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Immunization of Rabbits with Purified Nicotinic Acetylcholine Receptor

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Immunization of rabbits, rats and guinea pigs with nicotinic acetylcholine receptor from the electric organ of *T. marmorata* leads to a production of antibodies in these animals. Simultaneously a peripheral paralysis is seen, particularly pronounced in rabbits. In rats the symptoms are transient. The antibody titres in rabbits are correlated to the visible symptoms. The highest antibody titres are found in the most affected animals. Electrophysiological, immunological and morphological analysis indicate disturbances of the neuromuscular function, probably on the postsynaptic site. Thymus reactions may also be involved as a thymit has been found in the immunized rabbits.

Nicotinic acetylcholine receptor (nAChR) from the electric organ of *Torpedo marmorata* was purified using affinity chromatography[1,2] and shown to be a glycoprotein[3]. The predominant carbohydrate is mannose. Polyacrylamide gel electrophoresis shows two proteins with apparent molecular weights of 295 000 and 410 000 ($nAChR_I$ resp. $nAChR_{II}$). Both proteins bind [^{3}H]-acetyl α-neurotoxin from *Naja naja siamensis*. Injection into rabbits of small amounts of nAChR produces symptoms of muscle weakness in the animals, visible to the eye shortly after the appearance of circulating antibodies to nAChR in the rabbit serum, and increasing in severity as the antibody titre increases[4]; 27 out of 27 animals injected have produced such symptoms. Repetitive stimulation of the peroneal nerve shows marked fatigue in muscle response, the decrement being higher in animals with higher antibody titre. Single fibre EMG, allowing measurements of the variability, »jitter« in neuromuscular transmission time in single motor end plates showed increased jitter in all examined animals.

METHODS

At one week interval rabbits were injected into the thigh with 0.3 mg nAChR mixed with complete Freunds adjuvant. The animals were bled weekly and the antibody titres were determined with rocket-immunoelectrophoresis or with the indirect hemaglutination test[5]. The reciprocal of the highest serum dilution giving agglutination was recorded as the titre. Immunoglobulins were concentrated by precipitation with ammonium sulphate as described by Harboe and Ingild[6]. Fractionation of immunoglobulins on Sephadex *G-200* was performed according to Flodin and Killander[7] using 0.05 M tris buffer, pH=8.0 containing 0.146 M NaCl. The protein distribution was recorded at 280 nm with an Uvicord. The fractions were concentrated and tested immunoelectrophoretically (2 h, 10 V/cm) on agarose gels made 2% with respect to antiserum. 0.4—4 μg antigen was used in the electrophoresis.

For neurophysiological studies a Disa electromyograph with stimulator unit was used. Stimulation was made with a bipolar electrode over the peroneal nerve at the level of the fibular head. Stimulus strength was 50% above the stimulus for maximal muscle response. Stimulus frequence was 2, 5 and 10 impulses/s. For the two lowest frequences, 5—7 discharges were given. Initial amplitude of the response and relative amplitude decrease from the first to the fourth response was measured. The tests were repeated several times with 2—5 minutes between each. The variability (the jitter) in the neuromuscular transmission time in single motor endplates was measured by single fibre EMG as decribed by Ekstedt and Stålberg[8]. Intracellular recording of the miniature end plate potentials (MEPS) in intercostal muscle from immunized rabbits has been done using a method described by Elmquist *et al.*[9]. Autopsy of experimental animals was done and the following organs were examined by light microscopy; spleen, lung, heart, liver, muscles from legs and diaphragma, nerves from the legs. Light as well as EM microscopy were done on the motor endplate. Acetylcholinesterase (*AChE*) in neuromuscular junctions was stained according to Koelle[10] for comparison with normal junctions.

In collaboration with dr. F. Foldes, Department of Anesthesiology, Montefiore Hospital, New York, USA, rats and guinea pigs have been injected with 0.3 mg nAChR/kg. The diaphragm and m. gastrocnemius have been stimulated for 90 s at rate of 2 Hz in order to examine the curare sensitivity in these muscles.

