Reactivity of Myasthenia Gravis Serum γ-Globulin with Skeletal Muscle and Thymus Demonstrated by Immunofluorescence.* (29037)

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(Introduced by M. Landy)
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Van der Geld and Oosterhuis(1) reported that myasthenic sera reacting with both thymus and skeletal muscle lost all reactivity for these tissues after absorption with either thymus or skeletal muscle. These findings were interpreted as indicating the presence of an antigen common to skeletal muscle and thymus. With the antiglobulin consumption test used in those experiments, it was not possible, however, to localize the site of the reactive antigens. Accordingly, in the present study, it was sought to corroborate these findings with the use of a different technique and if possible to localize the site of the antigen-antibody reaction in these tissues.

Materials and methods. Patient sera were collected by Dr. H. J. G. H. Oosterhuis of the Neurological University Clinic, Wilhelmina Gasthuis, Amsterdam. Control sera were obtained from donors from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service. The indirect fluorescent staining method as employed in previous related studies was used(2). Calf thymus and rat diaphragm taken immediately after death were frozen in liquid nitrogen. Sections of 4 μ thickness were cut in a cryostat at −17°C and mounted on slides previously cleaned with dichromate and ethanol. The air dried sections were fixed for 10 minutes in acetone. Rabbit anti-human gamma globulin serum was twice precipitated with an equal volume of saturated $(NH_4)_2SO_4$. The albumin-free fraction was conjugated to fluorescein isothiocyanate by the method of Marshall *et al*(4). The conjugated fraction was purified by gel filtration with Sephadex G75 followed by 2 absorptions with acetone-dried horse liver powder according to the method of Johnson(5).

0.01 M phosphate buffered saline at pH 7.2 was used throughout the following procedures. After rinsing with phosphate buffered saline, the fixed tissue was treated as follows: 1. Incubation at room temperature for 30 minutes with one drop of serum diluted 1:4; 2. Washing with excess of phosphate buffered saline for 60 minutes with mild agitation and 6 changes of buffer; 3. Incubation at room temperature for 30 minutes with one drop of fluorescent anti-human gamma globulin serum; 4. Washing with phosphate buffered saline for 45 minutes with mild agitation and 4 changes of buffer. The sections were then covered with one drop of buffered glycerol and cover slip and examined under a Leitz-Ortholux microscope with ultraviolet light and appropriate filters.

It is emphasized that in this entire procedure, the crucial phase is the extensive washing required to remove non-antibody protein. The use of phosphate buffered saline at 37°C greatly enhances the effectiveness of the washing procedure. Especially with thymus, overall non-specific fluorescence (due to reaction with non-antibody protein) obscures the specific fluoresence. The use of serum dilutions 1/30 or 1/60 has been suggested as also helpful in circumventing the problem of non-specific staining(6). If forementioned precautions are taken, the specific apple-green fluorescence is made to stand out clearly against an autofluorescent blue background.

Results. The fluorescence in thymic tissue

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TABLE I. Incidence of Thymic Epithelial Cell Fluorescence.

Diagnosis	No. of sera	+	−
Myasthenia gravis	90	36	54
Rheumatoid arthritis	15	0	15
Myocardial infarction	20	0	20
Myopathies	15	0	15
Donors	70	0	70

was confined to large cells, usually in isolated groups of 3-6, located in the medulla. The fluorescence was strongest in the cell membrane and in cytoplasmic granulae which were best seen when viewed under high power (600 ×). It was evident that the search for these fluorescent cells had to be thorough and extensive inasmuch as only one such cell was encountered in the entire slide of some preparations. Conjugate controls alone (fluorescein conjugated rabbit anti-human γ-globulin) were negative.

Of a total of 90 myasthenic sera examined, 36 showed epithelial cell fluorescence. The control sera, all of which were negative, were obtained from 70 donors, 20 patients with myocardial infarction, 15 with rheumatoid arthritis, and 15 with myopathies. These findings are summarized in Table I. Our next step was to correlate the thymic fluorescence with the striational fluorescence of skeletal muscle which was first described by Strauss *et al*(7) and further characterized by Beutner *et al*(8). A very high degree of correlation was found. The few discrepancies observed were confined to sera in the weakly positive or equivocal range. These results are presented in Table II. As previously emphasized by van der Geld *et al*(9), the thymoma patients formed a distinct group; all thymoma sera were strongly positive in serum dilutions of 1:60 (represented in Table III).

