

Experimental autonomic neuropathy : An immunologically induced disorder of reflex vasomotor function

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Experimental allergic disorders of central and peripheral nervous systems are considered models of the acquired inflammatory demyelinating diseases of man. In these disorders the autonomic nervous system has not been studied histologically. Furthermore, the functional deficits of the autonomic nervous system that might result from such lesions have not been examined and no attempt has been made to produce an experimental disease with an antigen obtained from the sympathetic nervous system.

In this study we have tried to sensitize rabbits to an antigen extracted from human sympathetic nerves and ganglia and to compare the effects with those obtained by sensitization with sciatic nerve and Gasserian ganglia. Heating of the shaved back to produce reflex vasodilatation in the ear has been found to be a useful method for monitoring autonomic function in the rabbit. By the use of this test it was possible to demonstrate a specific deficit in vasomotor function of animals injected with sympathetic tissue antigen.

MATERIAL AND METHODS

Animals were placed in a wooden box with a wire mesh top. Fitting tightly over this was another box containing six 75 watt household bulbs, placed 6 inches above the wire mesh. The head and ears protruded from the boxes which fitted closely about the animal's neck so that no light was visible when the bulbs were switched on by a noiseless switch. Each ear was folded over a constantan copper wire thermocouple which was connected to a Grass polygraph. A temperature change of 0.6°C. in either ear produced a 1.5 cm. deflection of the recording pen. The hairs were clipped from the entire back and the skin exposed directly to light. To produce vasomotor paralysis 2.0 ml. of a 2% procaine solution was injected subcutaneously around the base of the right ear. This produced a marked increase in ear temperature which showed that the animal was not maximally vasodilated. When no increase in ear temperature occurred after the local anaesthetic the rabbit was considered not to be testable.

The experimental procedure was first to produce vasomotor paralysis in the right ear. Reflex vasodilatation was then studied in the left ear and was gauged by the change in ear temperature produced by heating of the trunk for 40 seconds. Each experiment was done at steady room temperatures ranging at various times between 18 and 21°C. This ensured that variations in ear temperature closely followed blood flow changes (Cooper, Cross, Greenfield, Hamilton, and Scarborough, 1949).

Aqueous extracts of human sciatic, sympathetic, and Gasserian ganglia were made. Each tissue was first pooled, then cut twice on a freezing microtome at 5 μ and suspended in 0.05M NaCl buffered with phosphate at pH 6.8-7.0. This was then homogenized in a Virtis homogenizer for one and a half minutes and spun at 20,000 r.p.m. for half an hour in a Spinco model E ultracentrifuge. The lipid layer was discarded. The clear supernatant was concentrated by pervaporation and dialysed against the buffered NaCl solution. Sympathetic tissue was also homogenized in the presence of 0.04% Na desoxycholate. The protein content of the extracts was determined by Lowry's method (Lowry, Rosebrough, Farr, and Randall, 1951).

Random bred female New Zealand rabbits weighing 2.3 kg. were used throughout, and 0.4 ml. of a 25% emulsion of human whole tissue antigen in mineral oil containing 3 mg./ml. of killed tubercle bacilli was given into the toe pads of the forepaws. Rabbits immunized with extracts received 5 mg. of protein in 0.4 ml. of an emulsion containing equal parts of aqueous extract in Freund's adjuvant distributed to the toe pads of all limbs. These animals were given an intravenous booster injection five weeks later with 2 mg. of the appropriate protein in 1 ml. of saline. Seven days later they were sacrificed and exsanguinated.

Pooled normal human sera, plasma, and concentrated extracts of sciatic, sympathetic, and Gasserian ganglia and liver were migrated electrophoretically in agar gel using the standard methods of Grabar and Burtin (1964). These were then tested against sera of immunized rabbits. Some sera were studied by simple diffusion in gel (Ouchterlony, 1958).