RESULTS

Rabbits injected with isolated nicotinic acetylcholine receptor mixed with Freunds' complete adjuvant developed antibodies after 2 injections of receptor protein and after three injections the same gradually increasing flaccid paralysis as described earlier[2,4], was seen. If not treated, death from respiratory paralysis occurs 7—14 days after the first visible symptoms. Neostigmine or Tensilon give a general, but transient relief, Tensilon having a dramatic effect. None of the control animals injected with AChE from *Torpedo marmorata* or with *Naja naja siamensis* α-neurotoxin in relevant concentrations showed any symptoms of muscle weakness, and antibodies against *Naja naja siamensis* neurotoxin[11] (kindly given to us by dr. S. Fuchs), do not crossreact with the purified receptor protein. Neurotoxin or AChE impurities as the cause of the observed symptoms are therefore unlikely.

The antibody titres have been followed continuously in 5 animals and correlated to visible symptoms. Fig. 1 shows the gradually increasing antibody

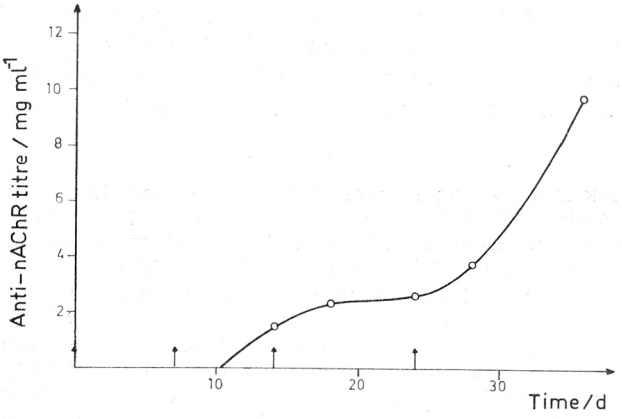

Fig. 1. Immunoresponse in serum of a rabbit injected with purified nAChR. ↑ time of injection (i. m.).

titre in one animal. In the examined animals the antibody titre varied between 2 500—3 500 μg/ml when the first symptoms were seen and 10 000—15 000 μg/ml when the animals were killed. These titres are higher than those quoted by Patrick *et al.*[12] and Sugiyama *et al.*[13]. However, Harboe and Ingild[6] have shown that antibody titres found by methods where antigen and antibody are allowed to react in free solution are 2—10 times lower than those found with techniques using reactions in a gel. Precipitation in free solutions was used by the authors quoted above. Repetitive stimulation of the peroneal nerve of rabbits shows successive fall in the amplitude of the compound muscle action potential[4] (Fig. 2). There is a relation between the antibody titre and the evoked muscle response, the decrement being higher as the antibody titres increase. Antibodies are always detected before the decrement. After Tensilon treatment the initial amplitude is increased and no reduction in response amplitude is seen on repetitive stimulation at the different frequencies. The conduction time from the stimulus site over the tibial anterior muscle is the same (1.4 μs) in control and sick animals and the nerve myelin sheat is normal in immunized animals.

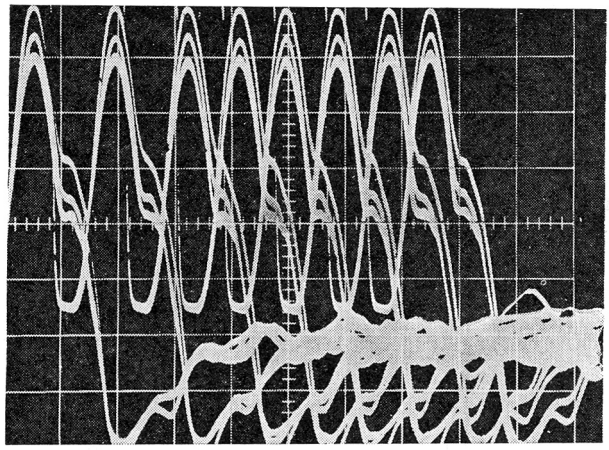

Fig. 2. Muscle action potentials recorded from tibialis anterior muscle of the immunized rabbit at nerve stimulation frequencies 5 Hz.

