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Circulating Immune Complexes Augment Severity of Antibody-Mediated Myasthenia Gravis in Hypogammaglobulinemic RIIIS/J Mice

Erdem Tüzün,* Benjamin G. Scott,* Huan Yang,* Bo Wu,2‡ Elzbieta Goluszko,3* Michelle Guigneaux,§ Stephen Higgs,§ and Premkumar Christadoss4*

Experimental autoimmune myasthenia gravis (EAMG) is severe in RIIIS/J mice, despite a significant B cell immunodeficiency and a massive TCR Vβ gene deletion. Severity of EAMG in RIIIS/J mice is greater than MHC-identical (H-2b) B10.RIII mice, suggesting the influence of non-MHC genes as an EAMG-potentiating factor in this strain. To delineate the role of deleted TCR Vβ genes in RIIIS/J mice, we obtained (RIIIS/J × B10.RIII)F1 (Vβb/c) × RIIIS/J (Vβb) backcross mice using Mendelian genetic methods and immunized them with acetylcholine receptor. EAMG susceptibility was not elevated in mice with Vβ genotype having 70% Vβ gene deletion. Next, we performed microarray analysis on 12,488 spleen cDNAs obtained from spleens of naive RIIIS/J and B10.RIII mice. In RIIIS/J mice, 263 cDNAs were overexpressed and 303 cDNAs were underexpressed greater than 2-fold, compared with B10.RIII mice. TCR gene expression was augmented, whereas NK receptor, C1q, and C3 gene expressions were diminished in RIIIS/J mice. RIIIS/J mice also had increased lymph node T cell counts, elevated serum anti-AChR Ab levels, and serum C3 and C1q-conjugated circulating immune complex levels. A direct correlation between increased serum C1q-conjugated circulating immune complex levels and disease severity was observed in RIIIS/J mice. The Journal of Immunology, 2004, 172: 5743–5752.

1 Experimental autoimmune myasthenia gravis (EAMG) is a T cell-dependent, Ab-mediated autoimmune disease of the neuromuscular junction (NMJ), which closely mimics human myasthenia gravis (MG) in its clinical and immunopathological manifestations (1). The autoantibodies to acetylcholine receptor (AChR) induce NMJ destruction and the loss of AChR in MG and EAMG. The production of Abs is dependent on MHC class II-restricted CD4+ T cell help (1–3). However, for the activation of AChR-specific CD4+ T cells and production of anti-AChR Abs, B7-CD28 and CD40-CD40L costimulatory molecule interactions are required (4, 5). Besides these costimulatory molecules, proinflammatory cytokines TNF-α, IL-6, IL-18, and IL-1β contribute to the activation of AChR-specific T and B cells and play a critical role in EAMG pathogenesis (6–10). One important mechanism by which anti-AChR Abs induce EAMG is the activation of a complement-mediated NMJ destruction. Proposed mechanism is largely based on experiments that revealed that IgG, C3, and membrane attack complexes (MAC) are colocalized in the NMJ of patients with MG (11, 12), and C5 gene knockout (KO) mice are highly resistant to EAMG (13). Moreover, we have recently shown that the classical complement pathway is responsible for C3 and MAC deposition at the NMJ, and is therefore essential for the development of EAMG in C57BL/6J (B6) mice (14).

We previously used the RIIIS/J mouse strain, which demonstrates increased EAMG incidence and develops more severe EAMG than the well-established EAMG-susceptible B6 strain (15). RIIIS/J mice, which have the largest known genomic deletion in TCR Vβ gene (13 of 21 Vβ genes) (16), are resistant to both experimental allergic encephalomyelitis (EAE) (17–19) and collagen-induced arthritis (CIA) (20–22). However, they are highly susceptible to EAMG, displaying more severe EAMG than B6 mice and the H-2-identical B10.RIII (H-2b) mice, suggesting that in RIIIS/J mice, non-MHC genes or factors influence disease severity. To study the non-MHC factors contributing to augmented EAMG severity in RIIIS/J mice, we delineated the role of deleted Vβ genes and analyzed the genetic, cellular, and immunopathological differences between RIIIS/J and B10.RIII mice. The increased severity of EAMG in hypogammaglobulinemic RIIIS/J mice turned out to be primarily associated with increased amounts of lymph node CD4+ T cells and C1q-conjugated circulating immune complex (C1q-CIC) levels.

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Materials and Methods

**AChR and mice**

AChR was purified from the electric organ of *Toxocera californica* by an α-neurotoxin affinity column (23). Seven- to 8-wk-old RIII/J, B10.RIII, and B6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All animals were housed in the mouse-abduction-free barn facility at the University of Texas Medical Branch and maintained according to the Institutional Animal Care and Use Committee Guidelines.

**Induction and clinical evaluation of EAMG**

For in vivo studies, all mice were anesthetized and immunized with 20 μg of AChR emulsified in CFA (Difco, Detroit, MI) s.c. at four sites (two hind footpads and two shoulders) on day 0. All of the mice were boosted with 20 μg of AChR in CFA s.c. at four sites on the back on day 30 (second immunization). For clinical examination, mice were left for 3 min on a flat platform and were observed for signs of EAMG. Clinical muscle weakness was graded, as follows: grade 0, mouse with normal posture, muscle strength, and mobility; grade 1, normal at rest, with muscle weakness characteristically shown by a hunchback posture, restricted mobility, and difficulty to raise the head after exercise, consisting of 20–30 paw grips on cage top grid; grade 2, mouse showed grade 1 symptoms without exercise during observation period on flat platform; grade 3, dehydrated and moribund with grade 2 weakness; and grade 4, dead.

