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MAST CELL SENSITIZING ANTIBODIES IN EXPERIMENTAL MYOSITIS

By

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Myasthenia gravis (M.gr.) is characterized by specific homologous and heterologous antibodies to striated muscles and myoid cells of the thymus detectable by immunofluorescence technique (1, 5, 6, 17, 20, 21), and by serological methods performed with muscle extracts, such as complement fixation (6, 9, 17, 19), agglutination of tanned red cells (3), and anti-globulin consumption (18). Mast cell sensitizing antibodies (MCSAb), characteristic for hypersensitive states (14, 15, 22), have been recently described in sera from patients with M.gr., using a muscle extract from Papio papio monkey as antigen, and it was possible to find a correlation between MCSAb and fluorescent antibodies to striated muscles and myoid cells of the thymus (8, 16). Similar MCSAb were detected in sera from rabbits immunized with monkey muscle extract (8, 16) and exhibiting an experimental disease characterized by flaccid paralysis and immunofluorescent antibodies to muscle striations and myoid cells of the thymus (7).

Experimental flaccid paralysis can also be induced by inoculation with acetylcholine receptors (ACR), which are considered to be involved in the pathogenesis of M.gr. (10, 11, 13). In these animals immunized with ACR purified from the electric organ of the *Electrophorus electricus* (eel), circulating antibodies for the eel electroplax, and positive skin tests for eel ACR were described, as well as the symptoms (10, 11, 13).

In the present work, we were interested in investigating whether animals immunized with eel ACR developed hypersensitivity and MCSAb to ACR, whether antibodies to striated muscle and myoid cells of the thymus could be detected in their sera, and whether there was any correlation between the different antibodies and the evolution of the disease.

For this purpose, guinea pigs and rats were immunized with eel ACR together with complete Freund's adjuvant (CFA). Hypersensitivity was studied by MCSAb, skin tests, and adherence of myofibrils to peritoneal macrophages or to lymphocytes from lymphatic ganglions and spleen. Antibodies to striated muscle and myoid cells of the thymus were examined microscopically on paraffin sections.

MATERIAL AND METHODS

Acetylcholine Receptors (ACR) were extracted from the electric organ of the *Electrophorus electricus* (eel). The electric organ was homogenized for preparation of crude membranes, which were collected by centrifugation at 7,000 *g*. The crude membranes were then resuspended, recentrifuged, mixed to Triton X-100, stirred and filtered; the detergent crude extract was purified by passage on an affinity column made of "cholinergic arms", dialyzed, concentrated, eluted in Triton X-100 detergent and further purified (19).

Animal Inoculation. Fourteen female, adult guinea pigs (Hartley) and 20 female, adult rats (Ch. Rivers) were bled and their sera kept at –20° C for further examinations. The ACR pure extract was mixed with equal amounts of CFA and diluted with Triton X-100 so that the final amount of inoculated pure "ACR" was 70 pmol per animal. The solution was inoculated into foot pads (seven guinea pigs and six rats), or into foot pads and paravertebral muscles (three guinea pigs and six rats). The animals were immunized by one inoculation (five guinea pigs and one rat), by two (five guinea pigs and five rats), by three (four rats) or even four inoculations (two rats). Controls (four guinea pigs and eight rats) were inoculated in the same way with Triton X-100 mixed with CFA. The samples of sera were withdrawn randomly 10 days after the first, second, third and fourth inoculations; total bleeding was performed in all cases during the final phase of the disease and in asymptomatic animals, before killing. Sera were kept at –20° C.

MCSAb were estimated by the direct mast cell degranulation (DMCD) for detection of attached MCSAb, and by indirect mast cell degranulation (IMCD) for examination of free MCSAb (14, 15, 16, 22).

