MONOCLONAL ANTIBODIES AGAINST DEFINED DETERMINANTS OF ACETYLCHOLINE RECEPTOR

Daria MOSHLY-ROSEN, Sara FUCHS and Zelig ESHHAR
Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot, Israel

Received 24 August 1979

1. Introduction

The nicotinic acetylcholine receptor (AChR) is a major autoantigen in the neuromuscular disease myasthenia gravis (MG) and experimental autoimmune myasthenia gravis (EAMG) [1–3]. Cellular and humoral immune responses to AChR have been demonstrated in both the human and the experimental diseases [1,2,4–7]. The involvement of anti-acetylcholine receptor antibodies in the pathogenesis of MG and EAMG has been proposed on clinical and experimental ground. Passive transfer of immunoglobulins from myasthenic patients [8] and from animals with EAMG [9] resulted in myasthenia gravis-like symptoms.

AChR is a complexed multideterminant immunogen which raises a heterogeneous immune response. In order to analyze the structure of the AChR molecule and for identifying the molecular entity which governs its myasthenic activity, large quantities of antibodies with defined specificity are required. Such antibodies also provide a useful tool for studying the role of antibodies in myasthenia and their mechanism of action. To this end we have used the method of lymphocyte hybridization developed [10]. Here we describe the preparation of monoclonal anti-AChR antibodies with restricted specificity towards defined determinants, secreted by hybridomas obtained by hybridization of anti-AChR antibodies producing cells with a myeloma line.

2. Materials and methods

AChR was isolated from the electric organ of *Torpedo californica* (Pacific Bio-Marine, Venice, CA) and was purified as in [11]. Reduced carboxymethylated AChR (RCM-AChR) was prepared by reduction and carboxymethylation of AChR in 6 M guanidine–HCl [12]. Trypsin-digested AChR preparation (T-AChR) was prepared as in [13]. α-Bungarotoxin (α-Bgt) was prepared according to [14]. Iodination of proteins with ^{125}I was performed by the chloramine-T method [15].

C57BL/6J mice were injected twice each with 10 μg Triton-solubilized AChR [16]. Sera of these mice contained high titers of anti-AChR antibodies ($\log_2$ hemagglutination titer 10–16). In order to enrich the number of the specific antibody forming cells, 40×10^6 splenic cells of 2 mice exhibiting muscular weakness characteristic to EAMG were transferred into lethally irradiated (800 R) syngeneic mice. Following cell transfer, the recipient mice were i.p. injected with 10 μg purified AChR. The spleens of the recipient mice were removed 5 days later, and fused with the P3-NSI/1-Ag4-1 (NS1) non-secreting plasmacytoma, in the presence of polyethylene glycol as in [17]. Anti-AChR antibody activity was tested in cultures exhibiting hybrid cell growth. The positive hybrid lines were further propagated in vitro and could develop antibody producing tumors upon i.p. inoculation into (C57BL/6 × BALB/c)F1 mice. Ascitic fluids developed in such mice were individually collected.

The immunoglobulin pattern of the ascitic fluids and of immune serum was analyzed by microzone electrophoresis on cellulose acetate strips [18]. The binding activity and specificity of the antibodies secreted by the hybridomas was analyzed by radioimmunoassay (binding and inhibition experiments) [12] using AChR, RCM-AChR and T-AChR as the radiolabelled antigens.

3. Results of discussion

In order to establish monoclonal antibodies with anti-AChR antibody activity, we fused spleen cells of AChR immunized mice with NS1 non-secreting plasmacytoma line. Growth of hybrid cells was observed in 50% of the initial culture cells. In 20 out of 39 independent cultures, anti-AChR antibody activity was detected by a radioimmunoassay with ^{125}I-labelled AChR. These positive hybrid lines were further propagated in vitro and in vivo, in the form of ascitic tumors.

