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Blockade of Acetylcholine Receptors: A Model of Myasthenia Gravis

Abstract. *In order to block acetylcholine receptors of muscle, the alpha toxin of the Formosan cobra (*Naja naja atra*) was given intravenously to rats. Electrophysiological and pharmacological changes typical of myasthenia gravis were recorded, including decremental responses to repetitive stimuli, curare sensitivity, neostigmine reversal, and posttetanic phenomena. This model supports the concept that a reduction of available acetylcholine receptors may play an important role in myasthenia gravis.*

Myasthenia gravis is a neuromuscular disorder manifested by weakness and fatigability of muscle. It is generally accepted that the abnormality involves the neuromuscular junction, but the exact site and nature of the defect are not yet settled (*1*). There has been considerable debate as to whether the nerve terminal (*2; 3*, p. 305) or the postsynaptic region (*4*) of the muscle, or both (*5*), are affected by the disease process. We have found an abnormality in the acetylcholine (ACh) receptors of neuromuscular junctions from myasthenic patients (*6*). By means of techniques which utilize α-[^{125}I]bungarotoxin binding to receptors, we determined that the number of available ACh receptors was reduced in myasthenic junctions, averaging 80 percent below normal. This finding raised the question of whether the receptor reduction per se could explain the physiopathological defects of myasthenia gravis. In our study we have compared the physiological effects produced by experimental receptor blockade in animals with those occurring naturally in myasthenia gravis.

In order to reduce the number of free receptors, we have used the α-toxin derived from the venom of the cobra *Naja naja atra* as a pharmacological blocking agent (*7*). Its specificity has been confirmed by its use in an assay for purified ACh receptor (*8*). Lyophilized venom was obtained from the Miami Serpentarium, and the α fraction was isolated and purified by the ion exchange chromatography method of Lee *et al.* (*9*).

Female Lewis rats, anesthetized with chloral hydrate (40 mg/kg), were prepared as follows for recording of evoked muscle action potentials. Tracheostomy was carried out, and the tube was connected to a Harvard respirator. The skin temperature of the rat was maintained at 35°C by means of an infrared lamp regulated by a telethermometer (Yellow Springs Instruments). In order to obtain reliable and consistent records of muscle action potentials, mechanically secure mounting of the animal and of the electrodes proved essential. The animal was strapped to a Lucite platform, and the right leg was taped to a metal rod. One surface recording electrode was secured over the mid-belly of the triceps surae (calf) muscle with collodion and tape, and the other was fastened over the Achilles tendon with a 9-mm Clay-Adams wound clip. After the right sciatic nerve was surgically exposed, the stimulator probe was gently hooked around it and clamped in position. The overlying skin was closed around the probe with pins and kept moist with mineral oil.

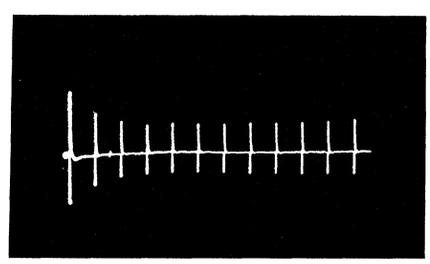

Fig. 1. Decremental response recorded in deltoid muscle of myasthenic patient (stimulus rate, 5 sec^{-1}).

Trains of stimuli were supplied by a Grass S-48 stimulator through a stimulus isolation unit. (The stimulus duration was 0.2 msec; the amplitude was 1.5 times maximal, usually 4 to 7 volts.) The muscle potentials were amplified by a differential amplifier (WPI Co. DAM-6) displayed on an oscilloscope (Tektronix), and recorded on Polaroid film for measurement.

Control records of the animal's responses to repetitive nerve stimulation were made at the start of each experiment. An injection of α-cobratoxin (12 to 20 μg) was then given intravenously, and recordings were made at intervals of approximately 30 to 60 minutes until the termination of the experiment, up to 14 hours later.

This method of giving a relatively large single dose of α-cobratoxin, followed by a long period of observation, was used to allow for adequate equilibration of the toxin throughout the neuromuscular junctional receptor pool. The toxin binds rapidly to receptors, but the toxin-receptor complex is slowly reversible (*10*). Although single evoked potentials decreased in amplitude for several hours after injection, they subsequently returned to normal or nearly normal levels (mean, 83 percent of original amplitude). All of the myasthenic features described below were present during the recovery phase, when the amplitude of single evoked potentials was approaching normal.

