the mean maximal disease score per group.

Hyperglycemia

Blood glucose was measured using a Beckman Glucose Analyzer II (Beckman Instruments, Brea, CA) as previously described (6). A mouse was considered diabetic when it presented a blood glucose level >13 mM.

Detection of antibodies by ELISA

Immune antibodies were measured using an ELISA assay in flat-bottom microtiter plates (Maxisorb; Nunc, Roskilde, Denmark) coated overnight with 50 ng/well of AchR or BSA in carbonate buffer at 4°C. The plates were blocked with 1% skim milk for 1 h at 37°C. To detect the antibody response to immunization, serum samples were diluted 1:500 in skim milk [natural autoantibodies were detectable only at lower dilutions (1:100) (1)]. The diluted sera were added to the plates and

incubated for 3 h at 37°C. Bound antibodies were detected using alkaline phosphatase-conjugated goat anti-mouse IgG or IgM (Jackson ImmunoResearch, West Grove, PA) together with Sigma's (St Louis, MO) substrate for alkaline phosphatase. After 30–60 min, absorbance at 405 nm was measured. To study the isotypes of the antibodies directed to AchR, the same protocol was repeated using isotype-specific secondary antibodies (PharMingen, San Diego, CA) conjugated to alkaline phosphatase.

T cell proliferative responses

Spleen cells were taken from immunized mice 25 days after the boost and 3×10^5 cells/well were cultured as previously described (6) in quadruplicate in round-bottom plates (Nunc; Nunc) for 72 h in the presence of different concentrations of either *T. californica* AchR or BSA. [³H]Thymidine (0.5 µCi 5 mCi/mmol; Amersham, Little Chalfont, UK) was added to the cultures for the last 18 h of incubation. Concanavalin A (Con A; Sigma) 1.25 µg/ml was used as a positive control. Thereafter, cells were harvested and the c.p.m. counted. The results are expressed as the stimulation index: the mean c.p.m. of cultures incubated with antigen divided by the mean c.p.m. of cultures incubated without any antigen.

IFN-γ release

Spleen cells were prepared from immunized mice 25 days after the boost and 3×10^5 cells/well were cultured as previously described (6) in quadruplicate in round-bottom plates (Nunc) for 72 h in the presence of different concentrations of either *T. californica* AchR or BSA. The supernatants were collected and analyzed for IFN-γ content by ELISA using an appropriate pair of capture and detecting mAb (PharMingen) according to the manufacturer's instructions. The results are expressed as pg/ml, based on a calibration curve constructed using known amounts of recombinant IFN-γ (PharMingen)

Statistical analysis

Student's *t*-test included in the Instat Package for Macintosh was used for statistical analysis of the data.

Results

Induction of EAMG in NOD/LtJ mice

Fifteen NOD/LtJ mice were immunized with AchR in CFA, boosted 1 month later and followed for clinical signs of the disease. In parallel, a group of 15 mice of the susceptible C57BL/6 strain was subjected to the same protocol of immunization as a positive control. None of the mice showed clinical signs of EAMG after the first immunization. However, 5 days after the boost (35 days after the first immunization), muscle weakness was detected in several immunized NOD/LtJ and C57BL/6 mice (Fig. 1). The mean maximal disease score (\pm SE) in the C57BL/6 group was 0.73 ± 0.12 , reproducing a previous report by others (7), and 0.47 ± 0.13 in NOD/LtJ mice. No significant statistical differences were detected in the incidence or the mean maximal score of these two groups. However, no sick animals were found in control groups made up of 15 NOD/LtJ or 15 C57BL/6 mice immunized with BSA in CFA under the same immunization scheme (data not shown). Fifteen days after the boost with AchR, the disease began to subside in the NOD/LtJ mice and it almost completely disappeared within the following 12 days.

NOD/LtJ mice spontaneously develop insulin-dependent diabetes mellitus (IDDM) (8); if untreated, their physical condition gradually deteriorates until they die. To exclude the possibility that the weakness resulted from the physical deterioration that follows the onset of diabetes and not from the induction of EAMG, we checked the blood glucose levels of the NOD/LtJ mice after the induction of EAMG. None of the NOD/LtJ mice used in the experiments described here manifested overt hyperglycemia during the 60 days that followed the boost (data not shown). Furthermore, BSA- and AchR-vaccinated NOD/LtJ mice manifested a significantly reduced incidence of hyperglycemia at the age of 12 months (0 and 25% respectively, $P < 0.0001$ compared to naive NOD/LtJ mice), an age when non-treated NOD/LtJ mice of our colony reach an incidence of diabetes of 85%. The arrest of the diabetogenic process is probably due to the use of CFA as an adjuvant for the induction of EAMG; CFA has been previously shown to inhibit the onset of hyperglycemia in NOD mice (9–11).

