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Immunology

In vivo therapy with monoclonal anti-I-A antibody suppresses immune responses to acetylcholine receptor

(autoimmune disease/*I* region/monoclonal antibodies/myasthenia gravis)

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ABSTRACT **A monoclonal antibody to *I-A* gene products of the immune response gene complex attenuates both humoral and cellular responses to acetylcholine receptor and appears to suppress clinical manifestations of experimental autoimmune myasthenia gravis. This demonstrates that use of antibodies against immune response gene products that are associated with susceptibility to disease may be feasible for therapy in autoimmune conditions such as myasthenia gravis.**

Susceptibility to experimental autoimmune myasthenia gravis (EAMG) is linked in part to the major histocompatibility complex (*H-2*) on chromosome 17 (1, 2). The immune response to acetylcholine receptor (AcChoR) has been mapped to the *I-A* subregion of the immune response gene complex (*I* region) within *H-2* (3). Recently it was shown that anti-I-A antibody has potent effects *in vivo* (4), including clinical prevention of the autoimmune disease experimental allergic encephalitis (EAE) (5). Because *I-A* regulates the expression of clinical EAMG and the immune response to AcChoR, we asked whether *in vivo* administration of anti-I-A antibody might decrease antibody and lymphocyte proliferation responses to AcChoR and whether it might prevent EAMG.

MATERIALS AND METHODS

Animals. SJL/J female mice were obtained directly from The Jackson Laboratory. C57BL/Ka (C57BL/6) male mice were bred in the animal facility of the Department of Radiobiology, Stanford University. Mice 6–10 weeks old were used.

Antigens. AcChoR protein was purified from *Torpedo californica*, provided by Maehr Marine (Monterey, CA) or Pacific Biomarine (Venice, CA), according to the method of Elliot *et al.* (6). The amount of AcChoR prepared was quantified by ^{125}I-labeled bungarotoxin (New England Nuclear) binding on DEAE-cellulose according to the method of Schmidt and Raftery (7). AcChoR was dialyzed for 12 hr with 0.1% phosphate-buffered sodium cholate. Tuberculin purified protein derivative was purchased from Connaught Laboratories (Willowdale, Ontario, Canada).

Antibodies. Monoclonal antibody from hybridoma 10-3.6 recognizes a public specificity (Ia.17) found on I-A^s cells and is an IgG2a (8). Monoclonal antibody BP107 recognizes I-A^{b,d,q,u,j} and is an IgG1. Monoclonal antibody BP107 does not crossreact with I-A^s in a binding assay (4). The hybridomas were maintained as ascites—monoclonal antibody 10-3.6 in BALB/c mice and BP107 in (SJL × BALB/c) mice. Both monoclonal antibodies had binding titers $>10^3$ on appropriate spleen cells.

Immunizations. In some experiments, soluble AcChoR was administered (50 μg in phosphate-buffered saline) in 500 μl intraperitoneally at 1-week intervals. To investigate anti-AcChoR antibody levels after administration of AcChoR in adjuvant and to induce clinical EAMG (2), emulsified AcChoR, 15 μg in complete Freund adjuvant (Difco), was injected intradermally among six sites on the back, the hind foot pads, and the base of the tail. Animals were reimmunized with this same regimen 4 weeks later (2). For lymphocyte proliferation experiments, mice were immunized in the hind foot pads with 7.5 μg of AcChoR emulsified in complete Freund's adjuvant containing mycobacterium H37Ra (400 μg/ml) in a volume of 100 μl.

