Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 9630–9634, December 1990
Neurobiology

Autoimmune preganglionic sympathectomy induced by acetylcholinesterase antibodies

(sympathetic nervous system/ganglionic transmission/ptosis/idiopathic orthostatic hypotension/complement-mediated cytotoxicity)

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Communicated by G. B. Koelle, September 10, 1990

ABSTRACT **Systemic injection of monoclonal antibodies to neural acetylcholinesterase in adult rats caused a syndrome with permanent, complement-mediated destruction of presynaptic fibers in sympathetic ganglia and adrenal medulla. Ptosis, hypotension, bradycardia, and postural syncope ensued. In sympathetic ganglia, acetylcholinesterase activity disappeared from neuropil but not from nerve cell bodies. Choline acetyltransferase activity and ultrastructurally defined synapses were also lost. Electrical stimulation of presynaptic fibers to the superior cervical ganglion ceased to evoke end-organ responses. On the other hand, direct ganglionic stimulation remained effective, and the postganglionic adrenergic system appeared intact. Motor performance and the choline acetyltransferase content of skeletal muscle were preserved, as was parasympathetic (vagal) function. This model of selective cholinergic autoimmunity represents another tool for autonomic physiology and may be relevant to the pathogenesis of human dysautonomias.**

At least two autoimmune disorders are caused by antibodies to exposed antigens of peripheral cholinergic synapses. In myasthenia gravis, the target is the nicotinic receptor of skeletal muscle (1–3). In the Lambert–Eaton myasthenic syndrome, it is the presynaptic voltage-gated calcium channel (4–7). Because acetylcholinesterase (AChE) is anchored in cholinergic synaptic membranes (8), this enzyme is accessible to immunoglobulins and might also be a target of autoimmunity.

We recently observed long-lasting eyelid drooping (ptosis) in rats injected with monoclonal anti-AChE antibodies that neither inhibit enzymatic activity nor reach epitopes in the central nervous system *in vivo* (9). With these antibodies we have created a model of autoimmunity to peripheral neuronal AChE, comprising striking and permanent damage to preganglionic sympathetic neurons without evident effect on other cholinergic systems. The characteristics of this syndrome were defined by physiological, biochemical, and morphological experiments.

MATERIALS AND METHODS

Antibody Injections. Murine IgG monoclonal antibodies ZR 2, 3, 4, 5, and 6, raised against rat brain AChE, were purified by chromatography on DEAE-Affigel Blue (Bio-Rad) as described (10). These antibodies avidly bound all molecular forms of AChE and showed no preferences for enzyme from particular tissues. Each antibody was directed against a different epitope of AChE, but none interacted with the active site or interfered with the catalytic activity of the enzyme (10). The antibodies chosen for these experiments had no *in vivo* access to their epitopes on brain AChE (9). The purified IgGs were injected as an equal-part mixture (total, 1.5 mg in 2 ml of 0.9% NaCl) into the tail vein of 300-g adult male Sprague–Dawley rats. Controls received identical amounts of normal mouse IgG or a murine anti-human AChE monoclonal IgG that did not recognize rat AChE.

Some rats were pretreated with cobra venom factor to deplete hemolytic complement. The protein, purified as described (11), was given in five i.p. doses of 83 μg, beginning 48 hr before IgG injection. A final dose was administered 24 hr after IgG.

Enzyme Assays. Dopamine-β-hydroxylase activity was measured in the presence of optimal concentrations of $CuSO_4$ according to the method of Molinoff *et al.* (12). Choline acetyltransferase activity (ChAT) was measured by the method of Fonnum (13), with choline-free blank samples (control experiments demonstrated that carnitine acetyltransferase contributed <5% of the activity assayed in ganglia or muscle). AChE was measured by the method of Ellman *et al.* (14) with ethopropazine (0.1 mM) as a selective inhibitor of butyrylcholinesterase.

Antibody Detection. Murine immunoglobulins in rat serum were measured as described (9) with a specific two-site enzyme-linked immunosorbent assay, based upon rat anti-mouse IgG (Boehringer Mannheim). Immune complexes of AChE and murine IgG were measured by adsorption on solid-phase protein A (9).

