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Immunological Relationship Between Acetylcholine Receptor and Thymus: A Possible Significance in Myasthenia Gravis

(autoimmune disease/neuromuscular junction/electric eel/immunologic cross-reaction/myoid cells)

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ABSTRACT **A defined immunological cross-reaction was observed between acetylcholine receptor fraction from the electric eel, *Electrophorus electricus*, and two calf thymus fractions. The cross-reaction was demonstrated on the cellular level by means of the lymphocyte transformation technique, and on the humoral level, by means of the microcomplement fixation assay.**

In the human disease myasthenia gravis both acetylcholine receptor at the neuromuscular junction and the thymus are affected, probably by an autoimmune mechanism. The immunological cross-reaction between acetylcholine receptor and thymic components may explain the association between endplate and thymus disorders in myasthenia gravis.

Evidence has recently been presented for the involvement of the acetylcholine receptor (AcChR) as an autoantigen in myasthenia gravis (MG). Fambrough *et al.* (1) demonstrated that there is a reduction of AcChR in the neuromuscular junctions of myasthenic patients. Other authors (2–4) have reported on the experimental induction of myasthenia in rabbits immunized with purified AcChR from electric fishes. We have reported (5–7) that lymphocytes from patients with MG were stimulated when cultured *in vitro* with a water-soluble AcChR fraction extracted from the eel *Electrophorus electricus*. In addition, we have recently found (Aharonov *et al.*, unpublished data) a humoral immune response to purified AcChR in sera of myasthenic patients. The above findings suggest that *in vivo* sensitization to self AcChR may occur in MG as a result of an autoimmune response. The muscular weakness characteristic of the disease would thus result from an "immunopharmacological" blockage of self AcChR on the post-synaptic membrane of the neuromuscular junction.

A model involving an autoimmune response to AcChR seems to explain adequately many clinical and physiological manifestations of the disease. However, any general model for the pathogenesis of MG must also take into account the involvement of the thymus in this condition. In most myasthenic patients either neoplastic or hyperplastic changes in the thymus are observed (8). Moreover, thymectomy was shown to be beneficial in the management of some patients (9). Immunological studies on myasthenic patients have demonstrated the presence of a humoral as well as a cellular immune response towards thymic tissues in some cases (10, 11), and a cytotoxic effect of myasthenic lymphocytes on cultured thymus cells has also been shown (12). Goldstein and others (13–16) have shown that animals immunized with a thymic extract developed an autoimmune thymitis as well as a partial defect in neuromuscular transmission.

The relation between the thymic disorder and the neuromuscular block is not yet understood. According to Goldstein (17) and Kalden *et al.* (15), the effect on neuromuscular transmission is mediated by a substance released from the thymus.

Another possibility is that an autoimmune response, as a result of antigenic alterations, may cause damage to both neuromuscular junctions and to the thymus (18). Both the neuromuscular junction and thymus may be involved concordantly, or one of them may be affected primarily and the other secondarily, due to immunological cross-reaction between them. In view of these possibilities and the recent observations concerning the role of AcChR in MG, we looked for an immunological cross-reaction between AcChR and thymus. Indeed, we have shown that AcChR fractions from electric organ tissue of the electric eel crossreact with components of calf thymus, both on the cellular and humoral levels of the immune response. Such a cross-reaction could provide a molecular basis for the association between neuromuscular block and thymic disorders in MG.

MATERIALS AND METHODS

Antigens. AcChR was solubilized from electric organ tissue of the electric eel, *Electrophorus electricus*, with 1% Triton X-100, by a procedure similar to that of Olsen *et al.* (19) with slight modifications (38). The Triton crude extract (designated AcChR I) was further purified by affinity chromatography on a *Naja naja siamensis* neurotoxin-Sepharose resin. The receptor was eluted from the resin with 1 M carbamylcholine; the dialyzed eluate was designated AcChR II. The preparations of acetylcholine receptor used in these experiments had specific activities of 3000–4500 pmol of toxin-binding sites per mg of protein. AcChR II was further chromatographed on an antibody column which was prepared by conjugating the IgG fraction from rabbit anti-acetylcholinesterase (AcChE) serum (20) to CNBr-activated Sepharose. This step was added in order to remove residual AcChE activity which was present in AcChR II. The AcChR fraction free of AcChE activity was designated as AcChR III.

Fresh calf thymus tissue was extracted with 0.1 M sodium phosphate buffer, pH 7.4, according to the method described by Trainin (21). The extract was centrifuged for 20 min at 2500 × *g* and the supernatant was designated as CT I. CT I

Abbreviations: AcChR, acetylcholine receptor; AcChE, acetylcholinesterase; MG, myasthenia gravis.