Enzyme-Linked Immunoadsorbent Assay (ELISA) for Anti-AcChoR Antibody. A microtiter ELISA was developed to measure the anti-AcChoR antibody levels (9). A standard reagent volume of 50 μl per microtiter well was always used. Unless otherwise specified, reagents were incubated in the wells for 2 hr at room temperature. Five micrograms of AcChoR diluted in bicarbonate buffer (pH 9.6) was added to each well of Immulon (Dynatech Laboratories, Alexandria, VA) microtiter plates. After incubation with the AcChoR, the plates were rinsed four times with a wash solution consisting of phosphate-buffered saline containing 0.05% Tween (polyoxethylene-20 sorbitan monooleate) and 0.05% NaN_3. It was determined experimentally that 5 μg of AcChoR per microtiter well was in excess of the amount of AcChoR needed to bind all of the anti-AcChoR antibody in a standard anti-AcChoR antiserum (defined below). Mouse sera were diluted in 0.01 M phosphate-buffered saline, pH 7.2/1.5 mM $MgCl_2$/2.0 mM 2-mercaptoethanol/.05% Tween-80/0.05% NaN_3 (PO_4-Tween buffer) and incubated on the plate. After the plate was washed, β-galactosidase-conjugated sheep anti-mouse antibody [$(Fab)_2$; Bethesda Research Laboratory] diluted 1:100 in PO_4-Tween buffer was added to each well. After a final washing, the enzyme substrate *p*-nitrophenyl-β-D-galactopyranoside at 1 mg/ml was added to the plate and the degree of substrate catalysis was determined from the absorbance at 405 nm after 1 hr, measured in an automated spectrophotometer.

In each assay, a standard antiserum was serially diluted and a plot of volume of standard antiserum versus absorbance was made. All test antisera were always diluted so that they were in the linear part of this standard curve. Pooled serum from SJL/J mice (bled 8 days after receiving three intraperitoneal immunizations with 50 μg of AcChoR at 2-week intervals) was used as the standard antiserum. Normal mouse serum at a dilution equal to that of the test antiserum was used to determine background values. Results are expressed in terms of the volume of the standard reference serum required to give an absorbance equal to that of 1 μl of the test serum.

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Abbreviations: EAMG, experimental autoimmune myasthenia gravis; AcChoR, acetylcholine receptor; EAE, experimental allergic encephalitis; ELISA, enzyme-linked immunoadsorbent assay.

Lymphocyte Proliferation. Seven days after immunization, mice were sacrificed and the popliteal lymph nodes were removed. Lymph node cells were dissociated in phosphate-buffered saline, washed three times, and resuspended in culture medium RPMI 1640 (GIBCO), supplemented with penicillin G (100 units/ml), streptomycin (100 μg/ml), 2-mercaptoethanol (30 μM), and 2% heat-inactivated horse serum. Cells (5×10^5) were cultured for 5 days in flat-bottomed Costar (Cambridge, MA) microtiter plates at 37°C in a humidified 5% CO_2/95% air environment. AcChoR protein (0.5 μg per well) and purified protein derivative (16 μg per well) were added to each well (in triplicate) and cultured for 5 days. [^{3}H]Thymidine was added 16 hr before the cells were harvested in an automated cell harvester. Thymidine uptake was expressed as

$$\Delta \text{ cpm} = \text{cpm with antigen} - \text{cpm without antigen.}$$

RESULTS

Reduction of Anti-AcChoR Antibody Titers. In order to determine whether administration of monoclonal anti-I-A antibody could decrease anti-AcChoR antibody titers in mouse strains that are susceptible to EAMG and are high responders to AcChoR, anti-I-A antibody was administered *in vivo* prior to immunization with soluble AcChoR or with AcChoR in complete Freund adjuvant. Anti-I-A antibody in ascites fluid was given intraperitoneally, 0.5 ml 1 day before and 0.5 ml 1 day after immunization. Administration of anti-I-A was repeated at the time of the secondary immunization. Animals were bled 1 week after secondary immunization, and titers were determined with an ELISA for anti-AcChoR antibody.

In the susceptible SJL/J ($H\text{-}2^s$) mouse, three groups were studied: group 1 received no monoclonal antibody, group 2 received monoclonal anti-I-A^b which does not bind to SJL/J cells, and group 3 received anti-I-A^s. After secondary intraperitoneal immunization of SJL/J mice with soluble AcChoR, anti-AcChoR titers were decreased by anti-I-A^s treatment, compared to treatment with the noncrossreactive anti-I-A^b or no monoclonal antibody. After secondary immunization (Fig. 1 *Left*), mean (±SD) anti-AcChoR antibody was $22.3 \pm 5.9 \times 10^{-3}$ μl with no treatment, $18.6 \pm 17.7 \times 10^{-3}$ μl with anti-I-A^b, and $0.2 \pm 0.4 \times 10^{-3}$ μl with anti-I-A^s ($P < 0.0001$ for I-A^s vs. no monoclonal and $P < 0.02$ for I-A^s vs. I-A^b). Similar results were obtained in three subsequent experiments. Thus it appears that, *in vivo*, treatment with anti-I-A antibody almost entirely prevents an antibody response to soluble AcChoR.