Morphology. For AChE histochemistry, tissues were fixed in 4% paraformaldehyde for 2 hr at 4°C, rinsed overnight in 10% (wt/vol) sucrose and exhaustively sectioned at 12 μm in a cryostat. All sections were stained for AChE by the method of Koelle and Friedenwald (15) as modified by Lewis (16) with 0.1 mM ethopropazine to inhibit butyrylcholinesterase.

For electron microscopy, superior cervical ganglia were fixed *in situ* by perfusion with 2% (wt/vol) glutaraldehyde and were postfixed in osmium tetroxide. Epoxy–araldite thin sections were stained with lead citrate and uranyl acetate. To count synaptic profiles, three sections taken at random from each ganglion were examined in their entirety at a magnification of 25,000 in a Philips 201 electron microscope.

Physiological Procedures. Blood pressure and heart rate were measured in rats anesthetized with pentobarbital, 45 mg/kg i.p., supplemented as needed to suppress responses to light pinching. After tracheal intubation, a heparin-saline-filled cannula was placed in the right carotid artery with care to avoid damage to adjacent nerves. With a Statham pressure transducer mean arterial pressure was monitored continuously, and heart rate was recorded at 5-min intervals.

Eyelid tension was measured in supine anesthetized rats by a strain-gauge recorder connected to the margin of the upper eyelid with fine thread. Baseline tension was set to 0.25 g. Presynaptic fibers to the superior cervical ganglion were

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Abbreviations: AChE, acetylcholinesterase; IgG, immunoglobulin G; ChAT, choline acetyltransferase; DMPP, dimethylphenylpiperazinium.

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exposed by gentle dissection, bathed in mineral oil, and placed over a bipolar platinum electrode. Supramaximal stimulation was applied in trains of 10 msec, 5 V, square-wave pulses at a standard frequency of 6 Hz.

Statistical Analysis. The SEM was used throughout as the measure of variation. Differences between means were evaluated by the two-tailed *t* test.

RESULTS

Over 30 rats in each group were observed closely for 8 hr after injection and periodically for as long as 15 mo. Control rats appeared unaffected by the injection of normal mouse IgG. On the other hand, all rats that received anti-AChE antibodies developed severe autonomic disturbance with signs of transient sympathetic activation followed by prolonged sympathetic depression. Surprisingly, these rats remained clinically normal in several other respects. There were no signs of muscle fasciculation or obvious motor dysfunction. Moreover, unlike anticholinesterase drugs, AChE antibodies caused no parasympathetic overactivity (lacrimation, salivation, bronchial secretion, or diarrhea).

The sympathetic abnormalities appeared very quickly. Piloerection and exophthalmos began 20 min after injection of AChE antibodies, peaked at 30–40 min, and then subsided. Next came ptosis (Fig. 1), a classic sign of sympathetic denervation (17). Ptosis began at 1 hr, was maximal by 2 hr, and continued indefinitely in every injected animal (12 rats observed for 3 weeks, four rats for 2 mo, two rats for 3 mo, and one rat for 15 mo). Serum antibody, on the other hand, was not detectable after 2 mo. Because AChE stores in nerve and muscle are replaced in a few weeks (18, 19), persistent ptosis must reflect permanent cellular lesions, not mere enzyme loss.