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was further centrifuged for 5 hr at 100,000 × g and the supernatant obtained following this centrifugation was designated CT II.

Pure AcChE was prepared according to Dudai *et al.* (22).

Immunizations. For preparation of antisera, rabbits were immunized with eel AcChR II fraction and with calf thymus fraction I (CT I). The immunogens were injected (0.5 mg per rabbit) intradermally at multiple sites in an emulsion with complete Freund's adjuvant. The animals were immunized twice with a 10-day interval between immunizations and were bled weekly.

For the lymphocyte transformation test, rabbits were injected in the hind foot pads with the following antigens: AcChR II, AcChR III (0.1 mg per rabbit), CT I (0.5 mg per rabbit), and eel AcChE (0.2 mg per rabbit) emulsified with complete Freund's adjuvant. Each antigen was injected into three to four rabbits.

Lymphocyte Transformation. Lymph node cells were diluted to a concentration of 8×10^6 to 10×10^6 cells per culture, and were incubated for 24 hr with various amounts of the test antigens according to the method described by Tarrab *et al.* (23). The antigens used were: AcChR fractions, calf thymus fractions and AcChE. Following incubation with the antigens, the cultures were incubated for an additional 24 hr with 0.1 μCi of [2-^{14}C]thymidine. Then the cells were collected and filtered as described previously (6). The results are expressed as stimulation indices (SI), namely the ratio of radioactivity (counts per minute, cpm) in tubes containing antigen to the radioactivity in antigen-free tubes (control), and they represent an average of values obtained in duplicate cultures and an average of three to four experiments. Stimulation indices higher than 2.0 were considered positive.

Microcomplement Fixation. Quantitative microcomplement fixation reactions were carried out as described by Levine (24).

AcChE Assay. AcChE activity was assayed by the method of Ellman *et al.* (25).

RESULTS

The immunological cross-reactivity between AcChR from the electric eel and calf thymus was studied by two techniques: the lymphocyte transformation test, which represents a cell-mediated immune response, and the microcomplement fixation assay, which measures humoral antibodies.

Lymphocyte Transformation. A defined cross-reaction between the AcChR and calf thymus was observed. Lymphocytes obtained from rabbits preimmunized with AcChR II were significantly stimulated when incubated in culture with either the homologous antigen or calf thymus extracts, CT I and CT II (see Table 1). Although the average stimulation indices obtained with the calf thymus extracts seem quite low, some animals gave stimulation indices as high as 4.0. These lymphocytes were also stimulated by eel AcChE. This latter response may be due to small amounts of AcChE present in the receptor preparation. Indeed, when lymphocytes obtained from rabbits preimmunized with AcChE-free AcChR (AcChR III) were employed, the response to AcChE was abolished. In addition, lymphocytes from rabbits preimmunized with purified AcChE were not stimulated *in vitro* with calf thymus extracts. This excludes the possibility of cross-reaction between the enzyme AcChE and the thymus. *Naja naja siamensis* α-neurotoxin, which was used for purifying the receptor by affinity chromatography, did not stimulate lymphocytes from rabbits preimmunized with AcChR.

TABLE 1. *Cellular cross-reactivity between AcChR and calf thymus extracts*

	Rabbits immunized with:		
	AcChR II	Calf thymus (CT I)	AcChE
Antigen in culture	Average SI*		
AcChR II	7.7	1.2	3.0 1.3†
Calf thymus (CT I)	3.0	5.3	1.3
Calf thymus (CT II)	2.5	3.5	1.3
AcChE	6.0 1.5‡	1.1	7.4
Human muscle	1.1	1.3	1.5

* The SIs given are those obtained with 0.5 μg of AcChR II or AcChR III, 50 μg of CT I, 100 μg of CT II and 5 μg of AcChE, as these amounts gave the highest stimulation.

† This value corresponds to the results obtained with lymphocytes from animals immunized with AcChE and challenged *in vitro* with AcChR III.

‡ This value corresponds to the results obtained with lymphocytes from animals immunized with AcChR III.

Lymphocytes from animals preimmunized with CT I were not stimulated by AcChR (Table 1). The inability to detect cross-reaction in this direction may be due to the low immunogenicity of the relevant determinant(s) in the thymus, which intrinsically appears to be weakly immunogenic.

No stimulation of lymphocytes from the above rabbits was observed when they were cultured with any of the following antigens: basic proteins of bovine central (26) and peripheral (27) nerve tissue, human aqueous muscle extract, and hen egg white lysozyme (a nonrelated protein). The stimulation indices obtained with the mitogens phytohemagglutinin and concanavalin A were about 30.