Antibodies to AchR

We studied the appearance of immune antibodies to AchR at different points during the immunization protocol: before immunization (day 0), and at 15 and 45 days after the first immunization. The conditions of the assay were adjusted to detect the production of immune antibodies beyond the background of pre-existing natural autoantibodies to AchR (1). Hence, to measure the antibodies induced by the immunization, we diluted the serum 1:500 and used skimmed milk for the blocking step; the detection of natural autoantibodies to AchR was done at a 1:100 dilution after blocking with BSA (1). Immune autoantibodies could also be detected at a dilution of 1:1000 (not shown). Figure 2 depicts the IgG and IgM antibody responses to the immunizing antigen, AchR, and to a control antigen, BSA. NOD/LtJ mice showed increased amounts of IgG antibodies to AchR after the first immunization, while no increase in AchR-specific IgG antibodies above background was yet detectable in C57BL/6 mice ($P < 0.05$).

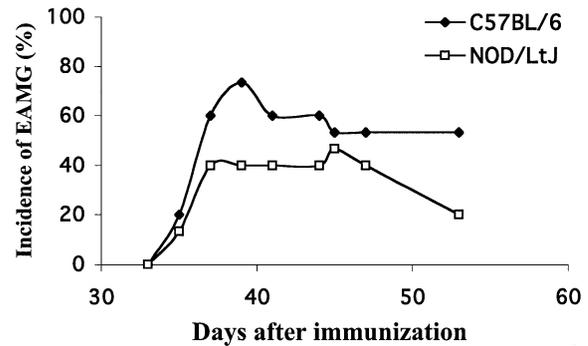


Fig. 1. NOD/LtJ mice are susceptible to EAMG. The incidence of muscle weakness is shown. Fifteen mice from each strain were immunized with *T. californica* AchR (10 μ g/mice) in CFA on day 0, boosted on day 30 and monitored on the following 23 days for signs of muscle weakness.

After the boost, however, both strains produced increased IgG antibodies to AchR, although the OD measurements were significantly greater in NOD/LtJ mice ($P < 0.05$). A small but significant induction of AchR-specific IgM antibodies was detected in NOD/LtJ mice at day 45. No significant amounts of IgG or IgM antibodies were detected against the control antigen BSA. Thus, mice of both strains mounted a strong and specific antibody response to AchR, although with different dynamics.

The clinical manifestations of EAMG are triggered by mechanisms thought to involve antibodies to AchR. These antibodies activate complement leading to focal lysis of the postsynaptic membrane (12), but the antibodies also have other effects such as down-regulation of the receptor (13,14). In the mouse, complement-fixing IgG isotypes are IgG2a and IgG2b. To characterize the antibodies to AchR, we studied their isotypes (Fig. 3). We found similar amounts of IgG2a and IgG2b in the NOD/LtJ and C57BL/6 strains, but we found higher levels of antibodies of the IgG1 subclass in NOD/LtJ mice ($P < 0.05$). Hence, NOD/LtJ mice produce complement-fixing anti-AchR antibodies in amounts comparable to those detected in the EAMG-susceptible C57BL/6 mice.

T cell responses to AchR

EAMG is a T cell-dependant disease and MHC class II genes influence susceptibility (2,15). Figure 4 shows that NOD/LtJ mice develop T cell responses at least as strong as those detected in C57BL/6 mice. No significant responses were detected against the non-related, control antigen BSA, and no differences between the strains were detected in T cell proliferation to Con A (data not shown). IFN- γ is thought to play a central role in EAMG by supporting the induction of complement-fixing antibodies (16); we therefore studied the secretion of IFN- γ by splenocytes upon *in vitro* stimulation with AchR. Spleen cells isolated from NOD/LtJ mice secreted IFN- γ in a dose-dependant manner and at levels comparable to those seen with spleen cells from C57BL/6 mice (Fig. 5).

Discussion

Natural antibodies can be defined as Ig binding specifically to self or foreign antigens, detectable in the serum of healthy