Affected muscles of patients with myasthenia gravis typically show a decrement in the amplitude of action potentials evoked by repetitive nerve stimulation (Fig. 1). Conventionally, a stimulus rate of 2 to 5 sec^{-1} is used, and the amplitude of the fourth potential is compared with that of the first (*11*). The first potential is usually normal or only slightly diminished and a decrement of more than 7 to 15 percent is considered to be consistent with a diagnosis of myasthenia.

In our experiment, we reproduced the clinical procedure by stimulating the sciatic nerve at 3 sec^{-1} and recording evoked muscle action potentials with surface electrodes placed over the calf muscles as described above. In control observations on 21 untreated

rats, none showed a decremental response, while a slight increase [mean (percent), 2.00 ± 0.44 S.E.M.] in amplitude was seen in 16.

After the α-cobratoxin was injected, a decremental response was present in all cases. It began within 1 hour, and reached a maximum ranging from 40 to 88 percent at 3 to 13 hours after injection [mean (percent), 64.33 ± 6.91 S.E.M.]. As described above, the amplitude of the initial potential declined for several hours after injection, but subsequently returned toward normal, in two instances surpassing the amplitude prior to injection. In every animal treated with cobratoxin, a marked decremental response (mean, 45.6 percent) was present at the time when the amplitude of the initial potential had returned to normal or near normal, thus reproducing the pattern in myasthenia gravis.

Myasthenic patients are abnormally sensitive to the effects of *d*-tubocurarine, becoming weak when given 1/20 to 1/10 of the usual curarizing dose (*12*). This has led to the use of a small dose of curare as a diagnostic test for myasthenia.

We tested four cobratoxin-treated rats for curare sensitivity by injecting 0.75 μg of *d*-tubocurarine intravenously (this is about 1/30 of the amount required in untreated control rats, to produce a decremental response at a stimulus frequency of 3 sec^{-1}). Three of the rats showed a marked exaggeration of the decrement recorded, while the fourth animal showed no change (*13*) at this dose [mean (percent), 242 ± 131 S.E.M.].

Both the weakness and the decremental responses in myasthenia gravis characteristically improve after treatment with anticholinesterase agents (*14*). We therefore injected neostigmine (2.5 to 7.5 μg) intravenously in four cobratoxin-treated rats. In all cases, the decremental response was reduced greatly, in two instances returning to normal [mean (percent), − 89.3 ± 5.73 S.E.M.].

The changes in neuromuscular transmission after tetanic stimulation are thought to be particularly characteristic of myasthenia gravis (*3*, p. 241). In practice, the nerve is first given a conditioning train of stimulation at a rate of 30 to 100 sec^{-1} for up to 10 seconds. Neuromuscular transmission is then tested by repetitive nerve stimulation at the usual rate of 3 sec^{-1}, applied at intervals thereafter. In myasthenic patients, muscle action potentials elicited 10 to 15 seconds after the conditioning stimulus show a less pronounced decrement ("posttetanic potentiation"). However, 2 to 4 minutes later, the decremental response becomes exaggerated ("postactivation exhaustion"). Neither posttetanic potentiation nor postactivation exhaustion are seen in normal humans (*15*) or in rats.

Nine rats treated with cobratoxin were tested for posttetanic changes. Eight showed an early improvement (at 30 to 40 seconds) in the decremental response [mean (percent), − 44.13 ± 12.09 S.E.M.]. All nine rats showed an enhanced decremental response (7 to 20 minutes) after the conditioning stimulation, although the change was small in two of the animals [mean (percent), 17.1 ± 5.15 S.E.M.]. Our findings are consistent with those seen in human myasthenia gravis, although the posttetanic cycle evolves more slowly in the experimental rats.

Miniature end-plate potentials (mepps) recorded from the intercostal muscles of patients with myasthenia gravis are reported to be reduced in amplitude (mean, 0.2 mv; normal, 1 mv) although normal in frequency (*2, 3*). In a previous study of Chang and Lee (*7*), with intracellular recording in vitro, the α-toxin of *Naja naja atra* produced a progressive reduction in the amplitude of miniature end-plate potentials in the rat diaphragm, eventually blocking these potentials completely at a sufficiently high concentration of toxin.