**RIA for anti-muscle AChR Ab**

The Ab response to mouse muscle AChR was measured by RIA, as previously described (23). The anti-AChR Ab levels were expressed in nanomoles of α-bungarotoxin binding sites bound per liter of serum for individual mice.

**ELISA for IgG isotypes**

Affinity-purified mouse AChR (0.5 μg/ml) was coated onto a 96-well microtiter plate (Dynatech Immunol 2; Dynatech Laboratories, Chantilly, VA) with 0.1 M carbonate bicarbonate buffer (pH 9.6) overnight at 4°C. The plates were blocked with 2% BSA in PBS at room temperature for 30 min. Serum samples were diluted 1/1000 in PBS/0.05% Tween 20 and incubated at 37°C for 90 min. After four washes, HRP-conjugated goat anti-mouse IgM and IgG isotypes (IgG, IgG1, IgG2b IgG2c, and IgG3 (Caltag Laboratories, San Francisco, CA)) dilution 1/500–1000 in PBS/0.05% Tween 20 were added and incubated at 37°C for 90 min. Subsequently, ABTs (indicator) solution in 0.1 M citric buffer, pH 4.3, in the presence of H2O2, was added and color was allowed to develop at room temperature in the dark. Absorbance values were measured at a wavelength of 405 nm, using a Molecular Devices (Sunnyvale, CA) EMAX microplate reader, and the results were expressed as OD values.

**ELISA for serum total IgG levels**

ELISA was performed in the same manner as mentioned above, except that the plates were coated with goat anti-mouse IgG (Chemicon International, Temecula, CA) with 0.1 M carbonate bicarbonate buffer (pH 9.6) overnight at 4°C. After incubation with serum samples, HRP-conjugated goat anti-mouse IgG Ab (Caltag Laboratories), directed to a different determinant than the primary coating Ab, was added in 1/1000 dilution in PBS/0.05% Tween.

**DNA microarray analysis of gene expression**

mRNA was extracted from five spleens each of RIII/J and B10.RIII mice. Spleens were pooled for each strain and then probed using one chip each for RIII/J and B10.RIII mice. Briefly, spleen cells were transferred to Eppendorf tubes, and 0.2 ml of chloroform was added. Tubes were mixed vigorously, incubated for 15 min at room temperature, and centrifuged at 12,000 rpm for 15 min at 4°C. The upper aqueous layer was removed, 0.5 ml of 50% isopropanol was added, and final mixture was incubated at room temperature for 10 min. After centrifugation at 12,000 rpm for 10 min at 4°C, samples were washed in 1 ml of 75% ethanol and centrifuged again at 7,500 rpm for 5 min at 4°C. Pellets were dissolved in 100 μl of diethylpyrocarbonate-treated water (Sigma-Aldrich, St. Louis, MO).

**MCU74Av2 mouse array was obtained from Affymetrix (Santa Clara, CA). The microarray covered 12,488 probes. First-strand cDNA synthesis was performed using total RNA (10–25 μg), a T7-cDNA oligomer, and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). Second-strand synthesis converted the cDNA into a dsDNA template for use in an in vitro transcription reaction. The cRNA or target RNAs were labeled with biotin during the in vitro transcription reaction. Biotin-labeled target RNAs were fragmented to a mean size of 200 bases to facilitate their hybridization to probe sequences on the gene chip array. Hybridization of gene chip array was performed at 45°C for 16 h in 0.1 M MES, pH 6.6, 1 mM sodium chloride, 0.02% EDTA, and 0.01% Tween 20. Array was washed using both nonstringent (1 M NaCl, 25°C) and stringent (1 M NaCl, 50°C) conditions before staining with PE streptavidin (10 μg/ml final). Gene chip array was scanned using a 2500 Gene Array Scanner and analyzed using the Microarray Suite 5.0 software (both Affymetrix).

The p values for detection of individual genes were calculated, and absent calls (detection p value >0.06) were removed. Before comparison analysis, a global normalization method was used to correct variations and normalize intensity levels. The comparison analysis was performed with Wilcoxon signed rank test to examine the hybridization intensity data from one gene chip and compare that with another gene chip. Relative changes in the expression level of each target RNA were noted. Among the significantly changed RNA levels, those with at least 2-fold increased or decreased average RNA levels were taken into consideration.

**Flow cytometry analysis**

Single-cell suspensions of lymph node cells were incubated for 30 min with one of the following anti-mouse Abs: PE-conjugated anti-CD4 and anti-CD19; FITC-conjugated anti-CD5 and anti-CD8 (all from BD Biosciences, San Diego, CA). PE- or FITC-conjugated rat isotypes were used for controls. Cells were washed twice and then were fixed with 2% paraformaldehyde and analyzed with a BD FACSanaly (BD Biosciences).

**Immunohistochemical staining for germinal centers**

Four-micrometer-thick sections of 10% formalin-fixed and paraffin-embdded spleens were prepared. Sections were deparaffinized and rehydrated. Endogenous peroxidase activity was quenched with 3% H2O2 in methanol. Sections were blocked for nonspecific binding with normal goat serum diluted 1/20 in DAKO (Carpenteria, CA) Ab diluent for 15 min. After rehydration, sections were done with DAKO Target Retrieval Solution in steam at 90°C. Sections were then cooled down on the bench top for 20 min, rinsed two to three times with distilled water, and transferred to TBS. Slides were then incubated for 30 min with peanut agglutinin (PNA)–biotin (Vector Laboratories, Burlingame, CA) diluted 1/250 in DAKO Ab diluents and washed, followed by a second incubation with streptavidin–HRP for 30 min. The LSAB2 system (K0758). Bound conjugates were visualized with DAKO liquid dianaminobenizidine substrate–chromogen for 5 min when a brown color for PNA-positive cells was obtained. For all staining steps, a DAKO Autostainer was used. Slides were counterstained for 2 min with Mayer's modified hematoxylin diluted 1/5 in distilled water.