The immunoglobulins secreted by many of the hybridomas displayed, by microzone electrophoresis, one sharp monoclonal band characteristic for each hybrid line, in contrast to the broad continuous immunoglobulin region observed upon electrophoresis of immune serum. The monoclonal immunoglobulin pattern of two representative hybrid lines is depicted in fig.1, in comparison with the pattern of anti-AChR immune serum. The immunoglobulin pattern and

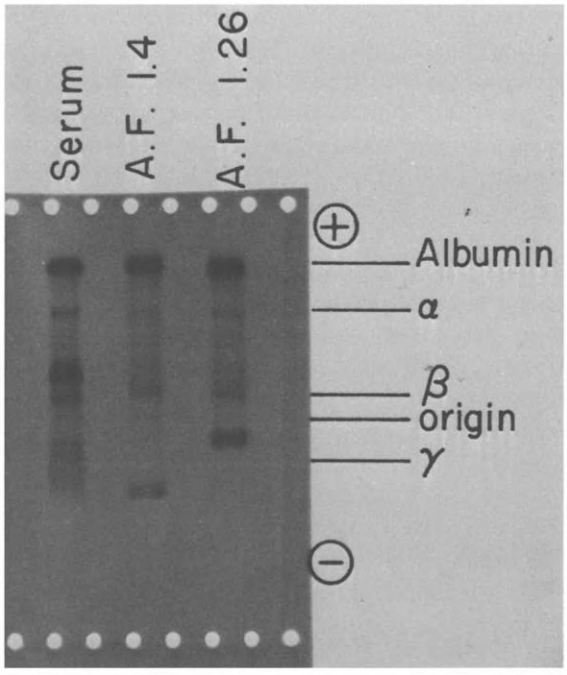

Fig.1. Microzone electrophoresis in barbital buffer (pH 8.6) on cellulose-acetate strips of mouse anti-AChR serum and of ascitic fluid 1.4 and ascitic fluid 1.26.

antibody activity in the positive hybridomas were stable during all transfer cycles.

For immunochemical analysis of the AChR determinants which are recognized by the various monoclonal antibody preparations, we have compared their activity towards the homologous antigen AChR with their activity towards two other receptor derivatives, RCM-AChR and T-AChR. RCM-AChR, which is a denatured AChR preparation obtained by reduction and carboxymethylation in 6 M guanidine–HCl, was shown to be devoid of pharmacological acetylcholine receptor activity [12]. It crossreacts with intact AChR but does not induce myasthenic symptoms upon immunization [12]. Moreover, RCM-AChR was shown to have suppressive effect on EAMG [19]. On the other hand, T-AChR obtained following tryptic digestion of AChR retains the pharmacologic and myasthenic activity of intact purified AChR [13]. As can be seen in table 1, anti-AChR sera from mice immunized with AChR contain antibodies binding to both RCM-AChR and T-AChR whereas, the antibodies produced by the hybridomas bound selectively only one of these antigens. The titration of two representative ascitic fluids (AF 1.4 and AF 1.26) and of an immune serum with AChR, RCM-AChR and T-AChR is depicted in fig.2. Restricted differential specificity of the ascitic fluids' antibodies is demonstrated also in inhibition experiments (fig.3). For instance, the binding of ^{125}I-labelled AChR to AF 1.26 is inhibited by RCM-AChR and not by T-AChR whereas the binding to AF 1.4 is inhibited by T-AChR and not by RCM-AChR (fig.3B,C). Notably, sera from immunized mice contain heterogeneous mixture of antibodies among which the anti-RCM-AChR antibody population appears to be a minor component (Fig.3A). The ability to select preferentially for one specificity, even a minor one, is one of the advantages offered by the lymphocyte hybridization method.

Since the original antibody forming cells were obtained from animals immunized with a detergent-solubilized AChR, it was of interest to check whether the hybridomas' antibodies recognize the receptor in its natural membraneous form as present in situ. To study this, we tested the binding activity of the antibodies with AChR-rich membrane fragments [20] isolated from *Torpedo* electric organ, and specifically labelled with ^{125}I-labelled α-bungarotoxin. All the hybridomas' antibodies were found to bind this

Table 1
Antibody titers in ascitic fluids (AF)

Sample	Antibody titer[a] towards		
	AChR	RCM-AChR	T-AChR
AF 1.3	1.6×10^{-6} M	0	4.0×10^{-8} M
AF 1.4	1.0×10^{-5} M	0	1.3×10^{-5} M
AF 1.15	1.0×10^{-9} M	0	1.0×10^{-8} M
AF 1.20	1.4×10^{-7} M	0	2.0×10^{-6} M
AF 1.24	1.6×10^{-6} M	0	2.0×10^{-6} M
AF 1.26	2.0×10^{-7} M	4.0×10^{-7} M	0
AF 1.32	1.4×10^{-5} M	0	1.8×10^{-5} M
AF 1.34	1.6×10^{-6} M	4.0×10^{-6} M	0
AF 1.39	3.1×10^{-7} M	8.0×10^{-7} M	0
Immune serum (pool)	9.0×10^{-8} M	5.3×10^{-8} M	4.0×10^{-7} M