Single fiber EMG allows measurements of the variability in neuromuscular transmission time in single motor endplates. The jitter was 10—30 μs in normal rabbits and was due to variability in the synaptic delays of the two motor endplates involved (Fig. 3). In immunized rabbits there was a marked increase in the jitter ($>$ 100 μs) and in some of the endplates partial impulse blockade was observed (Mattsson, Hilton-Brown, Stålberg and Heilbronn, to be published). The miniature endplate potentials (MEPS) in the intercostal muscle of healthy and experimental rabbits have been registered. The MEPS amplitude was found to be markedly decreased in the experimental animals. Only 10—20% of the normal value was found in all the examined animals (Fig. 4) (Elmqvist, Libelius, Mattsson and Heilbronn, to be published).

The antiserum and the isolated immunoglobulin fraction precipitated receptor protein, as measured by the [^{3}H]-acetyl-α-neurotoxin binding capacity of

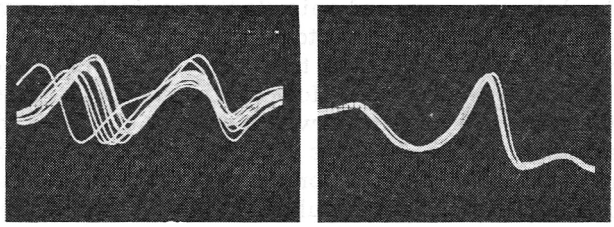

Fig. 3. Left: Increased pathological jitter in an immunized rabbit. Right: Normal jitter in a control rabbit.

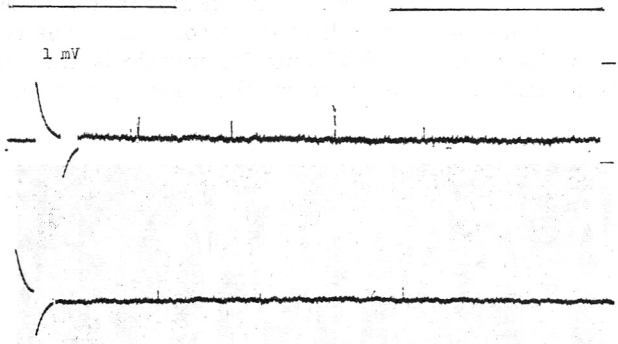

Fig. 4. MEPS amplitude in a normal rabbit intercostal muscle (above) and in an immunized rabbit (below).

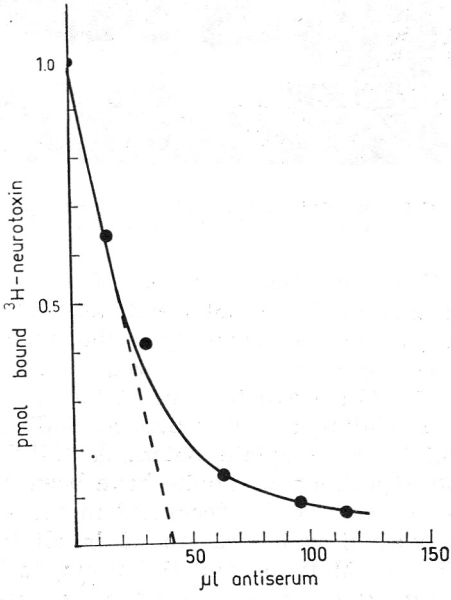

Fig. 5. Precipitation of purified nAChR by anti-nAChR from rabbit as measured by the [^{3}H] acetyl-α-neurotoxin binding capacity of the supernatant.