Thus, from the point of view of the electrophysiological abnormalities, we have shown that cobratoxin experimentally reproduces the characteristic features of myasthenia gravis, including (i) decremental response, (ii) curare sensitivity, (iii) prostigmine reversal, (iv) posttetanic potentiation, (v) postactivation exhaustion, and (vi) reduction in amplitude of miniature end-plate potentials. Since the α-toxin of *Naja naja atra* as used in our study is thought to block ACh receptors specifically (*7–9*), this suggests that a reduction of available receptors per se can account for the abnormalities of neuromuscular transmission in myasthenia gravis. However, it is not possible to determine the number of ACh receptor sites blocked by α-cobratoxin in the experimental animals by the use of α-[^{125}I]bungarotoxin binding techniques. The reversibility of α-cobratoxin allows it to be displaced from ACh receptors during the incubation with bungarotoxin and the washing steps inherent in the assay procedure.

Recent evidence indicates that the ACh receptors are located postsynaptically (*16*), and therefore it is reasonable to consider the myasthenic abnormality as being postsynaptic (*17*).

The question of how the receptor abnormality comes about in human myasthenia gravis is not yet resolved. Whether the receptor protein itself is defective, whether the receptor packing in the postsynaptic membrane is abnormal, or whether the receptor is

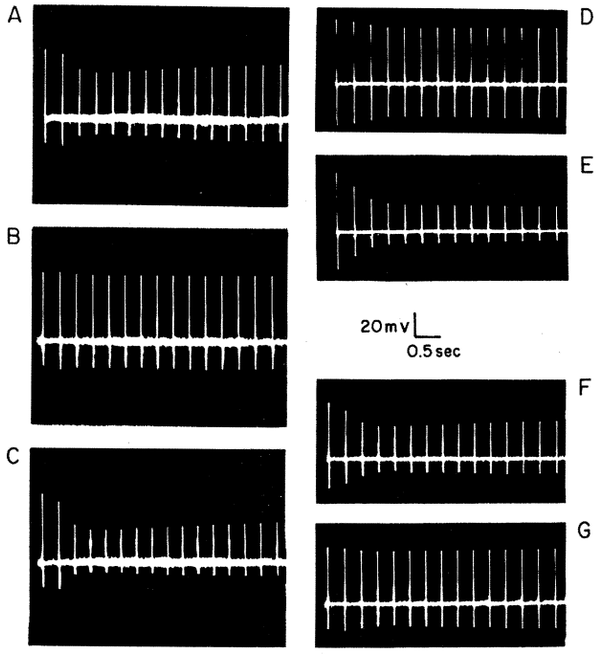

Fig. 2. In all records, test stimuli were supramaximal, at a rate of 3 sec^{-1}. (A) Response prior to tetanus. (B) Posttetanic potentiation; 40 seconds after tetanus (50 sec^{-1} for 5 seconds). (C) Postactivation exhaustion; 12 minutes after tetanus. (D) Response of another animal prior to curare. (E) Response 2 minutes after 0.75 μg of curare. (F) Response of animal prior to neostigmine. (G) Response 7 minutes after 2.5 μg of neostigmine.

damaged or blocked, perhaps by autoimmune mechanisms (*18*), must be elucidated by future investigation. However, our model provides support for the concept that the receptor abnormality may be of fundamental importance in myasthenia gravis.

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17. Desmedt has postulated a presynaptic defect, based chiefly on the presence of the post-activation exhaustion phenomenon in myasthenic muscles, and its absence in partially curarized muscles of cat or man (*3*, pp. 241 and 305). We are unable to explain why curare does not produce this phenomenon and α-cobratoxin does, although the marked differences in their pharmacokinetic properties may be significant in this regard. Since post-activation exhaustion is not well understood, no firm conclusions about the site of the underlying disorder should be based on its presence or absence. In our study we have merely concluded that specific blockade of ACh receptors is capable of producing the characteristic physiological phenomena of myasthenia gravis. The site of the lesion is inferred from present knowledge about the location of ACh receptors.
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Adenosine Inhibition of Lymphocyte-Mediated Cytolysis: Possible Role of Cyclic Adenosine Monophosphate

