FRACTIONATION OF ANTIBODIES TO ACETYLCHOLINE RECEPTOR ACCORDING TO ANTIGENIC SPECIFICITY

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1. Introduction

Acetylcholine receptor (AChR) is a major autoantigen involved in the neuromuscular disease myasthenia gravis (MG). Injection of experimental animals with purified AChR from electric fish leads to the elicitation of an immune response to this immunogen accompanied by a neuromuscular weakness, designated experimental autoimmune myasthenia gravis (EAMG) (reviewed [1–3]). Antibodies to AChR were shown to block the physiological activity of AChR [4–6] and to inhibit the binding of α-bungarotoxin to the receptor [7]. However, it is still not known which parts or immunogenic determinants in the AChR molecule are responsible for its myasthenic activity and whether or not these overlap with sites involved in the physiological function of the receptor. Detailed immunochemical analysis of AChR should shed light on these questions.

We have demonstrated that a denatured preparation of AChR (RCM-AChR, i.e., reduced and carboxymethylated AChR) cross reacts partially with anti-AChR antibodies by reacting with antibodies directed against only part of the antigenic determinants present in the intact receptor [8]. Moreover, RCM-AChR by itself elicits an immune response which is not associated with any myasthenic symptoms, and is capable of specifically suppressing EAMG [9]. Analysis of the specificity of the immune response elicited by AChR and RCM-AChR led us to propose that the denaturation of AChR destroys some structural antigenic determinants which are important for the induction of EAMG, and which may be located close to the toxin binding site [8,9]. AChR elicits antibodies against both myasthenic determinants in the AChR molecule which are involved in the disease and other antigenic determinants which are not involved in the disease. RCM-AChR cross reacts only with antibodies of the latter specificity and by itself elicits an immune response only to determinants other than the myasthenic ones.

Here we describe the fractionation of two antibody populations different in their immunological and physiological specificity from rabbit anti-AChR serum. The fractionation was achieved by using an immunoadsorbent of RCM-AChR bound to Sepharose. Both the adsorbed and unadsorbed subpopulations of antibodies bind to intact AChR, whereas only the subpopulation which does not bind to RCM-AChR blocks the in vitro binding of α-bungarotoxin to AChR.

2. Materials and methods

AChR was isolated from the electric organ of *Torpedo californica* (Pacific Bio-Marine, Venice, CA) and was purified as in [7]. Reduced carboxymethylated AChR (RCM-AChR) was prepared by reduction and carboxymethylation of AChR in 6 M guanidine–HCl [8], α-bungarotoxin (α-Bgt) was prepared according to [10]. Iodination with ^{125}I of these three proteins was performed by the chloramine-T method [11].

2.1. *Preparation of RCM-AChR–Sepharose immunoadsorbent and fractionation of antibodies*

RCM-AChR was bound to Sepharose 2B which

had been activated with CNBr [12]. CNBr-activated Sepharose (10 g) was added to a solution of RCM-AChR (8 mg) in 0.1 M $NaHCO_3$ (25 ml). The mixture was kept overnight at 4°C with gentle stirring and was then washed with PBS. The RCM-AChR–Sepharose immunoadsorbent was packed into a column and anti-AChR serum (2 ml) was chromatographed through it at room temperature. The unadsorbed antibody fraction which came through the column was designated effluent. Antibodies which were immunospecifically adsorbed to the column were eluted with 0.2 M NH_4OH and dialyzed against several changes of PBS (eluate). Both antibody fractions were adjusted with PBS to 10 times the original volume of the serum applied to the column.

2.2. *Immunization*

Rabbits (2–3 kg) were injected in the footpads and intradermally in 3–4 spots, each with 100 μg AChR emulsified with complete Freund's adjuvant. The animals were bled when myasthenic symptoms were observed (~30 days following immunization).

2.3. *Immunological assays*

For radioimmunoassay, the anti-AChR serum, or fractionated antibodies (0.1 ml in 10% normal rabbit serum) was incubated with the radioactive antigen (^{125}I-labelled AChR, ^{125}I-labelled RCM-AChR or ^{125}I-labelled α-Bgt-AChR) for 30 min at 37°C. Goat-anti rabbit immunoglobulin serum (0.1 ml) was added and the tubes were incubated for an additional 30 min at 37°C. The centrifuged precipitates were washed twice and counted in an autogamma scintillation counter. For inhibition experiments the antiserum (at a dilution that binds 20–40% of the radioactive antigen) was preincubated with different amounts of inhibitor for 30 min at 37°C and the assay was continued as above for binding.