In order to characterize esterases a modified Koelle and Friedenwald method was applied to immunoelectrophoretic and gel diffusion plaques as described

by Uriel (1963). The incubation medium consisted of butyrylthiocholine and ditetrazolium Cl mixed in a veronal buffered agar solution at pH 8.2. Antigen antibody precipitin arcs in which there was esterase activity stained blue.

Paraffin-embedded, haematoxylin- and eosin-stained samples of central and peripheral nervous systems and serial sections of both paravertebral sympathetic chains were examined. Tissues from rabbits sacrificed at various intervals after immunization and from healthy controls were studied.

RESULTS

A marked rise in temperature was seen in all 15 healthy control rabbits five to 10 seconds after infiltration with local anaesthetic about the base of the right ear. Thereafter small fluctuations in ear temperature were recorded. Illumination of the back produced a transient small fall in ear temperature followed by a sustained rise which outlasted the stimulus by 20 to 30 seconds and then returned to the baseline (Fig. 1). This reflex increase in ear

temperature was abolished by atropine and enhanced by eserine in each of two separate experiments.

VASCULAR REFLEXES IN IMMUNIZED ANIMALS Twenty-one rabbits given whole sympathetic tissue antigen were tested seven to 32 days after immunization. In 16 reflex vasodilatation in the ear was abolished and only small spontaneous temperature changes were seen (Fig. 1).

To determine the onset of the autonomic disturbance seven additional animals were immunized with sympathetic tissue antigen. They were tested on the day of immunization or two days later at which time the vascular reflexes were present in all. Five were tested again four days after immunization and all were found still to retain normal vasomotor function. Four were retested between six and eight days after injection and in three reflex vasodilatation was absent. By the tenth day six of the seven animals were affected and the last had no vasodilatation 14 days after immunization. The earliest appearance of the vasomotor abnormality was, therefore, six to eight days after the administration of the sympathetic antigen. Nine affected animals were retested at longer intervals, and four of these regained normal vasomotor function after two months. The results of vasomotor tests in rabbits immunized with a variety of antigens are given in the table.

All animals given sciatic and two given Gasserian whole tissue antigen had clinical evidence of allergic neuritis but all of these retained normal vasomotor function. Thirty-nine animals given sympathetic antigen remained clinically healthy. Some were temporarily incapacitated by swollen forelimbs and three rabbits had transient hindlimb paresis.

Immuno-electrophoresis The aqueous extracts of the various tissue antigens studied were found to be contaminated with serum proteins. Albumin, IgG (gamma 2-globulin), and several alpha and beta globulins were identified in immuno-electrophoresis using a commercial equine antiserum to human serum proteins (Hyland Laboratories) (Fig. 2). After absorption antisera from rabbits immunized with sympathetic tissue extract or whole sympathetic tissue contained an antibody which reacted with an antigen in human sympathetic extract but no reaction was seen with serum or plasma (Fig. 2). These antisera also failed to react with human sciatic, Gasserian ganglion, or liver extracts. The sympathetic tissue antigen appeared organ specific. The antibody to it could be absorbed out of the rabbit antisera by 0.17 mg. of sympathetic extract protein per millilitre of antiserum. Eight times this concentration of human sciatic, Gasserian ganglion, or liver extract protein did not alter it. In an aqueous extract of sympathetic ganglia treated with sodium