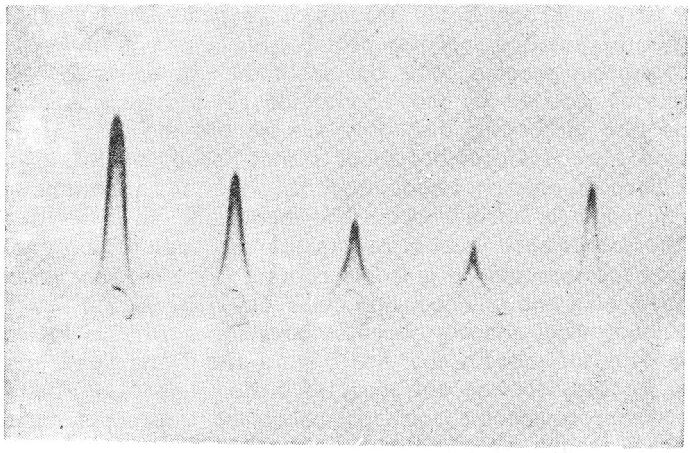

Fig. 6. Rocket immunoelectrophoresis of nAChR on an 1% agarose gel made 2% with respect to anti-nAChR. Wells from left: 1—3 nAChR 3.6, 1.8 and 0.9 μg resp. 4. isolated $nAChR_I$. 5. isolated $nAChR_{II}$.

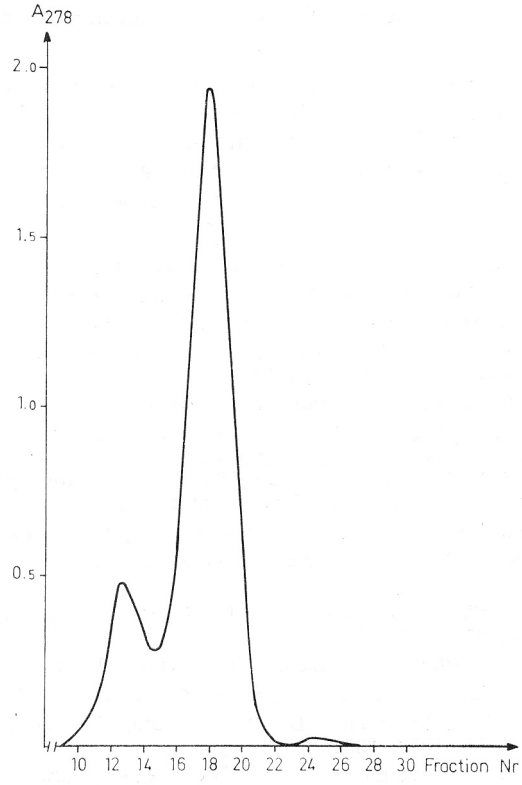

Fig. 7. Gel filtration of rabbit α-globulin on Sephadex G-200.

the solution, from a Triton X-100 extract of *Torpedo* electric organ as well as from a solution of purified receptor protein (Fig. 5). In immunoelectrophoresis the receptor protein reacted with the antibodies in one single line (Fig. 6). The two proteins isolated by polyacrylamide gel electrophoresis also reacted in one single line indicating that they are immunologically identical. It was not possible to adsorb antibodies from rabbit serum by mixing serum with freeze dried Torpedo electric organ suggesting that the immunologically active part of the molecule is buried within the membrane. In order to study which Ig class of the rabbit antiserum is responsible for the antibody activity rabbit γ-globulin was fractionated on a G-200 column (Fig. 7). The antibody activity was determined with immunoelectrophoresis. Only the second peak precipitated the antigen suggesting mainly receptor antibodies of the IgG type in the serum. In collaboration with dr. Aarli from the Department of Neurology, University of Bergen, Norway, the receptor protein has been crosstested against several sera from myastenic patients using an indirect haemagglutination test. No positive results were obtained.

No pathological signs were found in the spleen, lung, kidney, adrenal glands, liver and heart of the immunized rabbits. Muscle threads are occasionally atrophic or slightly inflamed, but no pronounced myocyte is observed. In some animals a relatively large number of eosinophilic granulocytes is seen among the lymphoid cells, possibly indicating a thymit. EM-microscopy on the endplate region indicates changes, the folding of the endplate region is reduced.