[a] Antibody titers were determined by radioimmunoassay using goat anti-mouse Fab for precipitating the Ab–Ag complexes

Titers are expressed as moles of antigen precipitated per litre of serum

bungarotoxin-labelled membrane bound receptor. This finding indicates that the antibodies secreted by the hybridomas are directed against antigenic determinants exposed also on the membrane-bound receptor, which make these antibodies suitable for in vivo studies. When tested with detergent-solubilized AChR, all the antibodies demonstrated similar titer when analyzed either with ^{125}I-labelled AChR or with ^{125}I-labelled α-bungarotoxin–AChR complex. This suggest that none of these hybridomas produced antibodies against the bungarotoxin binding site of the receptor. Gomez et al. [21] have recently described one monoclonal hybridoma, originating from AChR immune rat cells, with specificity towards the bungarotoxin binding site.

Our findings suggest that some of the hybridomas secret antibodies which recognize selectively conformational antigenic determinants [22] of the acetylcholine receptor, which are present in the trypsinated receptor and are absent in the denatured receptor. The myasthenic activity of the receptor molecule

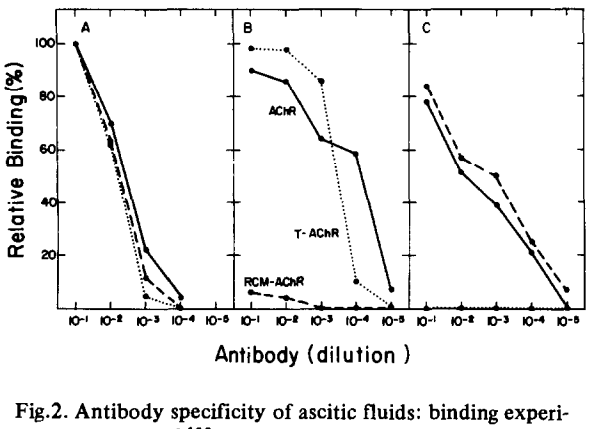

Fig.2. Antibody specificity of ascitic fluids: binding experiments. Binding of ^{125}I-labelled AChR (——) RCM-AChR (- - - - - - - - -) and T-AChR (.....) to: (A) C57BL/6J anti-AChR serum; (B) AF 1.4; (C) AF 1.26.

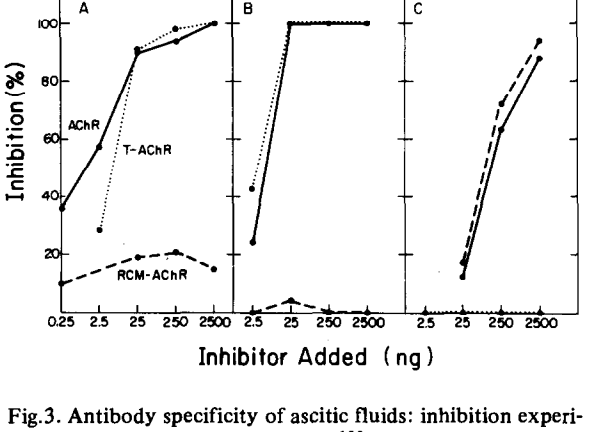

Fig.3. Antibody specificity of ascitic fluids: inhibition experiments. Inhibition of the binding of ^{125}I-labelled AChR to: (A) C57BL/6J anti-AChR serum; (B) AF 1.4; (C) AF 1.26 by AChR (——), RCM-AChR (- - - - - - - - -) and T-AChR (.....).

residues within this group of determinants. Thus it may be expected that antibodies directed against these specificities may have pathological effect associated with myasthenic activity. On the other hand, other hybridomas secret antibodies which recognize selectively RCM-AChR, which expresses the sequential antigenic determinants of the receptor molecule [12,22]. Such antibodies are probably not associated with any myasthenic activity and may even have a preventive or therapeutic activity [21]. The classification of the specificity of the various antibodies may be further assessed by using additional reagents and fragments of the receptor, representing defined antigenic regions.