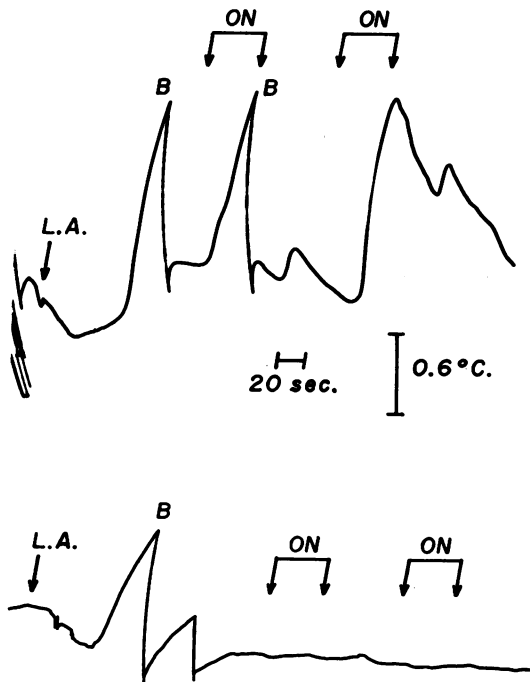


FIG. 1. Temperature recordings from rabbit ears before (above) and 10 days after (below) immunization with sympathetic tissue. At both times local anaesthetic (L.A.) produced a marked rise in ear temperature. Irradiation of the back (ON) produced a marked but transient rise in ear temperature before immunization; it had no effect 10 days later. B = Polygraph balance adjustment.

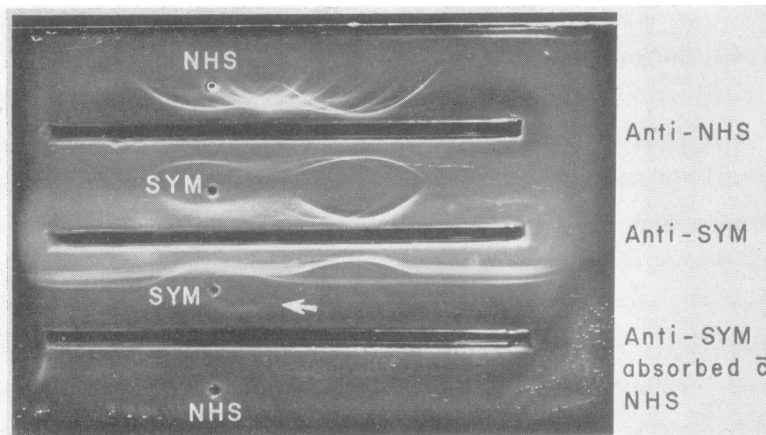


FIG. 2. Immunoelectrophoretic plate showing the reactions of normal human serum, sympathetic extract, and of serum from a rabbit with experimental autonomic neuropathy. Upper trough: horse antiserum to normal human serum proteins (Anti-NHS) shows several contaminating serum proteins in the sympathetic extract (SYM). Middle trough: the serum from an animal with experimental autonomic neuropathy (Anti-SYM) contains many antibodies to contaminating normal human serum proteins in the sympathetic extract (SYM). Lower trough: the same rabbit serum as in the middle trough but after absorption with normal

human serum 4:1. The antibodies to the contaminating normal human serum proteins have been removed by this procedure. The single remaining precipitin arc (arrow) reveals a specific sympathetic tissue antigen contained in the sympathetic extract (SYM). The excess normal human serum diffused as a front towards the middle trough and is responsible for the transverse extensions of the precipitin areas (arcs).

desoxycholate sympathetic tissue antigen was not revealed by a positive antiserum. Animals immunized with this extract did not develop antibody to sympathetic tissue antigen and had normal vascular reflexes (Table). Sera of rabbits immunized with Gasserian or sciatic antigens did not react with sympathetic extracts. Sympathetic tissue antigen migrated as a beta protein. It showed weak thiocholine esterase activity.

The tissues of the nervous system of 21 animals immunized with sympathetic antigen were examined. In 10 there was a mild infiltration of perivascular lymphocytes and mononuclear cells of the type seen in delayed hypersensitivity. These were located in the sympathetic ganglia. In 15 animals of this series similar infiltrates were found in the sciatic nerves (Fig. 3). *Encephalitozoon caniculi* infection was found in five animals. Nine animals given sciatic and one immunized with Gasserian antigen were examined and all showed the typical lesions of experimental allergic neuritis (Fig. 3). The

sympathetic chains of these animals were normal. In two of these rabbits lesions of encephalitozoon infection were seen in the brain. Ten healthy controls and animals immunized with Freund's adjuvant alone had no histological lesions.