**Immunohistochemical and immunofluorescence staining for B cell markers**

Seven-micrometer-thick spleen sections were frozen in liquid nitrogen, and stored at −80°C. Sections were allowed to air dry and then were fixed in cold acetone. Endogenous peroxidase activity and nonspecific binding were blocked with 3% H2O2 in methanol and 20% PBS in PBS, respectively. After washing with PBS, the sections were incubated overnight at 4°C in the presence of rat anti-mouse B220 or rat anti-mouse IgM Abs (both from BD PharMingen, San Diego, CA) (Abs diluted 1/400), followed by biotin-conjugated goat anti-rat IgG Ab (Kirkegaard & Perry Laboratories, Gaithersburg, MD) (diluted 1/500), avidin–HRP (BD PharMingen (diluted 1/500), and DAKO liquid dianaminobenizidine substrate-chromogen for development of a brown color. For detection of CD19+ B cells on the spleen tissue, frozen sections were incubated with FITC-conjugated rat anti-mouse CD19 Ab (BD PharMingen) (diluted 1/500) overnight at 4°C and then viewed in a fluorescence microscope (Olympus IX-70). Digital pictures were taken with a DP-11 digital camera.

**Measurement of serum C3 and C1q-CIC levels by ELISA**

Ninety-six-well microtiter plates (Dynex-Immulon 2; Dynatech Laboratories) were coated with goat Abs to mouse C3 (ICN Biomedicals/Cappel, Aurora, OH) in 0.1 M sodium carbonate buffer (pH 8.2) overnight at 4°C. The plates were then blocked with 2% BSA in PBS at room temperature for 30 min. Diluted (1/100 in PBS-0.05% Tween 20) serum samples (30 μl) were added and incubated at 37°C for 90 min. After four washes, HRP-conjugated goat anti-mouse C3 complement (ICN Biomedicals/Cappel) diluted 1/500 in PBS/0.05% Tween 20, was added and incubated at 37°C for 90 min. Subsequently, ABTS substrate solution in 0.1 M citric buffer (pH 4.3) in the presence of H2O2 was added, and color was allowed to develop at room temperature in the dark. Plates were read at a wavelength of 405 nm using a Dynatech ELISA reader, and the results were expressed as OD values. Serum C1q-CIC levels were determined with the same principle, with the difference that the plate was covered with goat Abs to...
Results

AChR-immunized RIIIS/J mice revealed increased incidence, severity, and earlier onset of EAMG compared with H-2-compatible B10.RIII mice

To induce EAMG, RIIIS/J, B10.RIII, B6, and (RIIIS/J × B6)F1 (F1) mice were immunized with AChR in CFA. Among all strains tested, RIIIS/J mice had significantly higher average clinical grades (Fig. 1), and the ratio of mice with severe (grade 3) EAMG was remarkably higher than that of other strains (Table I). One hundred percent of RIIIS/J, B10.RIII, and F1 mice developed EAMG, whereas the incidence of clinical EAMG was 92.8% in B6 mice (Fig. 1). Moreover, RIIIS/J mice developed disease significantly earlier than F1 and B6 mice (Table I), and 20% of RIIIS/J mice developed the clinical signs of EAMG before the second AChR immunization. MHC class II molecule is essential for the development of EAMG (2), and therefore, we immunized mouse strains with identical MHC class II alleles, I-Aa (RIIIS/J and B10.RIII mice), and also EAMG-susceptible B6 (I-Ab) mice. The significantly increased severity of clinical EAMG observed in RIIIS/J mice despite sharing the MHC class II allele I-Aa with B10.RIII mice suggests that non-MHC genes or factors influence disease severity in RIIIS/J mice. The high incidence and severity of EAMG in RIIIS/J mice make them an excellent new preclinical model for MG. The presence of EAMG-susceptible H-2b and H-2r alleles in the F1 mice did not augment disease, suggesting that H-2r homozgyosity and/or homozygosity in the non-H-2 gene(s) conferred increased EAMG susceptibility (as observed in RIIIS/J mice).

RIIIS/J mice exhibited an enhanced anti-AChR Ab response following AChR immunization, despite B cell deficiency and lower total serum IgG levels

Sera from individual mice were collected before immunization and 45 days after the first (15 days after the second) immunization with AChR in CFA. The anti-AChR IgG Ab response was measured by RIA and ELISA, and total serum IgG levels were detected by ELISA. Both RIIIS/J and F1 mice had significantly lower total serum IgG levels before and after immunization (Fig. 2A), and significantly higher serum anti-AChR Ab levels in response to AChR immunization (Fig. 2B), as compared with B10.RIII and B6 mice. These results indicate that the B cells of RIIIS/J mice may be responding vigorously to AChR immunization with a very restricted TCR Vβ repertoire due to the deletion of 70% of Vβ genes and possibly a restricted Ig repertoire because of partial B cell deficiency (Fig. 3). Furthermore, the B cell deficiency and reduced total serum IgG were not corrected in (RIIIS/J × B6)F1 progeny. A kinetic analysis of serum anti-AChR Ab levels (Fig. 2C) revealed that RIIIS/J mice have a lower IgM response as compared with B10.RIII mice throughout the experiment, and they lack the initial IgM peak observed in B10.RIII mice. These findings indicate that the higher serum anti-AChR IgG levels (Fig. 2B) of

### Statistical analysis

To determine the significance of the observed results, three statistical tests were used. Incidences of clinical EAMG were compared using the Fisher’s exact test, clinical scores were compared using Mann-Whitney U test, and all other parameters were compared using Student’s t test.