To test the ability of the anti-I-A^b monoclonal antibody (BP 107) to decrease anti-AcChoR antibody titers, C57BL/Ka (C57BL/6) ($H\text{-}2^b$) mice, which are also susceptible to EAMG (2, 10), were used. After primary and secondary immunization of these mice with 50 μg of soluble AcChoR intraperitoneally (Fig. 1 *Right*), antibody titers were $7.5 \pm 7.6 \times 10^{-3}$ μl in the no-treatment group, $6.2 \pm 4.8 \times 10^{-3}$ μl in the anti-I-A^s antibody

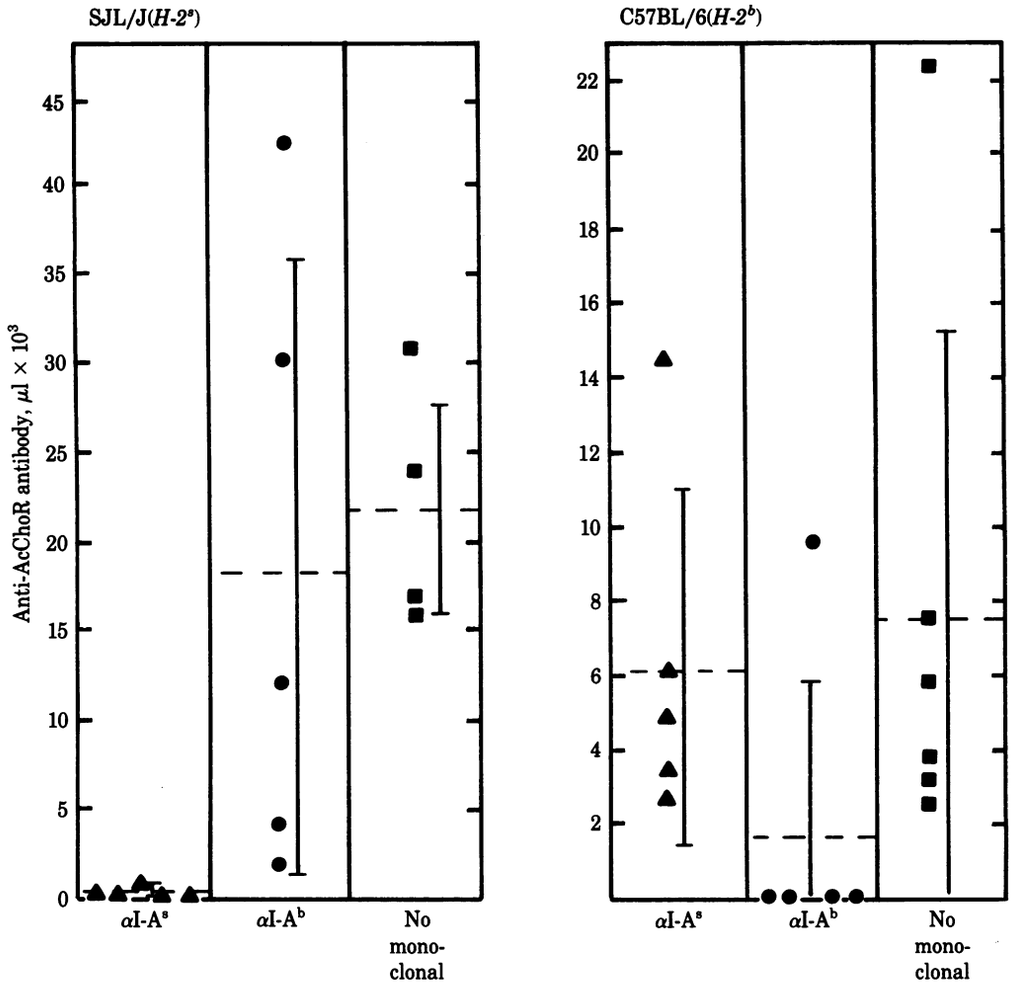

FIG. 1. Anti-AcChoR antibody titers after secondary immunization with soluble AcChoR. (*Left*) In individual SJL/J mice. (*Right*) In individual C57BL/6 mice.