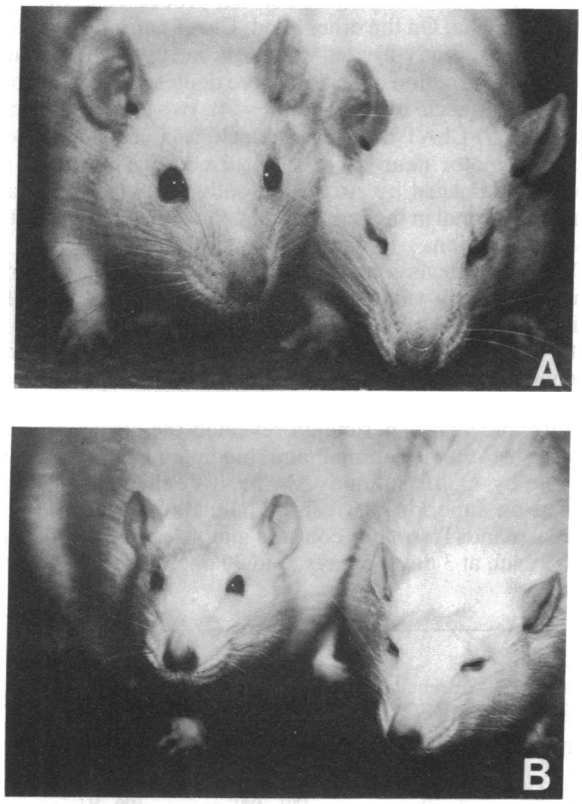

FIG. 1. Antibody-induced ptosis. (*A*) Twenty-four hours after tail-vein injection of 1.5 mg of monoclonal IgG antibodies reactive with neural AChE of rat (right) or of human only (left). (*B*) Same rats 1 yr later.

Tonic control of eyelid smooth muscle is adrenergic (20). Ptosis does not occur in rats with paresis induced by antibodies to nicotinic receptors of skeletal muscle (V.A.L. unpublished work), but it does follow sympathectomy (17). Thus a likely site for the ptosis-inducing lesion was the superior cervical ganglion. Ganglionic function was tested by recording palpebral tension while electrically stimulating the presynaptic input (Fig. 2). In control rats (n = 8), eyelid tension increased with preganglionic stimulation (peak response, 0.64 ± 0.07 g at 6 Hz). Direct stimulation of the ganglion elicited a similar response (0.7 ± 0.08 g). Eyelid contraction was also induced by dimethylphenylpiperazinium (DMPP), a selective agonist for nicotinic acetylcholine receptors of ganglia (ED_{50}, 50 μg i.v.; peak response, 0.5 ± 0.1 g). Eight rats with ptosis were tested 3–90 days after injection of AChE antibodies. None responded to preganglionic stimulation, but all responded normally to direct ganglionic stimulation (0.6 ± 0.12 g) and to DMPP (0.5 ± 0.12 g). We concluded that the postganglionic neurons were intact, but the preganglionic terminals were disabled or destroyed.

Another striking sign of autonomic disturbance, never seen in controls, was postural syncope. Starting 6 hr after injection, the rats reproducibly lost consciousness and stopped breathing within 30 sec when gripped about the thorax and held upright; they recovered quickly when placed on their backs. These observations suggested damage extending beyond the cervical sympathetic ganglia, including structures involved in cardiovascular regulation.

Heart rate and mean blood pressure were recorded acutely in supine anesthetized rats. There was no hemodynamic response to normal mouse IgG (1.5 mg i.v., three rats). Pressure and rate were also stable for 20 min after antibody injection (130 ± 9 mmHg, 346 ± 16 beats per min, n = 5). But at 40 min after injection (maximal piloerection) transient hypertension (+34 ± 11 mmHg, $P < 0.05$) and cardioaccel-

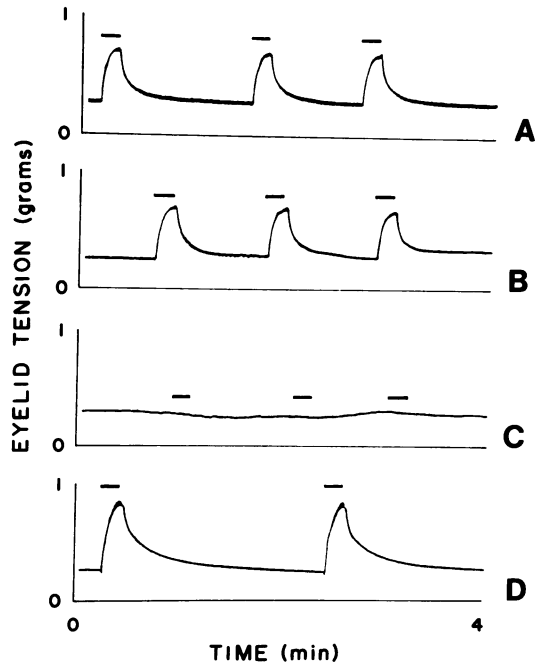

FIG. 2. Antibody-induced failure of ganglionic transmission. Eyelid tension in response to supramaximal electrical stimulation (horizontal bars) of superior cervical ganglion or preganglionic input. (*A*) Control eyelid tension (3 days after normal mouse IgG) upon preganglionic stimulation. (*B*) Control eyelid tension (3 days after normal mouse IgG) upon ganglionic stimulation. (*C*) Eyelid tension 3 days after anti-AChE antibodies upon preganglionic stimulation. (*D*) Eyelid tension 3 days after anti-AChE antibodies upon ganglionic stimulation.