2.4. *Effect of antibodies on the binding of ^{125}I-labelled α-bungarotoxin to AChR*

The assay of ^{125}I-labelled α-Bgt binding to AChR was performed as in [7]. The inhibition of the binding of toxin to AChR by specific antisera was measured by preincubation of AChR with increasing amounts of serum for 30 min at 37°C before the addition of ^{125}I-labelled α-Bgt. The degree of toxin-binding was determined relative to the binding obtained when ^{125}I-labelled α-Bgt was reacted with AChR before the incubation with the serum.

3. Results

3.1. *Fractionation and immunological analysis of two different anti-AChR antibody populations*

Antibody fractionation was performed on sera from rabbits which were immunized with AChR and had clinical signs of EAMG. Serum fractionation was performed as in section 2. Direct binding of the fractionated antibodies with labeled antigens was determined by a radioimmunoassay. As can be seen in fig.1A unfractionated anti-AChR serum binds to intact AChR (^{125}I-labeled AChR or ^{125}I-labeled α-Bgt-AChR) as well as to denatured receptor (^{125}I-labeled RCM-AChR). After this serum was chromatographed on RCM-AChR–Sepharose, the effluent does not bind RCM-AChR anymore, but binds to intact AChR (fig.1B). In contrast, the second antibody fraction eluted from the adsorbent (eluate) binds to both AChR and RCM-AChR (fig.1C).

A similar pattern of specificity was obtained in experiments in which inhibition of the binding of ^{125}I-labeled AChR to the different antibody fractions was studied. RCM-AChR is a weaker inhibitor than

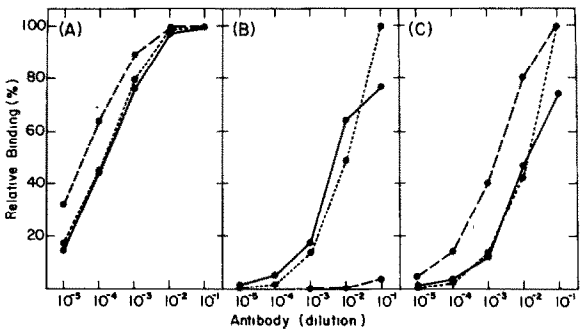

Fig.1. Binding of ^{125}I-labeled AChR (•———•), ^{125}I-labeled RCM-AChR (•– – – –•) and ^{125}I-labeled α-Bgt-AChR (• · ·•) To: (A), unfractionated anti-AChR serum; (B) effluent serum which did not bind to RCM-AChR–Sepharose; (C) eluted antibody fraction. The first dilution for the antibody samples (10^{-1}) is: for (A), a 1/10 dilution of the unfractionated serum; for (B) and (C), the respective antibody fraction after its total volume has been adjusted to 10-times the original volume of the serum applied to the column (see section 2).

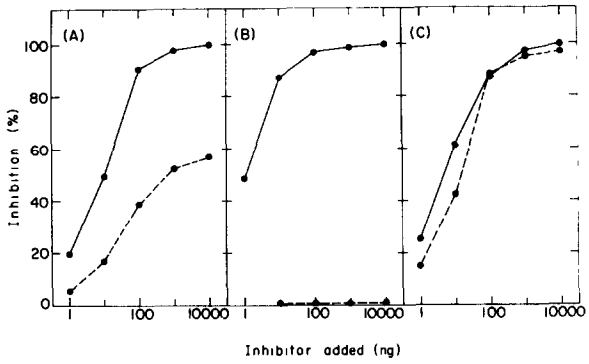

Fig.2. Inhibition of the binding of ^{125}I-labeled AChR to: (A) unfractionated anti-AChR serum; (B) effluent serum; (C) eluted antibody fraction, by unlabelled AChR (———) and RCM-AChR (– – – – –).