### Table I. Severe EAMG in RIIIS/J mice compared with B10.RIII and B6 mice

<table>
<thead>
<tr>
<th>Strains</th>
<th>Grade 1</th>
<th>Grade 2</th>
<th>Grade 3</th>
<th>p Values for Grade 3 (compared with RIIIS/J)*</th>
<th>Average Days of Onset after First Immunization</th>
<th>p Values for Onset of Disease (compared with RIIIS/J)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIIIS/J</td>
<td>10</td>
<td>50</td>
<td>40</td>
<td></td>
<td>35.2</td>
<td></td>
</tr>
<tr>
<td>B10.RIII</td>
<td>20</td>
<td>80</td>
<td>0</td>
<td></td>
<td>35.6</td>
<td>0.3899</td>
</tr>
<tr>
<td>B6</td>
<td>7.1</td>
<td>78.6</td>
<td>7.1</td>
<td>&lt;0.0001</td>
<td>37.9</td>
<td>0.0444</td>
</tr>
<tr>
<td>RIIIS/J × B6</td>
<td>50</td>
<td>37.5</td>
<td>12.5</td>
<td>&lt;0.0001</td>
<td>44.3</td>
<td>0.0004</td>
</tr>
</tbody>
</table>

* Disease incidences were compared using Fisher’s exact probability test.

* Average values of days between first immunization and onset of disease were compared using Student’s t test.

human C1q (Sigma-Aldrich) and HRP-conjugated goat anti-mouse IgG (Caltag Laboratories) was used as a secondary Ab.
RIIIS/J mice 2 wk after second immunization might be associated with augmented class switching from IgM to IgG, as compared with B10.RIII mice, starting from 5 days following the second AChR immunization. Additionally, RIIIS/J mice had lower serum anti-AChR IgG1, but significantly higher serum anti-AChR IgG2b and IgG3 levels as compared with B10.RIII mice.

**TCR Vβ gene deletion did not contribute to increased EAMG severity of RIIIS/J mice**

Initially, we hypothesized that Vβ gene deletion might have been contributing to increased EAMG susceptibility due to the deletion of T cells bearing specific Vβ genes involved in the regulation of

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**FIGURE 2.** Total serum IgG and anti-AChR Ab kinetic analysis in B10.RIII and RIIIS/J mice. Comparisons of A, total serum IgG vs B, anti-muscle AChR Ab levels of all mouse strains before and 45 days after the first AChR immunization and kinetics of serum levels of anti-AChR IgM and IgG isotypes (C). Asterisks on the top of the bars in A and B indicate p values for the comparisons between a specified strain of mice and B10.RIII mice separately for before- and after- immunization values, and in C, between RIIIS/J and B10.RIII mice for each time point. *, p < 0.05; **, p < 0.01; ***, p < 0.001 using Student’s t test. Bars indicate SEs. One representation of three independent experiments.
autoimmunity (15). In a previous study, we used congenic mice expressing \( \text{V}^\beta_2 \), \( \text{V}^\beta_3 \), and \( \text{V}^\beta_9 \) in the B10 background. The incidence of EAMG was increased in \( \text{V}^\beta_2 \)-bearing B10.TCR\( ^c \) mice, suggesting the possible linkage of \( \text{V}^\beta_2 \) genotype to EAMG susceptibility (15, 24). To confirm the role of \( \text{V}^\beta_2 \) genotype in EAMG, we performed Mendelian backcross genetics by making an F1 generation between RIIIS/J (\( \text{V}^\beta_2 \)) and B10.RIII (\( \text{V}^\beta_9 \)) mice and backcrossing them to RIIIS/J mice. According to the Mendelian genetics, the backcross segregated 1:1 \( \text{V}^\beta_2 \) and \( \text{V}^\beta_9 \) genotypes. The \( \text{V}^\beta_2 \) and \( \text{V}^\beta_9 \) mice were immunized with AChR and were screened for clinical EAMG. There was no statistically significant difference between the EAMG incidences of mice with \( \text{V}^\beta_2 \) and \( \text{V}^\beta_9 \) genotypes. Thus, the Mendelian genetic analysis ruled out the influence of the \( \text{V}^\beta_2 \) genotype in RIIIS/J mice as an EAMG susceptibility contributing factor (Table II).