DISCUSSION

The disorder produced by immunization with sympathetic tissue would not have been recognized without measurements of reflex vasomotor function. The pathways involved in normal reflex vasodilatation in the ear in response to heating of the skin elsewhere are not fully known. Holton and Rand (1962) have shown that direct electrical stimulation of the superior cervical ganglion of the rabbit produces in ear vessels an initial vasoconstriction followed by vasodilatation. The vasodilator phase was abolished by atropine and enhanced by eserine. This suggested that the efferent fibres causing vasodilatation were sympathetic cholinergic. Furthermore, histochemical studies of rabbit ear

TABLE

VASOMOTOR FUNCTION IN RABBITS IMMUNIZED WITH A VARIETY OF ANTIGENS IN FREUND'S ADJUVANT

Number of Rabbits	Antigen	Reflex Vasodilatation		
		Present	Absent	Not Testable
15	None	15	0	0
4	Adjuvant alone	4	0	0
15	Sciatic whole tissue	13	1	1
3	Sciatic aqueous extract	2	0	1
5	Gasserian ganglia whole tissue	5	0	0
28	Sympathetic ganglia whole tissue	2	23	3
8	Sympathetic ganglia aqueous extract	0	7	1
6	Sympathetic ganglia aqueous extract with Na-desoxycholate	6	0	0

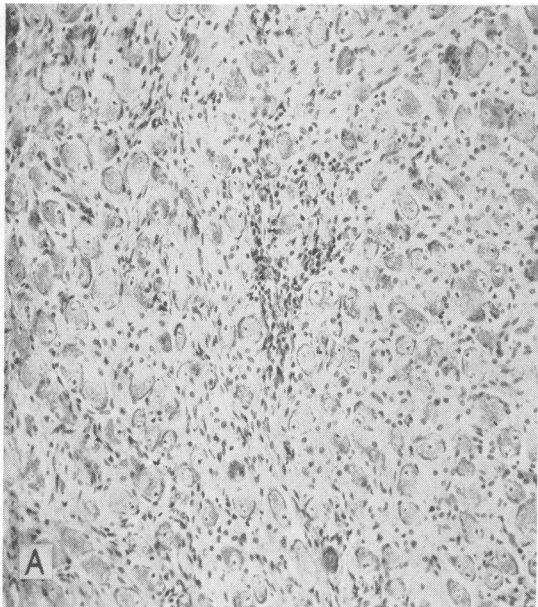


FIG. 3A

FIG. 3A. Sympathetic ganglion from animal with experimental autonomic neuropathy $\times 180$.

FIG. 3B. Posterior root ganglion from animal with experimental allergic neuritis $\times 280$.