Four receptor injected rats were compared with 4 others rats injected with the same amount of serum albumin and with 4 uninjected rats. The 4 receptor injected rats exhibited greatly increased curare sensitivity which was more marked in the diaphragm than in the gastrocnemius. The one guinea pig tested so far was also more sensitive to curare than the controls.

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DISCUSSION

W. N. Aldridge:

Do you consider the symptoms in the animal can be explained by the reduction of effective receptor molecules (shown by neurotoxin binding)?

R. D. O'Brien:

Why did you select intercostal muscles for a study of toxin binding? The animal is surviving well, so the intercostals are clearly in good order; why not study the leg muscles in which paralysis is evident?

M. E. Eldefrawi:

Your data on the binding of [^{3}H]-neurotoxin to muscles from normal immunized rabbits showed only 20% reduction in number of receptor sites. Do you think that such a reduction could explain the paralysis and death of the immunized rabbits and how do you compare your data with dr. Barnard's published values of at least 50% excess receptor (or spare receptor) found in rat diaphragms?

Ch. Mattsson:

The questions of Drs. Aldridge, O'Brien and Eldefrawi could be answered together. Our measurements were done on the intercostal muscles because MEPS were measured on this tissue and we wanted to be able to correlate the two. The animals were living and breathing when this was done, thus no complete block of neurotransmission there could be expected. Skeletal muscles, *e. g.* of the legs, might have shown greater block of receptors. In any case, Albuquerque *et. al.* (*Life Sci.* **12** (1973) 545) have shown that block of 25% of receptors impairs neurotransmission. Whether the reduction in functional receptors is the whole truth or not is too early to say. Certainly it seems to be an important part of the paralysis and the rabbits showed normal conduction in their motor nerves.

I. Silman:

What kind of symptoms do you see in AChR-immunized rats? Have you done EMG measurements on them?

Ch. Mattsson:

No EMG has so far been done on rats. Only visible symptoms have been observed. These symptoms are maximal after about 3 weeks and not so pronounced as in rabbits. However, in some experiments done together with dr. Foldes (Montefiori Hospital, New York), increased curare sensitivity was shown in these animals, particularly in the diaphragm.

B. Stamenović:

(a) Regarding the reduced amplitude of the MEPS in the immunized rabbits, what was the resting membrane potential of the impaired muscle fibres during the recording of MEPS? (b) When suggesting the postsynaptic change at the neuromuscular junction for the observed decrease in MEPS amplitude, have you any evidence of the unit size of MEPS as well as of the mean quantum content of EPP in such conditions, because the presented MEPS are very like those recorded by the Elmquist group in myasthenic patients.

Ch. Mattsson:

(a) The resting membrane potential was 80 mV in immunized as well as in control rabbits. (b) The mean quantum content has not yet been determined, only the MEPS amplitude which was 1 mV for controls and 0.2 mV or lower for immunized rabbits. It remains to see whether this is due to only postsynaptic effects or if presynaptic effects are involved as well. Iontophoretic application of ACh is currently studied.

SAŽETAK

Imunizacija kunića pročišćenim nikotinskim acetilkolinskim receptorom

Ch. Mattsson i Edith Heilbronn

Imunizacija kunića, štakora i zamorca nikotinskim acetilkolinskim receptorom električnog organa *T. marmorata*, uzrokuje stvaranja antitijela u tim životinjama. Utvrđena je istovremena pojava periferne paralize, koja je osobito jako izražena u kunića dok su u štakora simptomi prolazni. Titar antitijela u kuniću u korelaciji je s vidljivim simptomima. Elektrofiziološka, imunološka i morfološka analiza upućuju na poremećaj neuromuskularne funkcije, vjerojatno na postsinaptičkoj strani. Reakcije timusa mogle bi također biti uključene, budući da je u imuniziranim kunićima nađen jedan timit.

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