group, and $1.6 \pm 3.9 \times 10^{-3}$ μl in the anti-I-A^b group ($P < 0.05$ for anti-I-A^b compared to no monoclonal or anti-I-A^s antibody treatment). Thus, two different monoclonal anti-I-A antibodies effectively decreased anti-AcChoR antibody titers in the appropriate genetic strain.

After primary and secondary immunizations of SJL/J mice with 15 μg of AcChoR in complete Freund adjuvant (Fig. 2), antibody titers were $14.3 \pm 4.9 \times 10^{-1}$ μl in the no-treatment group, $14.1 \pm 4.7 \times 10^{-1}$ in the anti-I-A^b antibody group, and $9.0 \pm 4.0 \times 10^{-1}$ μl in the anti-I-A^s antibody group ($P < 0.01$ for anti-I-A^s compared to no monoclonal or anti-I-A^b treatment). Similar results were obtained in a second experiment with 10 mice in each group. Thus, anti-AcChoR antibody titers can be decreased with anti-I-A treatment even when mice are immunized with AcChoR in complete Freund adjuvant.

Reduction of AcChoR-Induced Lymphocyte Proliferation. To test whether cellular responses to AcChoR or to purified protein derivative would be influenced by treatment with anti-I-A antibody, SJL/J mice were given anti-I-A antibody 1 day before (0.5 ml) and 1 day after (0.25 ml) immunization with AcChoR. Draining lymph nodes were assayed 7 days later for lymphocyte proliferation responses. Mean (± SD) Δ cpm for AcChoR responses was 111,600 ± 31,800 for anti-I-A^b-treated mice, 67,800 ± 35,500 for untreated mice, and 40,300 ± 19,700 for anti-I-A^s-treated mice ($P < 0.05$ for anti-I-A^s vs. no treatment and $P < 0.0001$ for anti-I-A^s vs. anti-I-A^b treatment) (Fig. 3). Responses to purified protein derivative, expressed as mean (± SD) Δ cpm, were 122,570 ± 30,325 for anti-I-A^b-treated mice, 61,625 ± 21,639 for untreated mice, and 77,250 ± 36,393 for anti-I-A^s treated mice ($P < 0.15$ for anti-I-A^s vs. no treatment and $P < 0.01$ for anti-I-A^s vs. anti-I-A^b). Thus, proliferation responses to AcChoR were decreased but those to purified protein derivative were not after anti-I-A treatment, indicating that this treatment decreases cellular immunity to AcChoR but does not induce widespread suppression of responses to other antigens.

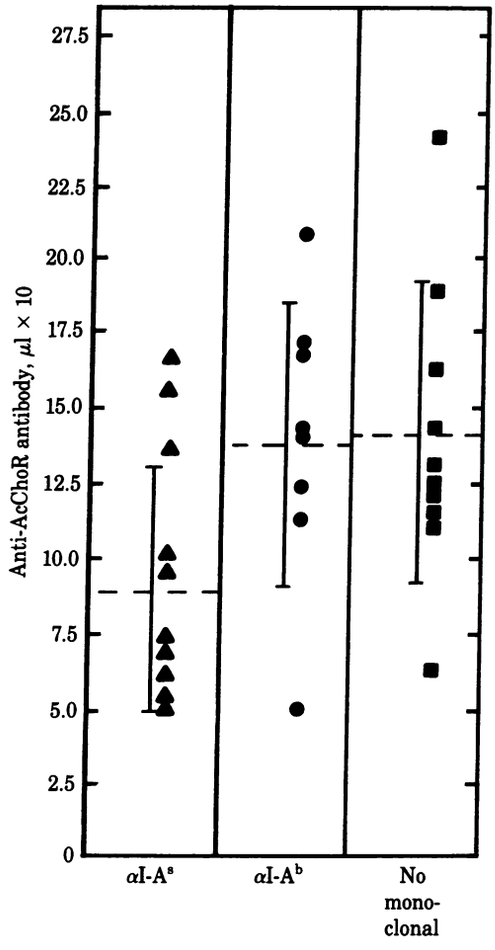

FIG. 2. Anti-AcChoR antibody titers in individual SJL/J mice after secondary immunization with AcChoR in complete Freund adjuvant.