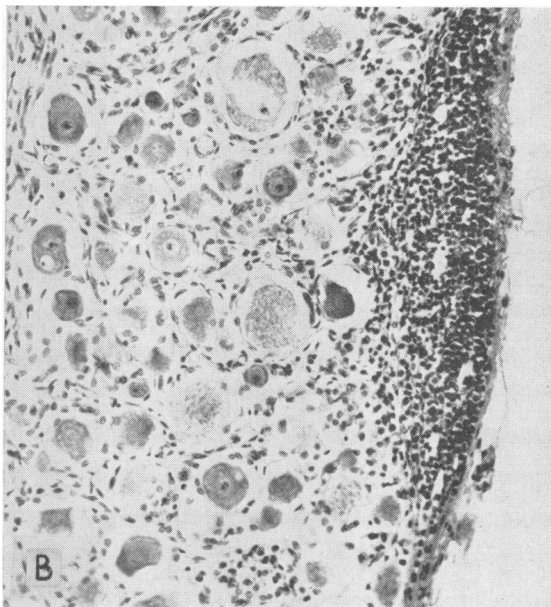


FIG. 3B

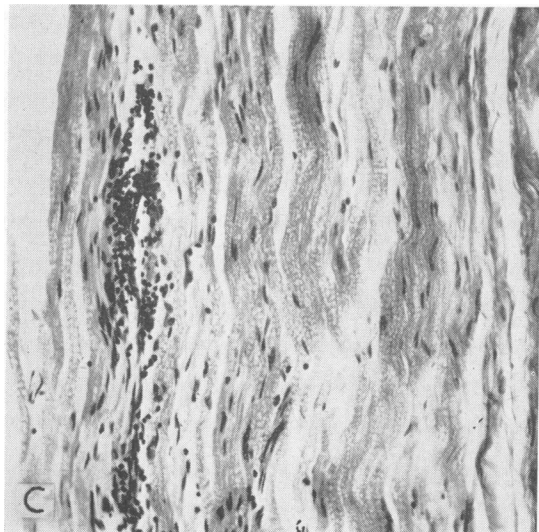


FIG. 3C

FIG. 3C. Sciatic nerve from animal with experimental autonomic neuropathy $\times 280$.

FIG. 3D. Sciatic nerve from animal with experimental allergic neuritis $\times 180$.

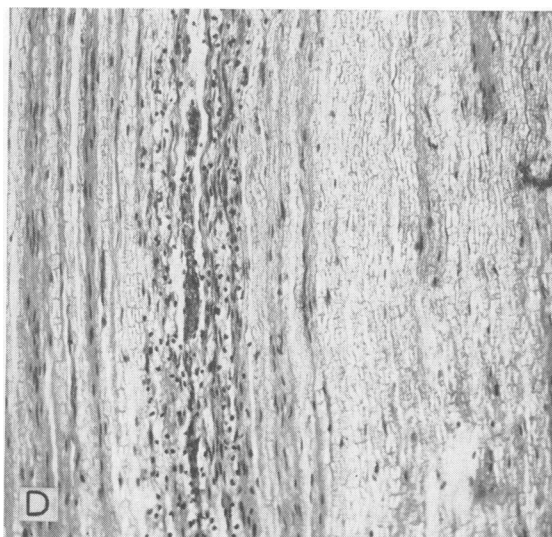


FIG. 3D

FIG. 3. The lesion in A is mild. Dense lymphocytic perivascular infiltrates are seen in B and C. The lesion in D is old, there are few lymphocytes, and fatty macrophages are prominent. Sections stained with haematoxylin and eosin.

perivascular nerve plexuses have shown that they have marked cholinesterase activity which could be reduced by cervical sympathectomy (Grant and Thompson, 1963). On the afferent side vasodilatation is likely to depend on neural structures rather than on warm blood because of the short latency and the prompt return of the blood flow to baseline levels after the cessation of the stimulus. Both direct sympathetic stimulation and brief heating of the skin produce identical blood flow changes, which are probably mediated by sympathetic efferents to the vessels.

Theoretically, a lack of normal reflex vasodilatation in affected animals could have been caused by a disturbance of the receptors in the heated skin of the back, by an affection of afferent nerves, central nervous structures or efferent fibres, or by an involvement of the blood vessels themselves. Vasodilatation in the ears of the affected animals could still be produced by local anaesthetic, even though the reflex response was absent. There is no evidence, therefore, for involvement of the vessel wall. There were no abnormalities of the exposed skin and it remained normally sensitive to pain, so that paralysis of the receptors was not the likely cause of the vasomotor disorder. Histologically, central nervous system lesions were not found and the mild perivascular lymphocytic infiltrates of the sympathetic ganglia in half of the affected animals did not seem an adequate explanation for the disease. By exclusion, therefore, the defect was probably related to involvement of the efferent sympathetic cholinergic fibres.

Experimental autonomic neuropathy was produced by administration of sympathetic tissue and not by that of any other peripheral nerve or sensory ganglia. This specificity, together with the technique of inducing the disorder, the duration of the incubation period, and the recovery after an interval of weeks, suggested that the disease was mediated by an immunological mechanism. Sympathetic ganglia are known to contain large amounts of cholinesterase and the antibody-antigen precipitates appeared to have esterase activity. This antibody, therefore, might have interfered with the function of sympathetic cholinergic efferents to the vessels of affected animals.