Augmented TCR and diminished NK receptor and complement gene expression of RIIIS/J mice in DNA microarray analysis

To identify the genetic determinants associated with increased EAMG severity in RIIIS/J mice, DNA microarray analysis was performed using the spleens of adult unimmunized RIIIS/J and B10.RIII mice. Among 12,488 cDNAs analyzed by microarray analysis, 263 were overexpressed and 303 were underexpressed greater than 2-fold in RIIIS/J mice as compared with B10.RIII mice. The most notable finding was the higher expression of TCR genes in RIIIS/J mice as compared with B10.RIII mice. This could be due to a compensatory increase in T cell numbers and/or TCR expression due to the deletion of \( \text{V}^\beta_2 \) genes. NK cell receptor and CD63 (monocyte-macrophage marker) genes were expressed less in RIIIS/J mice as compared with B10.RIII mice, suggesting that RIIIS/J mice might have a phagocytic cell deficiency. Interestingly, two complement proteins, C1q and C3, that are known to be important for EAMG induction (14) were underexpressed, and the complement inhibitor DAF-2 was overexpressed in RIIIS/J mice. There is a large amount of data regarding the significant role of proinflammatory cytokines in EAMG (6–10). However, neither microarray analysis nor cytokine measurements of serum samples or supernatants of AChR or immunodominant peptide \( \alpha_1 \& 46–162 \)-stimulated lymph node cells of AChR-immunized RIIIS/J mice revealed any significant alteration for any of the cytokines (IL-2, IL-6, IL-10, IL-18, and IFN-\( \gamma \)) tested (data not shown), suggesting that increased or decreased production of these cytokines between RIIIS/J and B10.RIII mice might not be associated with increased EAMG severity of RIIIS/J mice. However, TNF superfamily, member 7 (CD27) expression was increased more than 2-fold in RIIIS/J mice. Overall, the major identifiable susceptibility factor seemed to be increased TCR expression in RIIIS/J mice. The increased TCR expression correlates with augmented lymph node T cell counts in RIIIS/J mice (Fig. 3). Additionally, production of genes that might potentially be related with EAMG due to their importance in isotype switching (protein tyrosine phosphatase receptors and CD27), Ag presentation (cathepsin E), and cytokine network (IL-11R and IFN-induced genes) was also enhanced in RIIIS/J mice. Microarray data are freely available on the website Array Express, accession number: E-MEXP-86.

RIIIS/J mice had increased T cells and reduced B cells in their lymph nodes before and following AChR immunization

T and B cell ratios of the lymph nodes of RIIIS/J and B10.RIII mice were estimated by determining the expression of CD4, CD5, CD8, and CD19 molecules in the lymph node cells using flow cytometry analysis. As illustrated in Fig. 3, both unimmunized and immunized RIIIS/J and F1 mice had significantly increased amounts of T cells (CD4, CD5, and CD8\( ^+ \) cells) and decreased levels of B cells (CD19\( ^+ \) cells) as compared with B10.RIII and B6 mice, suggesting that this cell profile is an inborn characteristic of RIIIS/J mice and is only intensified, but not induced following AChR immunization. Not only the percentages of CD4\( ^+ \), CD8\( ^+ \) T cells, and CD19\( ^+ \) B cells, but also their absolute numbers were significantly augmented and diminished, respectively (data not shown). Therefore, increased TCR gene expression obtained in microarray data is more likely to be the consequence of increased T cell numbers rather than increased receptor expression or of both factors. Moreover, the elevated ratio of CD8\( ^+ \) T cells in F1 mice (with a moderate EAMG severity) was comparable to that of RIIIS/J mice, whereas RIIIS/J mice had exceedingly higher numbers of CD4\( ^+ \) T cells, suggesting that augmented CD4\( ^+ \) cell population and TCR gene expression could have contributed to heightened cellular and humoral immune response to AChR (i.e., IgG isotype switching (25)) and disease in RIIIS/J mice. It is very

### Table II. EAMG incidence in mice with \( \text{V}^\beta_2 \) and \( \text{V}^\beta_9 \) genotype derived from (RIIIS/J \times B10.RIII)\( F_2 \times RIIIS/J \) backcross

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of AChR-Immunized Mice</th>
<th>EAMG Incidence (%)</th>
<th>Grade 1 (%)</th>
<th>Grade 2 (%)</th>
<th>( p ) Value for Total Incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{V}^\beta_2 )</td>
<td>11</td>
<td>9.1</td>
<td>0</td>
<td>9.1</td>
<td>0.13</td>
</tr>
<tr>
<td>( \text{V}^\beta_9 )</td>
<td>12</td>
<td>33.3</td>
<td>25</td>
<td>8.3</td>
<td></td>
</tr>
</tbody>
</table>

**FIGURE 3.** FACS analysis of lymph node T and B cell counts in unimmunized and AChR-immunized mice, estimated by FACS analysis. Results are expressed as the individual percentage of positive cells for each marker. Percentages at the top of bars indicate the ratio of difference between RIIIS/J and B10.RIII mice for the specified markers. Results obtained for individual mice of each strain were compared using Student's \( t \) test. **, \( p < 0.01 \); ***, \( p < 0.001 \); ****, \( p < 0.0001 \); *****, \( p < 0.00001 \) (between RIIIS/J and other strains); and bars indicate SEs. Data represent one of three independent experiments.
interesting that RIIIS/J mice have \( \sim 50\% \) less CD19\(^+\) B cells as compared with B10.RIII and B6 mice. Despite the reduction in the lymph node B cells before and after immunization with AChR in CFA, the serum anti-AChR Ab levels were very high in RIIIS/J mice. Therefore, RIIIS/J mice are not deficient in self (AChR)-reactive B cells and rather have a higher frequency.