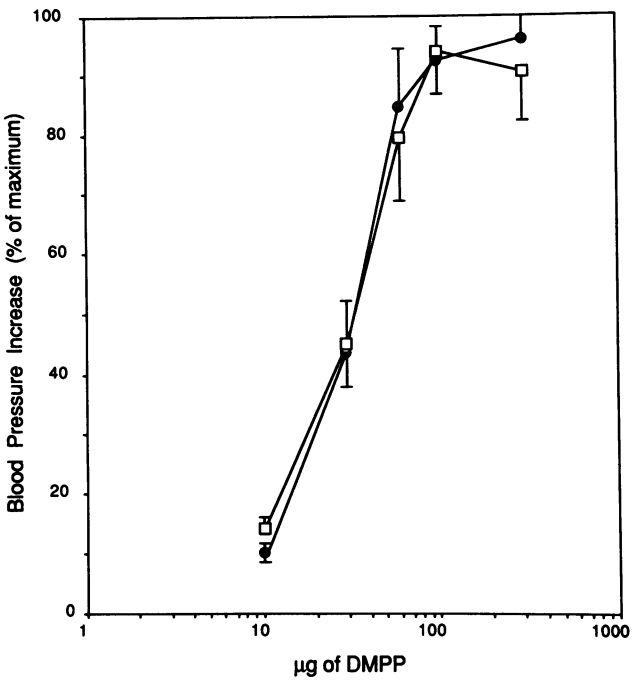

FIG. 3. Normal pressor function. Anesthetized rats were injected with the indicated doses of DMPP via the tail vein. Amplitude of the transient increase in mean blood pressure is shown as percentage of the maximal increase recorded. □, Control (n = 6); ●, 24 hr after anti-AChE antibodies (n = 5).

eration (+93 ± 13 beats per min, $P < 0.01$) occurred, despite the presence of anesthetic (21). By 100 min the heart rate was again normal, and blood pressure was 39 ± 11 mmHg below normal ($P < 0.05$).

Thereafter, blood pressure and heart rate were reduced in parallel (35% at 1 day, 25% at 3 weeks; six to nine rats per group, $P < 0.001$ in all cases). Atropine, 10 mg/kg i.v., did not raise blood pressure or heart rate, although it fully reversed the bradycardia induced by 3 μg of physostigmine. Hence, the cardiovascular effects of antibody did not reflect buildup of acetylcholine from impaired enzymatic hydrolysis.

Atropine-resistant hypotension and bradycardia might suggest damage to adrenergic neurons, but this was ruled out by another pharmacological experiment (Fig. 3). In control rats, DMPP raised blood pressure briskly, and the response was inhibited by 6 mg of hexamethonium, a ganglionic blocking agent. DMPP also raised blood pressure in rats rendered hypotensive by injection of AChE antibodies 24 hr earlier. Evidently, the postganglionic portion of the sympathoadrenal pressor system, including the nicotinic receptors of ganglia, was fully functional.

Parasympathetic function was also intact as shown by the chronotropic response to vagal stimulation. In four controls and four rats given AChE antibodies 3–5 days earlier, stimulation at 10 Hz slowed the heart by 50%, whereas stimulation at 30 Hz stopped it (data not shown). The vagal system thus appeared to resist antibody attack.

Table 1. Tissue AChE activity 72 hr after antibody injection

Antibody treatment	AChE activity, nmol/min per sample (n)			
	Ganglion	Adrenal	Diaphragm	Atria
Normal IgG	40 ± 3 (5)	19 ± 3 (5)	520 ± 12 (12)	157 ± 16 (5)
Anti-AChE	25 ± 2* (6)	11 ± 1* (6)	377 ± 13* (8)	116 ± 16 (6)

Ganglion is the superior cervical.
*$P < 0.01$.

Enzyme assays aided in defining the cellular targets of antibody-induced damage. AChE activity was reduced in most tissues, although the decreases were modest, even in ganglia (Table 1). Because the AChE antibodies were noninhibitory, the reductions reflected actual loss of enzyme. The distribution of the effects was consistent with antibody access to peripheral cholinergic synapses throughout the body. Three days after antibody injection, significant fractions of the tissue AChE were complexed with murine IgG (22 ± 3% in superior cervical ganglia, n = 6; 48 ± 4% in diaphragm, n = 4).