DMCD was carried out by mixing 0.05 ml peritoneal mast cells from the

immunized animals, with 0.05 ml antigen. The peritoneal mast cells were suspended in 199 M (12). Initially, the test was performed with purified ACR diluted with Triton X-100; since the Triton diluent alone was found to provoke, *in vitro,* lysis and degranulation of peritoneal cells from normal and inoculated animals, the purified ACR antigen was replaced by "crude membranes" diluted 1/100 in 199 M (see Material and Methods, first paragraph). Readings were taken after incubation of the mast cells with the antigen for 20 min at 37° C. The proportion of degranulated mast cells was established in several preparations, and the average compared with that in controls (same mast cells incubated only with 199 M, same mast cells incubated with other antigens, such as human globulin, and mast cells from normal animals incubated with the "crude membranes" antigen.

IMCD is based on the property of MCSAb to attach, *in vitro,* to normal mast cells which by further exposure to the specific antigen undergo degranulation (14–16, 22). The test was performed with peritoneal mast cells from normal rats; 0.05 ml serum was mixed with 0.05 mast cell suspension, and 0.05 antigen (crude membranes) was added to the mixture. Readings were taken after 20 min incubation at 37° C; count of degranulated mast cells and values were established as in the DMCD and compared with controls (normal mast cells and sera; normal mast cell antigen; normal mast cells with sera withdrawn before inoculation and antigen).

Skin tests were performed randomly on the 10th day after the first inoculation, by intradermal injection of 0.1 ml from a 1/100 dilution of pure ACR in Triton diluent (about 3.5 pmol in 0.1 ml). Two control inoculations consisting of 0.1 ml Triton dilution and 0.1 ml saline were given to the same animals. The control animals were tested with 0.1 ml Triton and 0.1 ml saline only. Skin reactivity of animals was examined 15 min after the injection, then every hour for 6 h, and reexamined after 24 and 48 h. Values were recorded of the diameter of the inflammation and congestion, and the intensity of the induration.

Adherence of myofibrils to peritoneal macrophages or leukocytes from the spleen or lymphatic ganglions of inoculated animals was performed according to the "Rosette formation" method (2). The cells were washed, resuspended in 199 M and mixed with a suspension of myofibrils. The mixture was left at +4° C for 18 h and drops of the mixture examined microscopically.

Preparation of myofibrils was performed according to the glycerinated myofibrils technique (4, 7) with slight modifications. In addition, in order to avoid possible alterations induced by glycerol treatment in the myofibrils., fresh, non-glycerinated myofibrils were prepared extemporaneously.

Indirect immunofluorescence tests were performed as described previously (5–8, 16). Detection of antibodies to striated muscle was done with cryostat sections of rat, guinea pig and monkey muscles, and for antibodies to myoid cells of the thymus, with cryostat sections from rat, guinea pig, calf and monkey thymus. Antibodies to the eel electroplax were evidenced on cryostat sections of eel tail.

The air-fixed sections were sensitized with the sera to be examined; antibodies were revealed by rabbit globulins treated with fluoresceine isothyocyanate (Inst. Pasteur Production) and directed against guinea pig (74411, Inst. Pasteur Production) or rat (74331 Inst. Pasteur Production) globulins. Microscopical examination was performed with Quartz iodine lamp and interference filter (5, 6, 7, 16).

Histological alterations were examined on paraffin sections from muscles, diaphragm, myocardium and thymus, stained with haematoxylin-eosine.

RESULTS

Evolution of the disease: The major symptoms of the experimental disease were preceded by restlessness and tremor, detectable only during handling. The initial, obvious symptoms were tremor and paresis of the hind legs, which soon evolved into intensive tremor and flaccid paralysis of all four legs; in the last phase of the disease, flaccid paralysis was generalized and intense respiratory distress occurred, accompanied by frequent hiccups. Death occurred soon after the above described symptoms. Most of the animals were killed during this phase. The evolution of the obvious symptoms took about 3–5 days in guinea pigs and 4–6 days in rats.

The onset of the disease occurred soon after the challenge inoculation (5–7 days for guinea pigs, 7–10 days for rats). A difference in reactivity was observed between the two species; guinea pigs were more sensitive than rats to ACR. In guinea pigs, disease was elicited by the first challenge injection, whereas two or three challenges were necessary for the onset of the symptoms in rats. Some of the rats were apparently healthy for a long time after repeated challenges.