**Spleen cells of RIIIS/J mice revealed suppressed surface markers for B cells at maturation stages and decreased amounts of germinal centers**

To further characterize the role of B cells in the secondary lymphoid organs of RIIIS/J mice, spleens and lymph nodes of AChR-immunized and unimmunized RIIIS/J, B10.RIII, and F1 mice were collected and flash frozen. The frozen sections were tested by immunohistochemistry or immunofluorescence for the presence of pro-B cell (B220, CD19) and immature B cell (IgM) markers and also germinal centers by PNA staining on paraffin-embedded sections. RIIIS/J mice revealed lower intensity of staining for all markers tested as compared with B10.RIII and B6 mice (Fig. 4), suggesting that B cells are deficient as a result of deficient production from the very early stages of maturation due to a not yet known mechanism. The reason for stronger B220 staining in Fig. 4 might be due to the fact that this molecule is also found on the surfaces of dendritic cells and NK cells. In contrast, LPS-induced B cell and Con A-induced T cell responses of spleen cells of RIIIS/J mice indicated that both B and T cells are capable of duly responding to mitogen stimulation (data not shown). PNA staining revealed an extraordinary circular staining pattern around the usual anatomical location for germinal centers in both RIIIS/J and B10.RIII mice, suggesting either different germinal center structure for these strains of mice or deficient B cell activation by T cells (Fig. 4). Nevertheless, in these mice, the average number of germinal centers was significantly lower than that of B6 mice (data not shown). Results obtained from lymph nodes of mice with or without AChR immunization and from spleens of mice without AChR immunization were highly comparable to the results obtained from spleen sections of AChR-immunized mice (identical germinal center architecture, but decreased staining intensity for all markers tested in unimmunized mice’s lymph node and spleen sections).

**RIIIS/J mice had remarkably higher serum C3 and C1q-CIC levels after AChR immunization, despite lower serum levels of C3 and C1q-CIC before AChR immunization**

As supportive data to the molecular analysis experiments, naive RIIIS/J mice had diminished serum C3 and C1q-CIC levels as compared with B6 mice (although this difference did not attain statistical significance). However, AChR immunization significantly augmented the production of both serum C1q-CIC (Fig. 5A) and C3 (Fig. 5B), particularly in RIIIS/J mice. Moreover, there was a significant correlation between clinical scores of RIIIS/J mice and their serum C1q-CIC (\( p = 0.0004 \) and \( r = 0.9698 \)) levels (but not C3 levels, tested by Spearman’s method for nonparametric correlations) (Fig. 5C). Given the reported importance of C3 in isotype switching, we suggest that the high C3 levels of F1 and RIIIS/J mice could have contributed to the high serum anti-AChR IgG levels in these two strains. In this study, we observed that: 1) serum C1q-CIC levels are augmented in AChR-immunized RIIIS/J...
As, AChR T cell epitope(s) appears to interact with multiple TCR.

Our results might also suggest that unlike myelin and collagen
creased EAMG susceptibility or serum anti-AChR Ab levels (30).

Therefore, it is conceivable that T cells bearing some of these V
molecules could have been involved in the regulation/suppression
increased spleen T cell frequencies (19), elevated complement
activation capacity (28), and serum C3 and C1q-CIC levels in our
studies following AChR immunization (Fig. 5). Similarly, it is also
noteworthy that they are resistant to T cell-dependent EAE (17–19)
and CIA (20–22), while being highly susceptible to Ab- and com-
plement-mediated EAMG, as reported in this work. Being highly sus-
cceptible to EAMG, while having a B cell immunodeficiency, makes
this mouse strain a potential candidate to study the association be-
tween autoimmune diseases and hypogammaglobulinemia.

There are interesting studies attempting to link the resistance of
RIIIS/J mice to EAE to certain genetic loci (17, 19). Moreover,
genetic linkage analysis suggested that the major loci controlling
CIA in crosses between RIIIS/J and B10.RIII mice were located
outside the MHC region (20), and similarly the gene(s) influencing
the severity of EAMG in RIIIS/J mice appears to be located out-
side of the MHC region. It is also plausible that rather than gene(s),
other defective immunological factors in RIIIS/J mice could have
contributed to increased disease severity.

Discussion

Inbred mouse strains with well-defined immune deficiencies or
augmented immune functions enable the characterization of resis-
tance or susceptibility factors of individual immunological dis-
ases. RIIIS/J mice constitute a special example in this respect due
to an immunodeficiency manifested as deletion of ~70% of TCR
Vβ genes (16), low B cell counts in the peritoneal cavity (26),
spleen (19) and lymph nodes (Fig. 3), low levels of serum IgM
(26), low Ab responses to T cell-dependent or -independent Ags
(27), and yet by their augmented immunological functions includ-
ing increased spleen T cell frequencies (19), elevated comple-
mement activation capacity (28), and serum C3 and C1q-CIC levels in our
studies following AChR immunization (Fig. 5). Similarly, it is also
noteworthy that they are resistant to T cell-dependent EAE (17–19)
and CIA (20–22), while being highly susceptible to Ab- and com-
plement-mediated EAMG, as reported in this work. Being highly sus-
cceptible to EAMG, while having a B cell immunodeficiency, makes
this mouse strain a potential candidate to study the association be-
tween autoimmune diseases and hypogammaglobulinemia.