Thus, monospecific antibodies appear to be a useful tool for structural and functional analysis of AChR; they may help to define the sites involved in the various biological activities related to AChR and to localize the various subunits in relation to the cell membrane. Moreover, they may enable the elucidation of the role of antibodies of defined specificity, in regulating the immune response to AChR and the disease resulting from it.

Acknowledgements

We thank Dr C. Milstein for providing the P3-NSI/1-Ag4-1 cell line, Mr D. Bartfeld for the samples of T-AChR and RCM-AChR and Mrs Michal Silverberg, for excellent technical help. This research was supported by grants from the Muscular Dystrophy Association of America, the United States-Israel Binational Science Foundation (BSF) and the Myasthenia Gravis Foundation. Z.E. is an incumbent of the Recanati Career Development Chair in Cancer Research.

References

[1] Fuchs, S., Tarrab-Hazdai, R., Nevo, D. and Bartfeld, D. (1978) in: The Biochemistry of Myasthenia Gravis and Muscular Dystrophy (Lunt, G. G. and Marchbanks, R. M. eds) pp. 189–216, Academic Press, London.

[2] Lindstrom, J. (1978) in: The Biochemistry of Myasthenia Gravis and Muscular Dystrophy (Lunt, G. G. and Marchbanks, R. M. eds) pp. 135–156, Academic Press, London.

[3] Drachman, D. B. (1978) New Engl. J. Med. 298, 136–142.

[4] Abramsky, O., Aharonov, A., Webb, C. and Fuchs, S. (1975) Clin. Exp. Immunol. 19, 11–16.

[5] Aharonov, A., Abramsky, O., Tarrab-Hazdai, R. and Fuchs, S. (1975) Lancet ii, 340–342.

[6] Lindstrom, J. M., Seybold, M. E., Lennon, V. A., Whittingham, S., Duane, D. D. (1976) Neurology 26, 1054–1059.

[7] Tarrab-Hazdai, R., Aharonov, A., Abramsky, O., Yaar, I. and Fuchs, S. (1975) J. Exp. Med. 142, 785–789.

[8] Toyka, K. V., Drachman, D. B., Griffin, D. E., Pestronk, A., Winkelstein, J. A., Fischbeck, K. H. and Kao, I. (1977) New Eng. J. Med. 296, 125–131.

[9] Lindstrom, J. M., Engel, A. G., Seybold, M. E., Lennon, V. A. and Lambert, E. H. (1976) J. Exp. Med. 144, 739–753.

[10] Köhler, G. and Milstein, C. (1975) Nature 256, 495–497.

[11] Aharonov, A., Tarrab-Hazdai, R., Silman, I. and Fuchs, S. (1977) Immunochemistry 14, 129–137.

[12] Bartfeld, D. and Fuchs, S. (1977) FEBS Lett. 77, 214–218.

[13] Bartfeld, D. and Fuchs, S. (1979) Biochem. Biophys. Res. Commun. in press.

[14] Clark, D. G., McMurchie, D. D., Elliot, E., Wolcott, R. G., Landel, A. M. and Raftery, M. A. (1972) Biochemistry 11, 1663–1668.

[15] Hunter, W. M. (1978) in: Handbook of Experimental Immunology (Weir, D. M. ed) pp. 14.1–14.40, Blackwell, Oxford.

[16] Fuchs, S., Nevo, D., Tarrab-Hazdai, R. and Yaar, I. (1976) Nature 263, 329–330.

[17] Eshhar, Z., Blatt, C., Bergman, Y. and Haimovich, Y. (1979) J. Immunol. 122.

[18] Hudson, L. and Hay, F. C. (1976) in: Practical Immunology, pp. 118–119, Blackwell, Oxford.

[19] Bartfeld, D. and Fuchs, S. (1978) Proc. Natl. Acad. Sci. USA 75, 4006–4010.

[20] Cohen, J. B., Weber, M., Huchet, M. and Changeux, J. P. (1972) FEBS Lett. 26, 43–47.

[21] Gomez, C. M., Richman, D. P., Berman, P. W., Burres, S. A., Arnason, B. G. W. and Fitch, F. W. (1979) Biochem. Biophys. Res. Commun. 88, 575–582.

[22] Sela, M., Schechter, B., Schechter, I. and Borek, F. (1967) Cold Spring Harbor Symp. Quant. Biol. 32, 537–545.