Spontaneous transitory remissions occurred in both species and were short in guinea pigs and prolonged in rats. Individual differences of reactivity were observed in both species.

MCSAb: DMCD with crude membranes was positive, and mast cells from immunized animals underwent strong and specific degranulation. The mast cells kept their sensitivity to this antigen for a long period of time, even several weeks or months after the last inoculation.

DMCD test performed with purified ACR was unsuccessful since most of the peritoneal cells from immunized controls and normal animals underwent degranulation or lysis with ACR diluted with Triton X-100 (see Material and Methods) as well as with Triton X-100 alone. In order to avoid these shortcomings, the purified ACR diluted in Triton X-100 was replaced by the "crude membranes" antigen (see Material and Methods).

IMCD test performed with peritoneal mast cells from normal rats was positive with sera from animals inoculated with ACR together with CFA; the values were higher with sera from animals which exhibited the experimental disease compared with sera from animals in which symptoms were not obvious. The antigen used in this test was the same as that used in the DMCD (crude membranes).

Skin tests performed randomly on the 10th day after the first inoculation revealed a delayed state of hypersensitivity.

Direct adherence of rat myofibrils to peritoneal macrophages or to lymphocytes from lymphatic ganglions and spleen were negative in all the examined cases. In order to see whether preparation of myofibrils had denatured their antigenic structure, fresh non-glycerinated myofibrils were used with the same results.

The indirect immunofluorescent test evidenced antibodies for the eel electroplax in all sera of animals immunized with ACR together with CFA; these antibodies were more prevalent in the sera of animals receiving several inoculations. Antibodies specific for band I and A of striated muscle as well as antibodies for myoid cells of the thymus were not detected, and only antibodies for the sarcolemma could be found in the immunized animals.

Microscopic examination of paraffin sections evidenced intensive changes in skeletal muscles, the diaphragm and, in a few cases, also in the myocardium. The histological picture showed mixed cellular infiltration, disorganization, segmental swelling, fragmentation and atrophy, as well as floccular, granular and hyaline degeneration of the muscle fibres (Fig.