This new disease differs in many ways from experimental allergic neuritis. The latter, like many other experimental disorders, appears to be an example of delayed hypersensitivity, and does not correlate with the presence of specific circulating antibodies, is characterized by extensive neurological deficits, and has as its pathological basis perivascular lymphocytic and mononuclear infiltrates with demyelination of nerve fibres; but vasomotor

function and sympathetic ganglia are normal. In experimental autonomic neuropathy, on the other hand, clinical disorders of any kind were negligible, cellular infiltrates and demyelination were rare, and vasomotor function was found to be impaired by special tests. In this disorder a tissue specific circulating antibody was found. Thus it may be assumed that our laboratory disease is due to a different mechanism. The few lesions of delayed hypersensitive type found in the sympathetic chain were unlikely to have been responsible for the disorder; however, lesions in the more peripheral parts of the autonomic nervous system have not been excluded. Some animals with experimental autonomic neuropathy had slight peripheral nerve lesions which were probably elicited by the small amount of myelin in the sympathetic antigen. This mild superimposed experimental allergic neuritis could have accounted for the minimal clinical signs of motor weakness of the limbs in a few of the animals.

Developmental arrest of the sympathetic paravertebral chain can be produced immunologically by anti-growth factor hormone (Levi-Montalcini and Booker, 1960). Animals given this serum soon after birth have been shown, when mature, to adapt poorly to cold. This arrest of growth of the sympathetic chain and its expected functional result has no apparent similarity to any disorders of autonomic function in man. The few known instances of the latter, which are manifest by orthostatic hypotension, disturbances of sweating, lacrimation, and salivation, have to date not been associated with known morphological alterations in sympathetic ganglia. In this respect experimental autonomic neuropathy resembles the human disease.

SUMMARY

A disorder of vasomotor function has been produced in rabbits immunized with human sympathetic ganglia and named experimental autonomic neuropathy. The abnormality appeared when rabbits were immunized with sympathetic tissue but not with sciatic or Gasserian ganglion antigen.

A circulating antibody which appeared specific to sympathetic tissue accompanied the experimental disorder. This antibody could be absorbed out of the immune sera by sympathetic but not by sciatic, Gasserian ganglion, or liver extracts. On electrophoresis it migrated as a beta protein.

This new disease stands in contrast to experimental allergic neuritis in which there are no abnormalities in vasomotor function or histological changes in sympathetic ganglia.

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REFERENCES

- Cooper, K. E., Cross, K. W., Greenfield, A. D. M., Hamilton, D. McK., and Scarborough, H. (1949). A comparison of methods for gauging the blood flow through the hand. *Clin. Sci.*, **8**, 217-234.
- Grabar, P., and Burtin, P., Editors (1964). *Immuno-Electrophoretic Analysis. Applications to Human Biological Fluids*. Elsevier, Amsterdam, London, New York.
- Grant, R. T., and Thompson, R. H. S. (1963). Cholinesterase and the nerve supply to blood vessels in the rabbit's external ear. *J. Anat. (Lond.)*, **97**, 7-22.
- Holton, P., and Rand, M. J. (1962). Sympathetic vasodilatation in the rabbit ear. *Brit. J. Pharmacol.*, **19**, 513-526.
- Levi-Montalcini, R., and Booker, B. (1960). Destruction of the sympathetic ganglia in mammals by an antiserum to a nerve-growth protein. *Proc. nat. Acad. Sci. (Wash.)*, **46**, 384-391.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.*, **193**, 265-275.
- Ouchterlony, Ö. (1958). Diffusion-in-gel methods for immunological analysis. *Progr. Allergy*, **5**, 1-78.
- Uriel, J. (1963). Characterization of enzymes in specific immune-precipitates. *Ann. N.Y. Acad. Sci.*, **103**, 956-979.