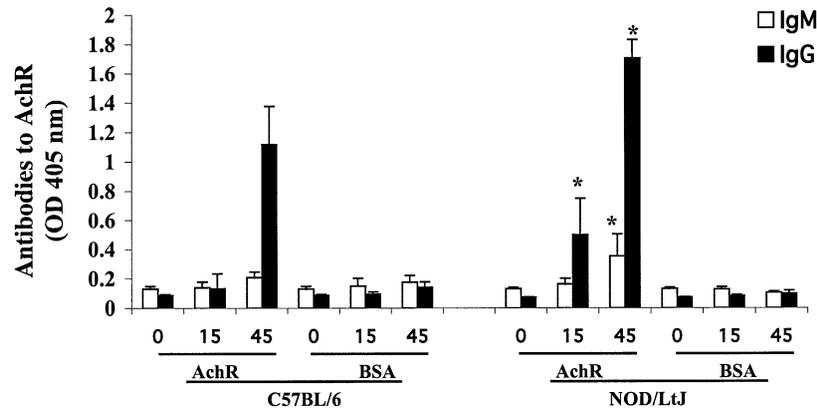


Fig. 2. Immune antibodies to AchR: kinetics of induction. Serum samples (15 per group at each time point) were collected before (day 0), and 15 and 45 days after the first immunization with AchR. IgG and IgM antibodies to AchR and to BSA were determined by ELISA using specific detection antibodies to analyze serum samples diluted 1/500. * $P < 0.05$ compared to matched samples taken from C57BL/6 mice.

individuals in the absence of immunization with the target antigen (17); immune antibodies, in contrast, follow known immunization. Note that this definition of natural antibodies does not imply anything about their origin. In this work, we studied the association between natural IgG autoantibodies and susceptibility to autoimmune disease (Table 1). Our model was EAMG in NOD/LtJ mice because these mice harbor natural IgG autoantibodies to AchR (1), but there are no reports of the susceptibility of these mice to EAMG. We found that NOD/LtJ mice are indeed susceptible to EAMG (Fig. 1); they mount B cell (Figs 2 and 3) and T cell responses (Figs 4 and 5), similar to those seen in EAMG-susceptible C57BL/6 mice. However, NOD/LtJ mice had a lower incidence, a lower mean maximal disease score and a shorter course of EAMG (Fig. 1), an observation that might be associated with their higher levels of anti-AchR IgG1 (Fig. 3); IgG1 might reflect higher levels of AchR-specific IL-4 production *in vivo* (18), a cytokine previously associated with the control EAMG (19). Nevertheless, our results support an association between natural IgG autoimmunity and susceptibility to the induction of a specific autoimmune disease. Indeed, C3H mice, a strain reported to be relatively resistant to EAMG (20–22), was found by us to lack natural IgG autoantibodies to AchR (not shown).

A healthy, mature immune system contains self-reactive B and T cells (23–27). It has been estimated that between 5 and 15% of splenic B cells activated *in vivo* can secrete natural antibodies (28). The repertoire of autoantibodies includes those of the IgG isotype, indicating that self-reactive T cells support their generation (27,29). How the self-reactive B cells became activated is still a matter of debate, but possible candidates are anti-idiotypic interactions (30) together with the direct recognition of autoantigens (31). Although immunization to foreign antigens cross-reactive with self is conceivable, the association of natural IgG antibodies with susceptibility to the induction of the particular autoimmune diseases would support the participation of the target autoantigen in the generation of specific natural autoantibodies.

Natural autoantibodies have been found in virtually every vertebrate (32), suggesting that they are positively selected and might play some role in body homeostasis. Indeed, natural autoimmunity is thought to perform several functions, which include the scavenging of metabolic waste (33), a first

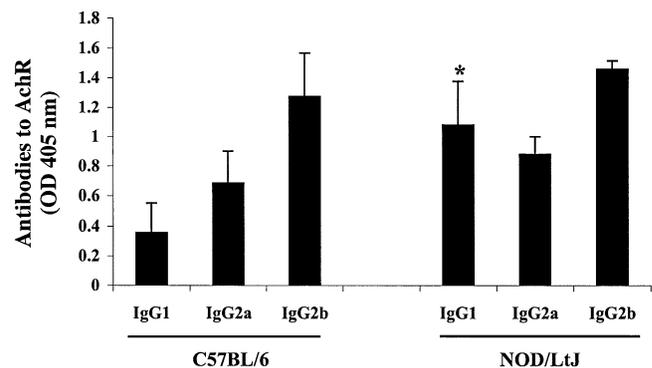


Fig. 3. Immune antibodies to AchR: isotypes. Serum samples (15 per group) were collected 45 days after the first immunization and analyzed by ELISA using isotype-specific detection antibodies. * $P < 0.05$ compared to C57BL/6 mice.

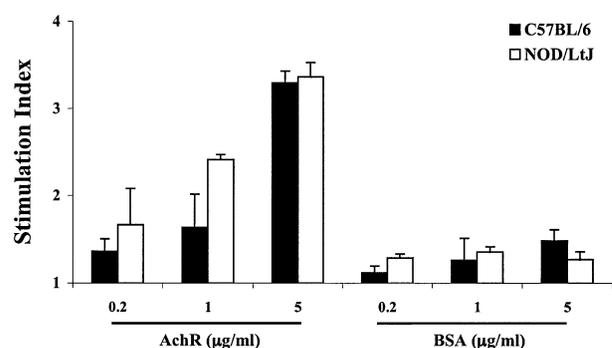


Fig. 4. Antigen-specific T cell proliferation. Four mice per group were sacrificed 55 days after the first immunization and spleen T cells were activated for 72 h with different concentrations of AchR or BSA. The results are presented as SI \pm SD; the average c.p.m. values of spleen T cells in the absence of antigen were 3016 ± 580 for NOD/LtJ mice and 2613 ± 234 for C57BL/6 mice.