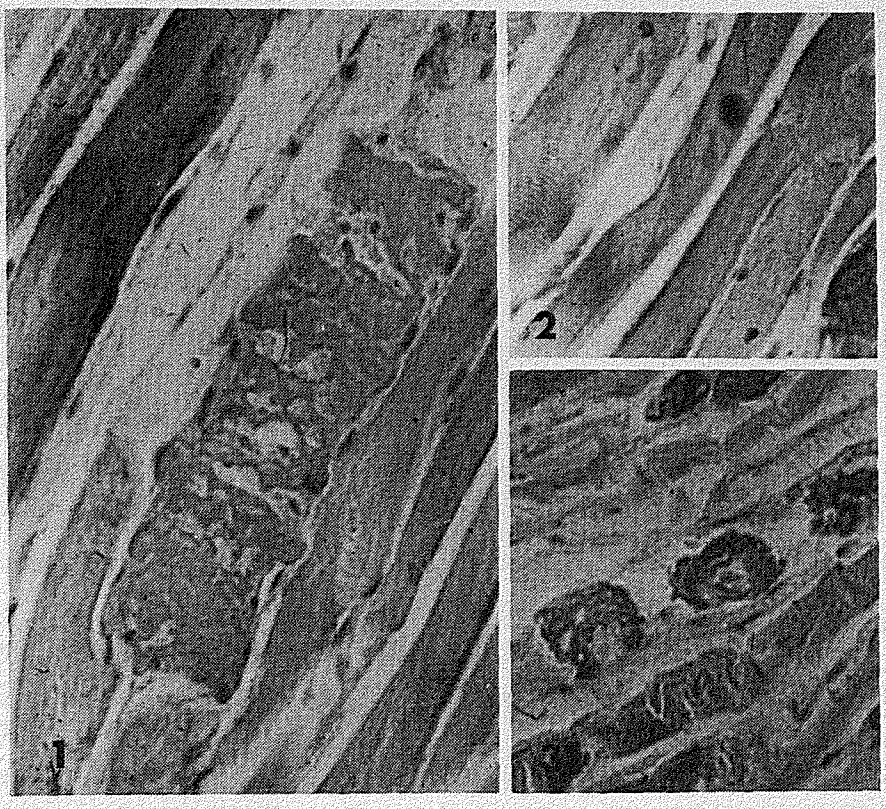


Fig. 1.

1. Muscle (guinea pig) obj. 45: Disintegration, segmental swelling, floccular degeneration. 2. Muscle (guinea pig) obj. 45: Segmental swelling, atrophy. 3. Diaphragm (guinea pig): Disintegration, segmental swelling, granular degeneration, atrophy, obj. 25.

1). These changes closely resemble those described in clinical polymyositis. The lesions were more intensive in animals with the experimental disease. The animals which did not present symptoms following immunization had slight muscle infiltration. Germinal centres were observed in the thymus of the immunized animals.

Controls inoculated with Triton X-100 together with CFA did not present muscular alterations, but a few germinal centres could be observed in their thymuses.

DISCUSSION

As can be seen from our results, inoculation with ACR together with CFA elicits, in guinea pigs and rats, a paralytic

syndrome with a strong hypersensitive state, as emphasized by the positive DMCD and IMCD tests.

Correlation could be observed between MCSAb and the evolution of the disease since values obtained with the IMCD were higher in animals with obvious symptoms compared with those showing no symptoms.

The lack of reactivity to striated muscle fibres and myoid cells of the thymus is contradictory, not only to the cellular and serological events in clinical M.gr., but also to the fact that antibodies to striated muscles and myoid cells of the thymus are produced by animals with flaccid paralysis following inoculation with muscle extracts.

Antibodies to sarcolemma can be explained by a probable common antigen for ACR and sarcolemma.

Another difference between this experimental disease compared with M.gr. and the experimental syndrome provoked by muscle extract inoculation, is the histological picture.

The intensive changes observed by us in muscles and diaphragm are different from those found in clinical M.gr., but strongly resemble those of clinical polymyositis. This is an immunological disease with muscular weakness, fatigability, hypersensitive aspects and strong muscular modification, but generally lacking in antibodies specific for striated muscles and myoid cells of the thymus.

According to these similarities between the "experimental ACR disease" and clinical polymyositis, it can be concluded that inoculation with ACR provokes a widely spread myositis in guinea pigs and rats which seems actually to be an experimental polymyositis with strong hypersensitivity to ACR as demonstrated by us through the DMCD and the IMCD tests.

SUMMARY

Mast cell sensitizing antibodies, direct mast cell degranulation and positive delayed skin tests were detected in guinea pigs and rats during experimental paralysis provoked by inoculation with acetylcholine receptors from the *Electrophorus elec-*

tricus. The animals also developed fluorescent antibodies to the sarcolemma, and massive myositis with histological changes in muscles similar to those characterizing clinical polymyositis.