Microcomplement Fixation. Antisera to AcChR II and to calf thymus fraction I (CT I) were elicited in rabbits. The antisera obtained were tested with both the homologous and heterologous antigens by the microcomplement fixation technique. As can be seen in Fig. 1A, antiserum to AcChR II cross-reacts with the two calf thymus fractions (CT I and CT II). No detectable complement fixation by antiserum to AcChR II was obtained with AcChE, with human aqueous muscle extract, or with *Naja naja siamensis* α-neurotoxin (not shown in the figure). Rabbit anti-CT I serum, which gave complement fixation with the two calf thymus fractions, also crossreacted significantly with AcChR (Fig. 1B). Preimmune sera did not fix complement with any of the antigens tested at antigen concentrations used for assaying the immune sera.

DISCUSSION

In the present study we have demonstrated a defined immunological relationship between thymic components and acetylcholine receptor fractions. Lymphocytes obtained from rabbits immunized with AcChR II were stimulated *in vitro*

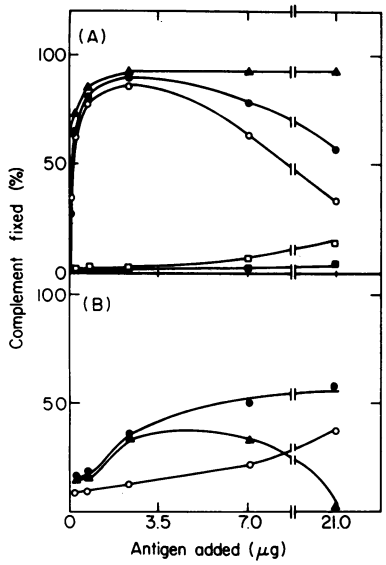

FIG. 1. Complement fixation reactions of anti-AcChR II (A) and anti-CT I (B). Serum dilution for both antisera was 1/800. The antisera were reacted with AcChR I (▲), CT I (●), CT II (○), AcChE (□), and human muscle extract (■).

both by the homologous antigen and by two calf thymus fractions (CT I and CT II). Similar cross-reactivity was demonstrated at the humoral level using antiserum against AcChR II.

The relationship between neuromuscular blockage and thymus alterations in myasthenia gravis is not yet defined. The immunological cross-reaction between AcChR and thymus demonstrated in this study may indicate that the association of neuromuscular block and thymitis in MG is the result of autoimmunization against an antigen shared by the neuromuscular endplate and by the thymus. According to the present study, the endplate and the thymus may be two separate, but immunologically related, target organs in MG. MG may thus be an autoimmune disease in which a breakdown of tolerance, both to self post-synaptic AcChR and to the related thymic component, results in a neuromuscular block as well as thymitis. Whether one of these loci is primarily affected and the other is secondarily affected due to cross-reaction between them, or both of them are affected concordantly, awaits further investigation.

If indeed the thymus is only one of the involved target organs and not the cause of the neuromuscular block, it appears possible that the thymic disorder may be the consequence of the disease rather than the cause of it. If this is the case, thymectomy should not necessarily eradicate the disease. It is possible that the improvement seen in some myasthenic patients following thymectomy depends on the general immunosuppression caused by this treatment. Indeed, some authors found a profound depression of lymphocyte reactivity and diminution of T cells in blood of myasthenics after thymectomy (11, 28–30), as would be expected taking into account the known immunosuppressive effect of thymectomy (31).

It was previously demonstrated that some thymic antigens are found also in other tissues and that thymic components crossreact with other antigens in the body (32). For example, an antigenic correlation has been demonstrated between human brain and thymus (33), and it is possible that this correlation may have some bearing on the cellular response to neural antigens which has been observed in some cases of MG (11, 34). Sera of myasthenic patients reacting with both muscle and thymus seem to contain antibodies directed against antigenic determinants shared by the two organs (10), and Aarli and Tönder (35) demonstrated the presence of anti-muscle antibodies in the sera of myasthenic patients.

The origin of the cross-reaction between thymus and muscle seems to stem from the "epithelial" thymus cells that are in reality "myoid" cells which have been shown to react with myasthenic sera (36). The electrogenic cell in electric organ is embryologically derived from muscle fibers which retain many of the pharmacological and physiological features of the postsynaptic membrane of skeletal muscle. In particular, the nicotinic AcChR in the electric organ tissue from *Electrophorus electricus* shows very similar specificity towards cholinergic agonists and antagonists as the same receptors in skeletal muscle (37), and crossreacts immunologically with AcChR from other species (2, 3). Therefore, it is possible that the AcChR from the eel crossreacts with AcChR on the membrane of the thymic "myoid" cells or with another related component present in the thymus.

Although the results presented here were obtained with not completely purified AcChR preparations, we have now obtained preliminary results of a similar nature using highly purified AcChR from electric organ tissue of *Torpedo californica*.