Effects on Clinical EAMG. The effect of *in vivo* anti-I-A treatment on clinical EAMG in SJL/J mice was assessed. Myasthenia symptoms included a characteristic hunched posture with drooping of the head and neck, exaggerated arching of the back, splayed limbs, abnormal walking, and difficulty in righting. Mild symptoms were present only after a standard stress test involving swimming (10). Weakness was ameliorated (unless mice were moribund) within 5–10 min of administration of neostigmine bromide (0.0375 mg/kg) and atropine sulfate (0.015 mg/kg) intraperitoneally. Clinical disease was apparent in 11 of 19 control animals (7 of 9 treated with anti-I-A^b and 4 of 10 untreated); 2 of 10 anti-I-A^s-treated animals were mildly myasthenic (χ^2 with continuity correction = 4.28, $P < 0.03$ for anti-I-A^b vs. anti-I-A^s treatment; χ^2 with continuity correction = 2.48, $P < 0.12$ for both control groups vs. the anti-I-A^s-treated group).

DISCUSSION

These experiments indicate that *in vivo* therapy with monoclonal antibody to *I-A* gene products partially suppresses both cellular and humoral responses to AcChoR and appears to suppress clinical EAMG. The decrease in anti-AcChoR titers observed with *in vivo* anti-I-A therapy is greater than that achieved with other experimental therapies for myasthenia gravis such as cyclophosphamide and bone marrow transplantation in rats (11) or plasmapheresis and azathioprine in humans (12, 13). The present experiments with anti-I-A antibody treatment revealed a 40% reduction of anti-AcChoR antibody titers after a severe challenge with AcChoR in complete Freund adjuvant, injected at multiple sites to induce disease. It is clear, however, that anti-I-A antibody can prevent an anti-AcChoR antibody response almost completely after soluble immunization, a condition perhaps resembling natural autoimmunization more closely.

These results extend earlier observations on the influence of anti-I-A antibodies *in vivo*. These effects include prolonged graft survival (14), regulation of immunity to tumors (15, 16), inhibition of schistosome granuloma formation (17), potentiation of antibody responses (18), and inhibition of T-helper cell induction (19). More recently, haplotype-specific suppression of antibody responses to antigens under *Ir* gene control (4), and prevention of clinical EAE (5) have been accomplished with monoclonal anti-I-A antibody.

Therapy with monoclonal anti-I-A^s, unlike other current therapies for myasthenia gravis, had some antigenic specificity. Whereas cellular proliferation responses to AcChoR—an antigen clearly under *Ir* gene control (1–3)—were decreased, the responses to purified protein derivative—which is not under *Ir* gene control—were in the I-A^s-treated animals not significantly different from those in the untreated animals. It is not clear why the response to purified protein derivative is not at least partially suppressed. No diminution in the purified protein derivative response after anti-I-A treatment has been observed in EAE as well (unpublished data).

As other investigators have observed (20–22), the frequency of clinical myasthenia in mice is relatively low. Explicit correlations between degrees of severity of clinical EAMG and anti-