REFERENCES

1. Beutner, E. H., Witebsky, E., Ricken, D. & Adler, R. H. (1962): Studies on autoantibodies in Myasthenia gravis. J.A.M.A. *182,* 46–58.
2. Boyden, S. V. (1964): Cytophilic antibody in guinea-pigs with delayed hypersensitivity. Immunology *7,* 474–483.
3. Djanian, A. Y., Beutner, E. H. & Witebsky, E. (1964): Tanned cells haemagglutination test for detection of antibodies in sera of patients with Myasthenia gravis. J. Lab. clin. Med. *63,* 60–70.
4. Duc, H. T. (1970): Etude immunologique du muscle locomoteur du Papio-papio, Thesis, Inst. Pasteur, Paris.
5. Eyquem, A. & Duc, H. T. (1971): Etude immunopathologique sur la Myasthemie. 2nd National Congress of Medical Microbiology, Bucharest.
6. Eyquem, A. & Duc, H. T. (1972): Immunopathologie de la Myasthemie. Ann. Inst. Pasteur *123,* 115–116.
7. Eyquem, A., Duc, H. T., Hermet, J. & Basseterre, S. (1976): Anticorps anticellules myoides du thymus et anti muscle strie chez le lapin et le rat aprés immunisation anti muscle et anti thymus. Ann. Immun., in press.
8. Eyquem, A., Vardinon, N., Basseterre, S. & Schwartz, J. (1975): Mast cell sensitizing antibodies in Myasthenia gravis, II. European Immunology Meeting, Sept. Amsterdam.
9. Grob, D. & Namba, T. (1963): Complement fixation by muscle nucleoproteins and serum of patients with Myasthenia gravis and other diseases. J. clin. Invest. *42,* 940–947,
10. Lennon, V., Lindstrom, J. & Seybold, M. (1975): Experimental autoimmune Myasthenia gravis: cellular and humoral immune responses. 5th Conference on Myasthenia Gravis, (May 30–31) New York. Ann. N.Y. Acad. Sci., in press.
11. Lindstrom, J., Lennon, V., Seybold, M. & Wittingham, S. (1975): Experimental autoimmune Myasthenia gravis: biochemical and immunochemical aspects. 5th Conference on Myasthenia gravis, (May 30–31) New York. Ann. N.Y. Acad. Sci., in press.
12. Morton, H. J., Morgan, J. F. & Parker, R. C. (1963): In Parker R. C. (ed.): Methods of tissue culture, pp. 74–75. Huebner, P. P. Inc., New York.
13. Patrick, J. & Lindstrom, J. (1973): Autoimmune response to acetylcholine receptor. Science *180,* 871–872.
14. Schwartz, J., Klopstock, A., Zickert-Duvdevani, P. & Honig, S. (196[illegible] Detection of hypersensitivity by indirect mast cells degranulation. Int. Arch. Allergy *26,* 333–339.

15. Schwartz, J. & Vardinon, N. (1966): *In vitro* prevention of direct mast cell disruption by specific antibody. Int. Arch. Allergy *30*, 67–74.
16. Schwartz, J., Eyquem, A. & Vardinon, N. (1975): Mast cell sensitizing antibodies in Myasthenia gravis, I. Immunological Communications *4*, 243–250.
17. Strauss, A., Seegal, B. C., Hsu, K. C., Burkholder, P. M., Nastuk, W. L. & Osserman, K. E. (1960): Immunofluorescence demonstration of a muscle binding, complement fixing serum globulin fraction in Myasthenia gravis. Proc. Soc. exp. Biol. (N.Y.) *105*, 184–191.
18. Strauss, A., Deitch, A. & Hsu, K. C. (1961): Further observation on localization of a muscle binding, complement fixing serum globulin fraction in Myasthenia gravis. Fed. Proc. *20*, 38–42.
19. Sugiyama, H., Benda, P., Meunier, J. C. & Changeux, J. P. (1973): Immunological characterisation of the cholinergic receptor protein from *Electrophorus electricus*. FEBS Letters *35*, 124–128.
20. Van der Geld, H., Feltkamp, T. E. W., Van Loghem, J. J., Oosterhius, H. J. G. H. & Biemond, A. (1963): Multiple antibody production Myasthenia gravis. Vox Sang. (Basel) *8*, 373–379.
21. Van der Geld, H., Feltkamp, T. E. W. & Oosterhuis, H. J. G. H. (1964): Reactivity of Myasthenia gravis serum globulin with skeletal muscle thymus demonstrated by immunofluorescence. Proc. Soc. exp. Biol. (N.Y.). *115*, 782–785,
22. Vardinon, N., Levanon, M. & Schwartz, J. (1967): The immunological value of the mast cell disruption inhibition test. Acta allergol. *22*, 28–38.

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