ChAT, a marker of cholinergic neuronal cytoplasm but not a direct target for AChE antibodies, was affected more variably from tissue to tissue than was AChE. ChAT activity vanished from the superior cervical ganglion 3 days after injection and was substantially depleted in the stellate ganglion and adrenal gland (Table 2). The enzyme loss was comparable to that observed 3 days after transection of the preganglionic fibers, and similar changes occurred in the thoracic, lumbar, and coeliac ganglia (data not shown). Nearly identical results were also obtained at 1 day and 1 week after antibody injection. These results implied that antibody effects were not limited to the target enzyme but included lysis of cholinergic neuronal processes in the sympathetic ganglia. On the other hand, ChAT activity in skeletal muscle in antibody-treated rats was not altered. Because unilateral transection of sciatic nerve depleted ChAT activity in hindlimb muscle by 93 ± 2% (n = 4), two conclusions may be drawn: (*i*) ChAT of normal muscle was primarily associated with motor neurons. (*ii*) Motor neurons were not significantly damaged by AChE antibody. ChAT activity also remained normal in the atria, consistent with the preservation of vagal function.

Other enzyme assays were directed at the status of adrenergic neurons in superior cervical ganglia. Five days after antibody injection, ganglionic dopamine-β-hydroxylase activity was 80 ± 4% of the control value (n = 9). Therefore, adrenergic neurons, for which the enzyme is a marker, were largely spared.

Morphological experiments revealed the structural counterparts of the functional and biochemical abnormalities induced by AChE antibody. AChE histochemistry was performed on superior cervical and stellate ganglia as well as adrenal glands from four controls and three antibody-treated rats (1 each at 3 days, 5 days, and 90 days after injection). In

Table 2. Tissue ChAT activity 72 hr after antibody injection

Treatment	n	Ganglia		Adrenal*	Diaphragm†	Atria†
		Superior cervical*	Stellate*			
Normal IgG	10	24 ± 2	32 ± 6	26 ± 3	490 ± 53	110 ± 20
Anti-AChE	12	0.4 ± 0.1‡	3 ± 0.4‡	3 ± 0.6‡	510 ± 82	120 ± 15
Normal IgG + CoF	2	24, 31	37, 48	30, 31	430, 450	130, 140
Anti-AChE + CoF	2	50, 62	41, 60	8, 20	380, 390	106, 92

Cobra venom factor (CoF) was given to deplete hemolytic complement.
*nmol/hr.
†nmol/hr per gram of wet weight.
‡$P < 10^{-5}$.

every case, there was striking and selective loss of AChE activity in ganglionic neuropil but not in nerve cell bodies, just as after surgical denervation (Fig. 4). In the adrenal medulla, AChE-rich presynaptic fibers disappeared within 3 days of antibody injection. Infiltrations of inflammatory cells were not seen.

The effects of AChE antibodies on cholinergic synapses in superior cervical ganglia were further examined by electron microscopy (Fig. 5). In control ganglia (n = 3), vesicle-laden terminals juxtaposed to pre- and postsynaptic densities were readily identified at an apparent abundance of 50 ± 7 per μm^2. Although the morphometric data were not corrected for potential overlap between sections, they differed radically from those obtained 1 week after antibody administration. The ganglia from antibody-treated animals (n = 3) were devoid of synaptic triads and, indeed, of any processes containing small, clear vesicles. No other structural abnormalities were evident, however, such as cytoplasmic vacuolization of neuronal cell bodies, residues of synaptic specializations, or lacunae.

Experiments with purified anti-complementary factor from *Naja naja* cobra venom clarified the mechanism of immunosympathectomy (Table 2). In rats depleted of hemolytic complement by cobra venom factor (11), antibody injection caused no ptosis, and the sympathetic ganglia lost no ChAT activity. Complement activation is obviously essential for the immunological lesion.