Both the striational and the epithelial cell fluorescence were abolished by previous absorption of the positive sera with striated muscle. However, absorption with brain, spinal cord, kidney and thyroid, did not affect the striational or thymic fluorescence. Absorption of positive sera with thymic tissue did not abolish epithelial cell or striational muscle fluorescence completely. The failure to remove this activity completely is attributed to the paucity of epithelial cells in calf thymus and to the high sensitivity of the indirect immunofluorescent technique.

TABLE II. Comparison of Thymic Epithelial Cell Fluorescence and Striational Fluorescence.

	Reactivity	
	+	−
Thymic fluorescence	36	54
Striational "	34	56

Discussion. Germinal center reaction in the thymus accompanies about 70% of cases of myasthenia gravis (Castleman)(10). This reaction appears identical histologically with that seen in the spleen of animals intravenously injected with antigens and in lymph nodes draining the local sites of injected antigens. Direct injection of antigens in the exposed thymus of guinea pigs produced germinal centers and antibody containing cells (Marshall and White)(11).

TABLE III. Myasthenia Associated with Thymoma.

		Immunofluorescence	
Patient	Serum dilution 1:60	Thymus	Muscle
G 3		+++	+++
G 40		+++	+++
G 47		+++	+++
G 86		+++	+++
G 90		++++	++++
F 124		+++	+++
G 142		++++	++++
G 143		++++	++++
G 165		+++	+++

Owing to the complete lack of reactivity of the gland to parenterally administered antigens, the presence of a blood-thymus barrier has been postulated. Using fluorescein-conjugated anti-human 7S γ-globulin, White and Marshall(12) could localize γ-globulin containing cells in 6 thymuses obtained from patients with myasthenia gravis. Hess *et al* (13) observed in one case of myasthenia gravis that there were γ-globulin binding properties common to thymoma tissue and myasthenic muscle. These miscellaneous observations suggest that in myasthenia gravis the thymus may have embarked upon an immunological reaction against antigen in the gland. We postulate that by infection, soma-

tic mutation or neoplastic proliferation, the epithelial cells undergo an antigenic change and that this event in turn initiates an auto-immune process in the thymus which is very rich in immunologically competent cells.

Our demonstration, however, of multiple antibodies in the sera of patients with myasthenia is suggestive of an auto-immune disease based on disturbed tolerance(9). The presence of epithelial cells in a thymoma has previously been interpreted by Iverson(14) as suggestive of a clinical association with myasthenia gravis. Lattes(15) has analyzed 107 primary tumors of thymus of which 30 occurred in patients with myasthenia gravis. In the cases of myasthenia gravis, whenever non-neoplastic tissue was available for histological study, germinal centers were demonstrated.

Generalized lymphadenopathy which often characterizes systemic response to antigen is not a feature of myasthenia gravis. The fact that immunohistological activity, with the possible exception of lymphorrhages in skeletal muscle, is strictly confined to the thymus suggests that this organ is the focal site of origin of cells associated with myasthenia gravis.

Our data suggest that antibody produced by this postulated mechanism is cross reactive with skeletal muscle. A thymic origin for antibody against skeletal muscle is therefore suggested.