line of defense against viral and bacterial infection (34,35), the control of autoimmune responses (36,37), and the repair of the central nervous system following trauma (38). However, an immune system that naturally harbors self-reactive clones is potentially dangerous, and so regulatory mechanisms of

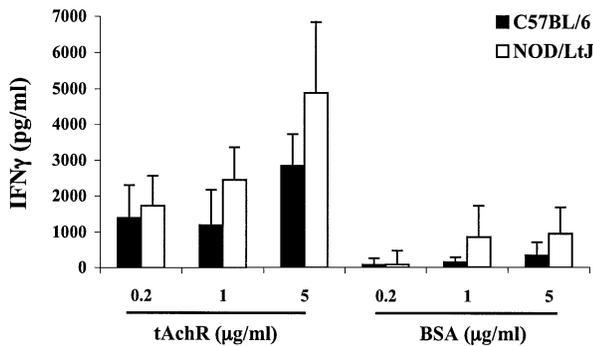


Fig. 5. Antigen-specific IFN- γ secretion. Four mice per group were sacrificed 55 days after the first immunization, their spleen cells activated for 72 h with different concentrations of AchR or BSA and the amount of IFN- γ released to the medium quantified using a cytokine-specific capture ELISA. The results are presented as the mean \pm SD.

control have evolved based on anti-idiotypic interactions (39) and regulatory cells (40–42). The elimination of these regulatory mechanisms leads to the development of spontaneous autoimmune disease (43), demonstrating that the natural self-reactive clones contained in a healthy immune system are potentially pathogenic.

After reviewing the literature describing natural autoantibodies in humans, Lacroix-Desmanzes *et al.* concluded that the natural autoantibodies found in healthy individuals may be indistinguishable from the autoantibodies found in autoimmune disease in terms of V gene usage, extent of mutations, affinity and specific reactivity (27). It is clear, however, that patients with autoimmune disorders produce increased amounts of specific autoantibodies (44); thus the differences between natural and disease-associated autoimmunity might be quantitative rather than qualitative. Autoimmune diseases can therefore be viewed as the consequence of uncontrolled natural autoimmunity, possibly due to defects in natural regulatory mechanisms. This interpretation is compatible with the concept of the immunological homunculus (45,46) which, in contrast to the clonal selection theory (47), builds on the observation that autoimmune disease is usually focused on the relatively limited number of self-antigens that are also targeted in natural autoimmunity.

The NOD/LtJ mouse can be used as a model to study the link between natural autoimmunity, its regulatory mechanisms and autoimmune disease. In pathogen-free laboratory conditions, NOD/LtJ mice spontaneously develop a high incidence of IDDM; the other autoimmune disorders to which NOD/LtJ mice are susceptible (Table 1) do not develop spontaneously but are induced by immunization with specific antigen in an adjuvant. Thus, regulatory mechanisms must operate to keep these other autoreactivities under control. However, appropriate immunization of NOD/LtJ mice with antigen in adjuvant can overcome these putative regulatory mechanisms and lead to the induction of experimental autoimmune encephalomyelitis (EAE) (48), systemic lupus erythematosus (SLE) (49) or EAMG (this manuscript); a suitable adjuvant can break regulation. Interestingly, the induction of SLE in NOD/LtJ mice seems to inhibit the spontaneous autoimmune process that leads to IDDM (49). Thus, the repertoire of natural self-reactive IgG

reflects not only the presence of self-reactive clones, but also highlights the complexity of the regulatory mechanisms that operate to control disease.

Early detection of humans prone to develop autoimmune disorders could lead to early diagnosis or preventive therapies, particularly useful in disorders where the onset of symptoms is a late event in the natural history of the disease. IDDM, for example, becomes evident only after >80% of the insulin-producing β cells have been destroyed. However, newly designed immunotherapies can stop and even revert the autoimmune attack if administered in time (50); the ability to arrest autoimmune disease highlights the need for methods to identify individuals at risk of developing autoimmune disorders. In the future, natural autoantibodies together with genomic analysis might provide useful tools to determine the predisposition of a human to develop a particular autoimmune disease.

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Abbreviations

AchR	acetylcholine receptor
CFA	complete Freund's adjuvant
Con A	concanavalin A
EAE	experimental autoimmune encephalomyelitis
EAMG	experimental autoimmune myasthenia gravis
IDDM	insulin-dependent diabetes mellitus
NOD	non-obese diabetic
SLE	systemic lupus erythematosus

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