Another requirement for immunosympathectomy, possibly related to the activation of complement, was the need for concurrent administration of multiple AChE antibodies. All five antibodies had access to ganglia *in situ* (IgG-complexation of AChE ranged from 5% to 25%). Yet four of the antibodies induced no discernible sympathetic dysfunction when injected alone in a dose of 1.5 mg, and the fifth induced only mild and transient ptosis. In comparison, equal part mixtures of *any three antibodies* (total 0.9 mg of IgG per injection), caused marked ptosis along with >95% depletion of ChAT activity from superior cervical ganglia.

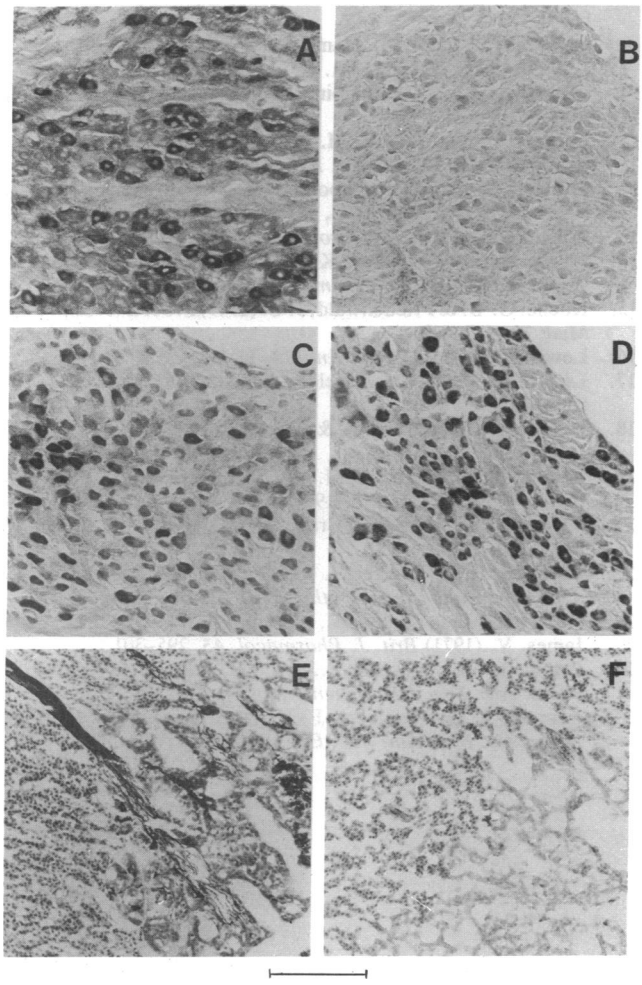

FIG. 4. Depletion of presynaptic AChE. Representative sections stained for AChE with 10^{-4} M ethopropazine as pseudocholinesterase inhibitor. (*A*) Normal superior cervical ganglion. (*B*) Background control with 10^{-5} M physostigmine. (*C*) Ganglion 3 mo after AChE antibodies. (*D*) Ganglion 1 week after surgical denervation. (*E*) Normal adrenal. (*F*) Adrenal 3 days after antibody injection. (Bar = 200 μm.)