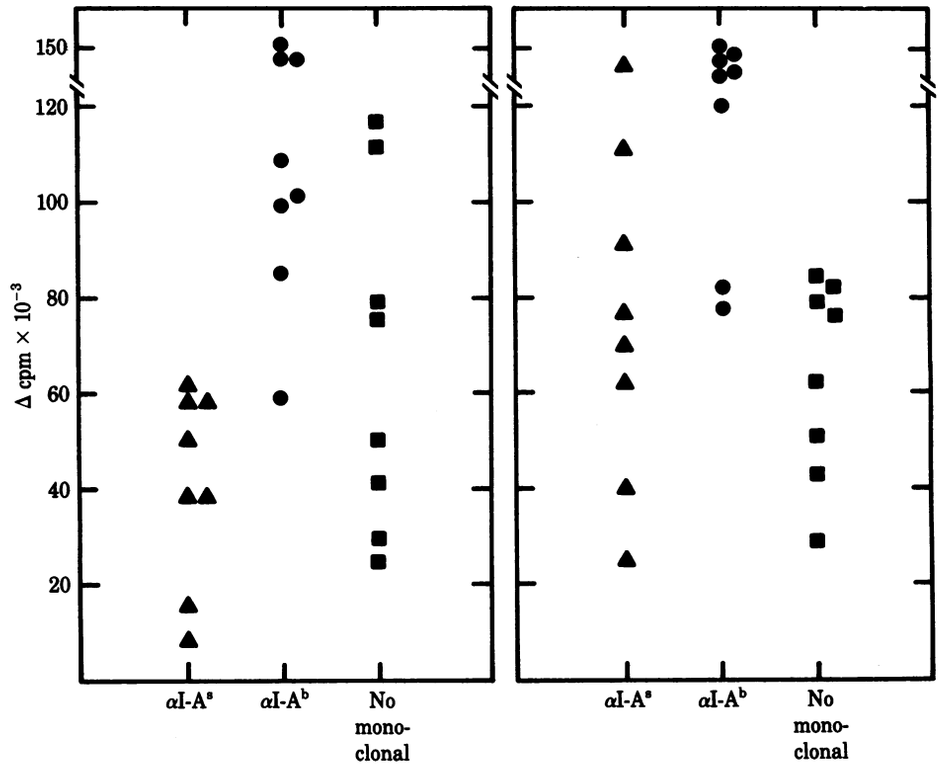

FIG. 3. *In vitro* lymphocyte proliferation responses in individual mice. (*Left*) AcChoR responses. (*Right*) Responses to purified protein derivative.

AcChoR titers (1, 2, 10), lymphocyte proliferation responses to AcChoR (3, 20), or amplitude of electromyographic decrement to repetitive stimulation (22) have not been established in murine EAMG. Until these relationships are proven, the assessment of putative therapy of clinical EAMG must be interpreted cautiously.

At present, the mechanism of action of anti-I-A antibody is unknown. The way in which anti-I-A decreases cellular and humoral responses to AcChoR may involve blockade of antigen-presenting cells which have been shown to play a critical role in both proliferative and antibody responses to AcChoR (23). Another possibility, not mutually exclusive, is that treatment with anti-I-A activates suppressor cells for AcChoR. Administration of anti-I-A both *in vitro* and *in vivo* has been shown to induce suppressor cells in various systems (24–26). A suppressor T cell for anti-AcChoR antibody responses is induced after anti-I-A treatment *in vivo* (unpublished data).

Myasthenia gravis is one of several human autoimmune diseases linked to *HLA-D* (27, 28). In myasthenia gravis, antibodies to AcChoR impair neuromuscular transmission by mediating loss of AcChoR in the postsynaptic membrane (29, 30). Any therapeutic regimen aimed at suppressing myasthenia gravis ideally should attenuate the antibody responses to AcChoR while leaving the rest of the patient's immune system intact.

Therapy with antibody to *Ir* gene products may be helpful in diseases linked to specific *Ir* genes. This type of therapy may be haplotype specific, leaving the action of the allelic gene at a heterozygous locus intact (4). Antibody to *Ir* gene products may block the immune response to several disease-inducing epitopes on a self-antigen. It may be necessary to suppress the response to several distinct epitopes on AcChoR in order to treat myasthenia gravis effectively (31). Therapy with anti-idiotypic antibody to a single disease-inducing AcChoR determinant, even if totally effective in suppressing the response to that determinant, might be unsuccessful in treating the disease (32, 33).

These experiments indicate that *in vivo* therapy with monoclonal antibody to *I-A* gene products, the murine counterpart of HLA-D antigens, partially suppresses both cellular and humoral responses to AcChoR. Therapy of myasthenia gravis may be feasible with antibodies to products of immune response genes that regulate the response to AcChoR.

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