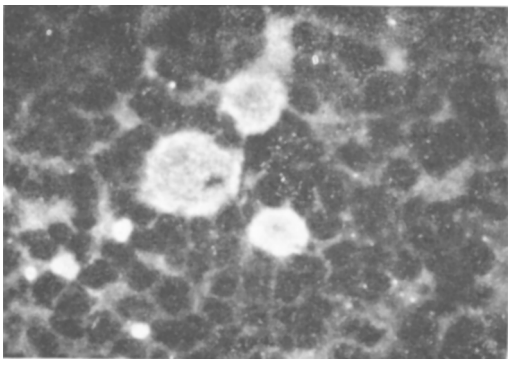

FIG. 1. Section of calf thymus treated with thymoma serum (G 143) in dilution 1:60 and fluorescein conjugated rabbit anti-human gamma globulin. Note strong immunofluorescence of cytoplasmic granules in 3 epithelial cells. Smaller fluorescent areas are blue-white or pale yellow and represent autofluorescent granules in other cell types. (Magnification 400 ×)

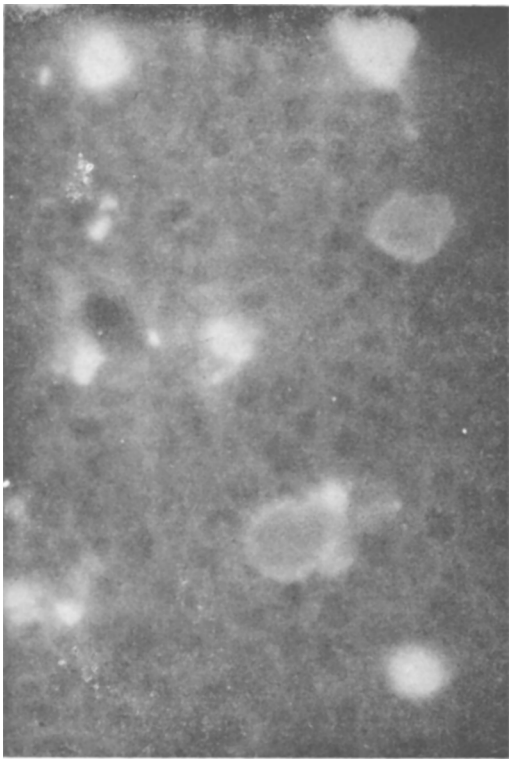

FIG. 2. Section of calf thymus treated with control serum dilution 1:4 and fluorescein conjugated rabbit anti-human gamma globulin. Two large epithelial cells devoid of immunofluorescence are seen to the right of center.

Experiments are in progress to test the reactivity with thymus tissue of sera from patients with other auto-immune diseases. We also performed additional immunofluorescent studies with calf spleen and lymph node and examined 8 sera from myasthenics with known reactivity for thymic epithelial cells. No similar reactivity was demonstrated for cells in either spleen or lymph node.

Summary. Sera from patients with myasthenia gravis (36 out of a total of 90) showed reactivity with epithelial cells in the thymus by means of the immunofluorescence technique. This antibody was cross reactive with skeletal muscle. All 9 sera of patients whose myasthenia was associated with a thymoma showed strong reactivity in serum dilutions of up to 1:60. Antigenic change in epithelial cells is postulated as a possible mechanism

initiating an auto-immune response in the thymus.

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Hypotensive Activity of 3-(N-methyl-t-butyl-amino)-1-bromo-3-methyl-1-butyne Hydrochloride. (29038)

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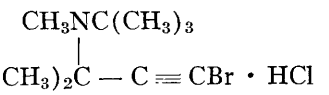
In searching for agents that lower blood pressure, much attention has been given to substances whose principal action is ganglionic blockade. Since these agents block all autonomic ganglia to a varying degree, search continues for a compound having more specific blood pressure lowering action, but with a minimum of ganglionic block.

A series of haloethynyl derivatives has been prepared(1), of which 3-(N-methyl-t-butyl-amino)-1-bromo-3-methyl-l-butyne hydrochloride (Lilly no. 37157) has exhibited good hypotensive activity with minimum ganglionic blockade in animals. This paper presents data obtained in the pharmacologic evaluation of this compound.



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Methods. Hypertension was produced in male Long-Evans rats by a modification of the Goldblatt procedure(2). Control blood pressures were taken by a microphonic manometric technique(3) before oral administration of 20 mg/kg of the drug or an equivalent volume of 0.9% saline. Following drug administration, blood pressure was determined every hour for 7 hours and the mean change over the 7-hour period was calculated for 3 hypertensive rats. The average maximum depression of blood pressure was also recorded.