TCR Vβ gene deletion does not contribute to RIIIS/J disease
severity

RIIIS/J mice (TCR Vβ6 haplotype) have a genomic deletion of
~130 kb of DNA encoding 13 Vβ genes (Vβ 5.2, 8.3, 5.1, 8.2, 5.3,
8.1, 13, 12, 11, 9, 6, 15, and 17) of the 21 known Vβ genes (16).
Therefore, it is conceivable that T cells bearing some of these Vβ
molecules could have been involved in the regulation/suppression
of the autoimmune response to AChR, and thus played a role in
EAMG susceptibility of RIIIS/J mice. However, mice with Vβ6
haplotype in F1 × RIIIS/J backcross failed to reveal an increased
EAMG susceptibility. In accordance with these results, previous
studies have shown that the use of multiple Vβ genes is required
in EAMG pathogenesis (29, 30), and the absence of some of the
Vβ genes does not cause a significant change in EAMG induction.
Although TCR Vβ6 and Vβ8 have been shown to be preferentially
used during the in vivo response to AChR (29, 30), neither deple-
tion of Vβ6+ T cells nor the genetic deficiency of Vβ6 gene can
prevent the immune response to AChR or clinical development of
EAMG (30). Moreover, Vβ8 transgenic mice do not reveal in-
creased EAMG susceptibility or serum anti-AChR Ab levels (30).
Our results might also suggest that unlike myelin and collagen
Ags, AChR T cell epitope(s) appears to interact with multiple TCR
Vβ repertoire and can induce an immune response even in the
absence of a large group of Vβ genes. It has also been suggested
that autoimmune diseases might have been encountered in the
course of primary immunodeficiencies due to the abnormal han-

ding of certain Ags (i.e., superantigens) (31), and AChR might be
acting as a superantigen in RIIIS/J mice.

Elevated TCR gene expression and CD4+ T cells

The most significant factor revealed in microarray gene analysis
was the elevated TCR expression in the spleen tissues of RIIIS/J

FIGURE 5. Serum C1q-CIC (A) and C3 (B) levels of all strains of mice before and after AChR immunization. In RIIIS/J mice, serum C1q-CIC
test levels are correlated with the clinical severity of EAMG (C). *, p < 0.05; **, p < 0.01 using Student’s t test; bars indicate SEs. One representation
of three independent experiments.
mice, presumably reflecting higher spleen T cell counts, as shown in a previous report (19). Although our studies showed both increased CD4+ and CD8+ T cells, the latter subtype of cells does not seem to be an important part of increased EAMG severity, because F1 mice also had relatively increased CD8+ T cells in their spleens. Plausibly, increased CD4+ T cells might have been induced increased EAMG severity in RIIIS/J mice by also increasing production of cytokines relevant to EAMG induction. However, in several experiments, we could neither show any difference in cytokine levels in serum samples or supernatants of AChR-exposed lymph node cells of RIIIS/J mice (data not shown), nor any alteration in the expression of any of the cytokines tested in microarray analysis.

**B cell immunodeficiency and autoimmunity in RIIIS/J mice**

By using both immunohistochemistry/immuno-fluorescence methods and FACS analysis on the secondary lymphoid tissues of mice (Figs. 3 and 4), we showed that RIIIS/J mice have lower B cell counts, which is consistent with lower serum IgM levels (Fig. 2C) observed in this strain. PNA staining is used for detection of germinal centers, and in cases of disturbed T cell-mediated B cell activation, PNA staining of splenic tissue may be significantly reduced (32, 33). This might be indicative of the fact that both RIIIS/J and B10.RIII mice have a problem with T cell-dependent B cell activation. However, underexpression of B cell markers for earlier B cell maturation stages (CD19, B220) might suggest that B cell deficiency of RIIIS/J mice might not be only due to deficient activation, but also due to decreased B cell production. Increased elimination of B cells by either apoptosis or necrosis still remains a possibility. However, FACS analysis on the lymph node cells obtained from AChR-immunized RIIIS/J and B10.RIII mice did not reveal any increase in necrotic (14.28 vs. 10.45%) or apoptotic (37.32 vs. 36.13%) cells (assessed by 7-amino actinomycin D and annexin-V staining, respectively) in RIIIS/J mice as compared with B10.RIII mice. A deficiency of B cells has been proposed to cause autoimmune diseases due to a consequential decrease of complement receptors, which are important for immune complex clearance and the elimination of autoreactive B cells (34). Although MG is not frequently observed in B cell-immunodeficient cases, several Ab-dependent, complement-mediated autoimmune diseases such as MG (i.e., hemolytic anemia, idiopathic thrombocytopenic purpura, autoimmune glomerulonephritis) are detected in these patients (31). RIIIS/J mice might thus constitute a good model for research on B cell immunodeficiency syndromes (e.g., common variable immunodeficiency), in which hypogammaglobulinemia and complement-mediated autoimmune diseases occur together (31). Augmented EAMG in partial B cell-deficient RIIIS/J mice suggests that partial B cell deletion alone should not be attempted in the clinical trial of MG, and rather specific depletion of anti-AChR-producing B cells should be tried.

**TCR Vβ gene deletion suppresses cell-mediated autoimmunity, and B cell deficiency augments B cell-mediated autoimmunity**