Abstract. *The in vitro destruction of tumor cells by specifically sensitized mouse lymphocytes was inhibited by adenosine; this inhibition was markedly potentiated by the presence of an inhibitor of adenosine deaminase. The inhibition of cytolysis by adenosine was accompanied by a rapid elevation in lymphocytic adenosine 3′,5′-monophosphate (cyclic AMP) concentrations. Both the inhibition of cytolysis and the elevation of cyclic AMP were reversed by prolonged incubation of the lymphocytes in the presence of adenosine or, more rapidly, by removal of the adenosine. Low concentrations of adenosine also caused an elevation of cyclic AMP in human lymphocytes, and this effect of adenosine may contribute to the lack of immune response associated with adenosine deaminase deficiency.*

Recently there have been reports (*1*) of a number of children with combined immunodeficiency disease associated with a deficiency of adenosine deaminase (E.C. 3.5.4.4). A chance association of these two rare defects would appear to be highly improbable. Evidence against the possibility of the deletion of a single genetic locus involving both adenosine deaminase production and the immune response has been presented (*2*). Green and Chan (*3*) have reported that established fibroblasts or lymphoid cells die in the presence of 10^{-4} to $10^{-5}M$ adenosine. They found that pyrimidine nucleotide concentrations were markedly depressed and that the toxic effects of adenosine were prevented by pyrimidine sources, such as uridine. This led them to speculate that the association of combined immunodeficiency disease and adenosine deaminase deficiency was the result of adenosine-induced pyrimidine starvation of lymphoid cells. Adenosine has been reported to cause an increase in cyclic AMP in a variety of cell types (*4*), and elevated concentrations of cyclic AMP in the effector lymphocytes are known to be inhibitory to lymphocyte-mediated cytotoxicity (LMC) (*5*). Our studies with cytotoxic lymphocytes have shown that adenosine is an inhibitor of LMC and that its inhibitory activity is correlated with elevated lymphocytic cyclic AMP.

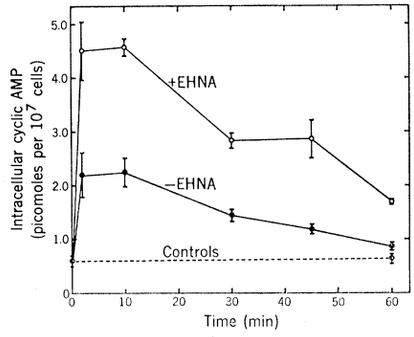

Fig. 1. Cyclic AMP concentrations in peritoneal exudate lymphocytes as a function of time after addition of adenosine (37.5 μM) ± EHNA (7.9 μM) to the cell suspensions. The cells (1×10^7 in 5.0 ml of buffer solution) were incubated for 30 minutes at 37°C, at which time ($t = 0$) adenosine or EHNA plus adenosine were added to the incubation mixtures. Duplicate control incubations were prepared with and without EHNA (7.9 μM); these two types of controls yielded identical cyclic AMP values. Each point represents the mean ± standard error of the mean (S.E.M.) for four determinations.

The in vitro model for lymphocyte-mediated immunity we have used has been described (*6*) and is based on the model reported by Berke *et al.* (*7*). Briefly, the target cell is a mouse ascites leukemia, EL4, which multiplies rapidly and kills C57BL mice after intraperitoneal injection. However, intraperitoneal injection of these cells into an allogeneic strain of mice, such as CD-1, results in the production of sensitized lymphocytes and ultimate rejection of the tumor by an unknown mechanism.

Cytotoxic peritoneal exudate cells were obtained from CD-1 mice after rejection of the EL4 cells, by repeated lavages of the peritoneal cavity. Nonadherent peritoneal exudate lymphocytes were obtained by passing the crude population of cells over a glass wool column and used in both the in vitro cytolysis and the cyclic AMP experiments. These cell populations contained over 90 percent small to medium-size lymphocytes. While the exact proportion of cytolytic lymphocytes is not known, Sullivan *et al.* (*8*) have reported that more than 80 percent of the peritoneal exudate lymphocytes obtained from a number of strains of mice after intraperitoneal injection of