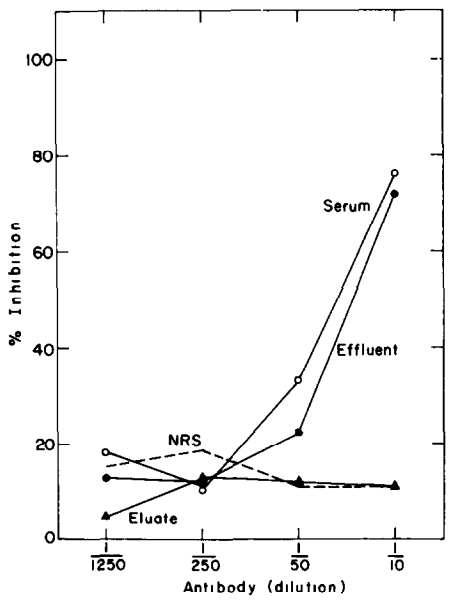

Fig.3. Inhibition of the binding of ^{125}I-labeled α-Bgt to AChR by: unfractionated anti-AChR serum, (o———o); effluent serum (•———•); eluted antibody fraction (▲———▲), and normal rabbit serum (– – – –).

AChR of the binding of ^{125}I-labeled AChR to unfractionated anti-AChR and inhibits only part of the binding (fig.2A) [8,9]. However, there is no inhibition at all by RCM-AChR of the binding of ^{125}I-labeled AChR to the effluent serum whereas AChR is a good inhibitor (fig.2B). There is no difference in the extent of inhibition by AChR and RCM-AChR of the binding of ^{125}I-labeled AChR to the eluted antibodies (fig.2C). This latter population of antibodies (eluate) is the one which is common to the intact and denatured receptor, whereas the subpopulation of antibodies in the effluent recognizes only the intact receptor and does not cross react with the denatured receptor.

3.2. *Effect of the fractionated antibodies on the binding of bungarotoxin to AChR*

Antibodies to AChR were shown to block the physiological activity of AChR and this reaction is possibly associated with the pathogenesis of autoimmune myasthenia gravis [4–6]. These antibodies also inhibit the in vitro binding of α-bungarotoxin to AChR [7,8,13].

Comparison of the effect of anti-AChR serum with that of the fractionated antibodies, on the binding of ^{125}I-labeled α-Bgt to AChR shows that the effluent antibodies which do not bind to RCM-AChR are those which block the binding of α-bungarotoxin to AChR (fig.3).

4. Discussion

Acetylcholine receptor is a multisubunit high molecular weight protein [7,14–16] containing a variety of immunopotent determinants. Thus, immunization with such an immunogen leads to the production of antibodies of heterogeneous antigenic specificities. It is likely that antibodies of only limited defined specificities are involved in the pathogenesis of EAMG. Zurn and Fulpius [13] have shown that in an AChR-immunized rabbit there was a relative increase in the level of antibodies which block toxin binding to the receptor concomitantly with the onset of EAMG, and implied that this antibody subpopulation might play a role in the appearance of the paralysis observed in this disease.

Here we have fractionated two antibody subpopulations from sera of AChR-injected rabbits, by using a RCM-AChR–Sepharose immunoadsorbent. Antibodies which were not adsorbed by the immunoadsorbent (effluent) were directed to determinants which are present only on the intact AChR (fig.1,2).

Only this antibody population blocks toxin binding to the receptor (fig.3) and it is possible that it represents the antibodies which are relevant in causing the neuromuscular damage in animals with myasthenia gravis [13]. If so it may be possible to transfer EAMG to normal rabbits by this antibody subpopulation. The antibody subpopulation which is bound to the immunoadsorbent, binds equally AChR and RCM–AChR (fig.1,2) and does not block the binding of toxin to the receptor (fig.3). Such a specificity was observed in rabbits immunized with RCM-AChR [8] and was predominant also in rabbits in which EAMG was prevented or reversed by RCM-AChR injection [9]. These antibodies are probably directed exclusively against sequentional antigenic determinants [17] in AChR. Transfer of this subpopulation of antibodies to myasthenic rabbits may lead to suppression of the disease. We have not succeeded in transferring myasthenia to rabbits by unfractionated rabbit anti-AChR serum (Tarrab-Hazdai and S.F., unpublished data), but this may result from the fact that in unfractionated serum antibodies of physiologically opposite functions (blocking and non-blocking) are present.

Further immunochemical analysis of AChR, isolation of the different antigenic determinants and the respective specific antibodies may help for a detailed structural analysis of AChR and for characterizing the exact specificity of antibody involved in the pathogenesis of both experimental and human myasthenia.

Acknowledgements

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