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1. Fambrough, D. M., Drachman, D. B. & Satyamurti, S. (1973) *Science* **182**, 293–295.
2. Patrick, J. & Lindstrom, J. (1973) *Science* **180**, 871–872.
3. Sugiyama, H., Benda, P., Meunier, J.-C. & Changeux, J.-P. (1973) *FEBS Lett.* **35**, 124–128.
4. Heilbronn, E. & Mattson, Ch. (1974) *J. Neurochem.* **22**, 315–317.
5. Abramsky, O., Aharonov, A., Webb, C., Teitelbaum, D. & Fuchs, S. (1974) *Isr. J. Med. Sci.* **10**, 1167–1168.
6. Abramsky, O., Aharonov, A., Webb, C. & Fuchs, S. (1975) *Clin. Exp. Immunol.* **19**, 11–16.
7. Abramsky, O., Aharonov, A., Teitelbaum, D. & Fuchs, S. (1975) *Arch. Neurol.*, in press.
8. Castleman, B. (1966) *Ann. N.Y. Acad. Sci.* **135**, 496–504.
9. Papatestas, A. E., Alpert, L. J., Osserman, K. E., Osserman, R. S. & Kark, A. E. (1971) *Amer. J. Med.* **50**, 465–474.
10. van der Geld, H. W. R. & Strauss, A. J. L. (1966) *Lancet* **i**, 57–60.
11. Field, E. J., Bates, D., Shaw, D. A., Griffin, S. G., Shenton, B. K. & Smith, K. (1973) *Lancet* **ii**, 675–676.
12. Mori, R. & Kawanami, S. (1973) *Lancet* **i**, 210.
13. Goldstein, G. (1968) *Lancet* **ii**, 119–122.
14. Goldstein, G. & Hofman, W. W. (1971) *Res. Publ. Ass. Nerv. Ment. Dis.* **49**, 241–252.
15. Kalden, J. R., Williamson, W. G., Johnston, R. J. & Irvine, W. J. (1969) *Clin. Exp. Immunol.* **5**, 319–340.
16. Kawanami, S. & Mori, R. (1972) *Clin. Exp. Immunol.* **12**, 447–454.
17. Goldstein, G. (1974) *Nature* **247**, 11–14.
18. Rule, A. H. & Kornfeld, P. (1971) *Mt. Sinai J. Med., New York* **38**, 538–572.
19. Olsen, R. W., Meunier, J.-C. & Changeux, J.-P. (1972) *FEBS Lett.* **28**, 96–100.
20. Gurari, D., Silman, I. & Fuchs, S. (1974) *Eur. J. Biochem.* **43**, 179–187.

21. Trainin, N. (1974) *Physiol. Rev.* **54**, 272–315.
22. Dudai, Y., Silman, I., Kalderon, N. & Blumberg, S. (1972) *Biochim. Biophys. Acta* **268**, 138–157.
23. Tarrab, R., Sulica, A., Haimovich, J. & Sela, M. (1971) *Eur. J. Immunol.* **1**, 231–236.
24. Levine, L. (1967) in *Handbook of Experimental Immunology*, ed. Weir, D. M. (Blackwell, Oxford), pp. 707–719.
25. Ellman, G. L., Courtney, D., Andres, V., Jr. & Featherstone, R. M. (1961) *Biochem. Pharmacol.* **7**, 88–95.
26. Hirshfeld, T., Teitelbaum, D., Arnon, R. & Sela, M. (1970) *FEBS Lett.* **7**, 317–320.
27. London, Y. (1971) *Biochim. Biophys. Acta* **249**, 188–196.
28. Taylor, R. B. (1965) *Nature* **208**, 1334–1335.
29. Metcalf, D. (1965) *Nature* **208**, 1336.
30. Schechter, B., Nir, E. & Levine, S. (1974) *Is. J. Med. Sci.* **10**, 1170.
31. Miller, J. F. A. P., Mitchell, G. F. & Weiss, N. S. (1967) *Nature* **214**, 992–997.
32. Schlesinger, M. (1972) *Progr. Allergy* **16**, 214–299.
33. Greaves, M. F. & Brown, G. (1974) *Lancet* **i**, 455.
34. Kott, E. & Rule, A. H. (1973) *Neurology* **23**, 745–748.
35. Aarli, J. A. & Tönder, O. (1970) *Clin. Exp. Immunol.* **7**, 11–21.
36. van der Velde, R. L. & Friedman, N. B. (1966) *Fed. Proc.* **25**, 661.
37. Hall, Z. W. (1972) *Annu. Rev. Biochem.* **41**, 925–952.
38. Ahazonov, A., Kalderon, N., Silman, I. & Fuchs, S. (1975) *Immunochemistry*, in press.