RIIIS/J mice are capable of producing high amounts of anti-AChR Abs, despite their lower serum total IgG levels, which might be a consequence of the restricted TCR Vβ and/or Ig repertoire, and could lead to defective tolerance induction to self Ag and thus emergence of self-reactive T and B cells. However, RIIIS/J mice are not highly susceptible to other induced autoimmune diseases such as EAE and CIA. These two diseases are predominantly mediated by T cells, while EAMG is an Ab- and complement-mediated disease. We can argue that the deficiency of B cells and possibly restricted Ig repertoire could augment Ab-mediated diseases, while the restricted TCR Vβ repertoire could suppress the cell-mediated diseases such as EAE or CIA due to the deletion of specific Vβ+ cells involved in the infiltration of the target tissues, myelin in EAE, and synovium in CIA. Another important topic about Abs is how RIIIS/J mice can produce increased amounts of anti-AChR IgGs, while their anti-AChR IgM levels are lower than other strains. In Fig. 2C, RIIIS/J mice are shown to make a less intense and a quicker peak IgM response as compared with B10.RIII mice, and it is also seen that at the same time, their IgM levels are decreasing, while their serum IgG levels are rising in a significantly steady state manner, eventually exceeding the IgG...
level of B10.RIII mice. Therefore, a reasonable explanation to the above question is that the switching of RIIIS/J mice to IgG production is much more quick and efficient than other strains. Nevertheless, increased anti-AChR IgG production of RIIIS/J mice might not be the reason for increased EAMG susceptibility because of the generally accepted concept about the lack of correlation between anti-AChR Ab levels and EAMG or MG severity and also high serum anti-AChR IgG levels observed in F1 mice, which demonstrate less susceptibility. Previously, it has been postulated that RIIIS/J mice are giving a more Ab-dependent and less T cell-mediated response to the Ags because of their Vβ deletion, and thus developing weaker CIA (22). However, in our model, the lymph node cells of RIIIS/J mice gave a more prominent (but not significantly) lymphocyte proliferation response to AChR or immunodominant peptide (el46–162) stimulation than other strains (stimulation index for AChR-stimulated RIIIS/J lymph node cells was 3.2 vs 2.7 for B10.RIII mice), and also the mitogen response of lymph node T cells of RIIIS/J mice to Con A stimulation was equal to those of B6 and B10.RIII mice (data not shown). This discrepancy might be due to differences in the pathogenesis of EAMG and CIA and/or Ags used for immunization.

C3, C1q-CIC, and EAMG

It is remarkable that C3, which seems to be produced to a smaller degree in RIIIS/J mice before immunization (as revealed by both ELISA studies and microarray analysis), achieves a giant leap in production after immunization and serum C3 levels of RIIIS/J mice surpass that of other strains (Fig. 5). C3 and other complement components are known to be highly important for EAMG induction and pathogenesis. We have previously shown that C3 (14) and C5 KO mice (13) are highly resistant to EAMG induction by AChR immunization. However, it is difficult to attribute increased EAMG severity to serum C3 levels, because F1 mice, which have moderate disease severity, have equivalent serum C3 levels. Our previous unpublished studies have shown that serum C3 levels are not strictly associated with EAMG incidence and severity, and highly EAMG-resistant strains may have serum C3 levels comparable to those of EAMG-susceptible B6 mice. Therefore, serum C3, possibly, is not the most significant or sole contributor of increased EAMG susceptibility in RIIIS/J mice, although it is rather plausible that it plays a very important role in EAMG pathogenesis. It is notable that two strains (RIIIS/J and F1) with higher serum C3 levels also have higher serum anti-AChR levels. These high Ab levels might have been accomplished by isotype switching enhancing effect of C3. C3 is known to have two major roles in enhancing Ab production: 1) binding to CD35/21 receptors of B cells and dendritic cells in the secondary lymphoid organs together with the Ag, and 2) assisting isotype switching. In the absence of C3, B cells undergo apoptosis because of inadequate Ag stimulation (35, 36). To our knowledge, previous studies performed to explore these mechanisms in more depth have been performed either using C3 KO mice or immunizing mice with C3-conjugated Ags (37, 38). However, both attempts either eliminate or activate both mechanisms. Reminiscent of C3 KO mice, RIIIS/J mice have low lymph node and spleen B cell numbers. Our results show that with lower B cell numbers and a less intense primary immune response, but increased serum C3 levels, RIIIS/J mice are still capable of producing high AChR-specific IgG levels, which might be due to enhancement of isotype-switching effect by C3 or other yet unknown molecules. In this respect, CD27, which was overexpressed in microarray analysis, might be of particular interest for future studies, because this molecule is known to be important for isotype switching.

Elevated serum C1q-CIC levels of RIIIS/J mice might be a better explanation for increased EAMG severity because serum C1q-CIC levels are higher in RIIIS/J mice than all other three strains (Fig. 5). Increased serum C1q-CIC levels might be increasing disease severity by increasing the efficiency and magnitude of Ab-Ag interactions at the NMJ. We recently showed that the classical complement pathway is crucial for induction of EAMG, and C4 KO mice are highly resistant to EAMG (14). Elevated immune complex levels might also be facilitating better complement activation, increased C3 and MAC production at the NMJ, and thus more intense NMJ destruction. To our knowledge, this is the first study showing an association between serum C1q-CIC levels and clinical severity of EAMG. We predict that serum C1q-CIC measurement in human MG patients could predict clinical severity and response to treatment.

Concluding remarks

Based on studies that RIIIS/J mice are resistant to certain autoimmune diseases while being highly susceptible to EAMG, we propose the below hypothesis: the increased EAMG severity of RIIIS/J mice might be due to increased amounts of CD4+ T cells and increased complement activation capacity. Despite lower B cell counts, AChR triggers a very strong immune response in RIIIS/J mice, and B cells of RIIIS/J mice are capable of producing high amounts of anti-AChR IgGs. These Ags conjugate with C1q to form C1q-CICs, which can potentially activate T cells (39), explaining significant increase in CD4+ cells in RIIIS/J mice after AChR immunization. Activation of the complement cascade by C1q increases the production of C3, and C3 further increases Ab production. This vicious circle between T cells, C3, and IgGs (Fig. 6), combined with a highly active complement system, stimulates the production of higher amounts of immune complexes and C3, which eventually destroy NMJ and thus increase EAMG severity. We believe that our findings and further studies testing the above-mentioned predictions might aid in understanding mechanisms underlying the pathogenesis of autoimmune diseases observed during the course of B cell immunodeficiency syndromes.

References