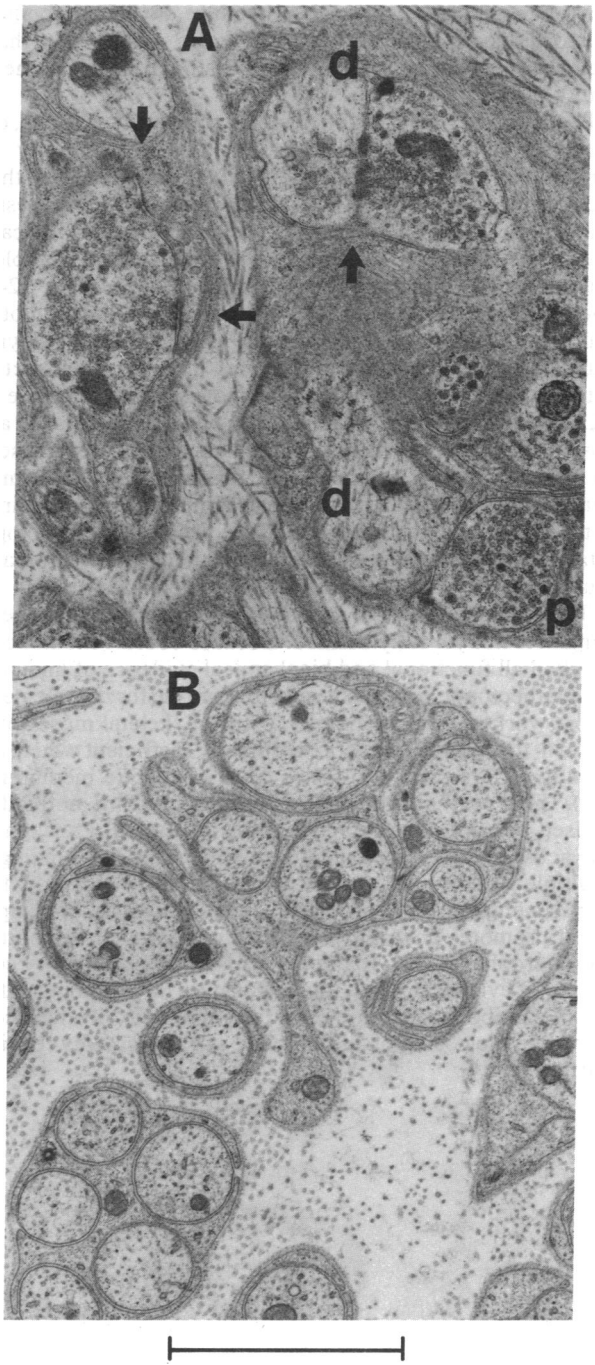

FIG. 5. Loss of synaptic triads in superior cervical ganglia. (*A*) Control (arrows, axodendritic synapses; d, dendrite; p, preterminal axon). (*B*) Section 1 week after injection of AChE antibodies. (Bar = 2 μm.)

DISCUSSION

We demonstrate that antibodies to neuronal AChE can permanently ablate preganglionic sympathetic nerve terminals while sparing postganglionic cells. The evidence for selective destruction of preganglionic terminals included the following: (*i*) failure of ganglionic transmission with no loss of activation by direct electrical and pharmacological stimuli; (*ii*) obliteration of preganglionic cholinergic markers with retention of a marker of postganglionic adrenergic neurons; and (*iii*) loss of morphologically identifiable terminals with preservation of principal ganglion cells.

Destruction of the presynaptic input to adrenal medulla as well as the input to sympathetic ganglia presumably reflects the anatomical and developmental relationship of these structures. Because of this combined damage, AChE antibodies should induce more complete adrenergic dysfunction than does anti-nerve growth factor (17) or chemical sympathectomy (22, 23).

The apparent preservation of motor and vagal functions, on the other hand, contrasts markedly with the severity of sympathoadrenal dysfunction. This contrast implies that AChE-rich structures in other cholinergic systems resist damage by AChE antibodies. Preliminary results indicate that the antibodies reach the motor endplate and complex with most of the external AChE forms at this site (24). Therefore, the relative sparing of muscle endplates, motor neurons, AChE-bearing adrenergic neurons, and parasympathetic neurons cannot be explained in terms of restricted antibody access. Variations in the molecular architecture of AChE and its topological relation to plasma membranes are possible determining factors. For example, the neuromuscular junction has rich deposits of asymmetric AChE forms, densely packed in the synaptic basal lamina. This reservoir of extracellular antigen may divert the focus of immunologic attack away from the cell surfaces of the motor neuron and muscle.

The mechanism of the immunologic lesion in sympathetic ganglia is not yet fully defined. Because cobra venom factor blocked all functional and biochemical evidence of immunosympathectomy, we assume an effector role for complement. From work on the nicotinic receptor of skeletal muscle it is known that complement activation is inefficient when autoantigens are sparsely distributed in synaptic membranes (25). This mechanism may explain why AChE antibodies caused ptosis only when given in combination. Presumably the activation of complement is facilitated by IgG binding to multiple epitopes of neuronal AChE. In the rat model of experimental myasthenia gravis, initial activation of complement promotes phagocytosis of the postsynaptic membrane by macrophages (11). In our experiments, inflammatory cells were not evident in sympathetic ganglia 3 days or longer after AChE-antibody injection. The possible role of such cells earlier in the course of autoimmune sympathectomy requires further study.

Preganglionic immunosympathectomy is attractive for experiments on circulatory control mechanisms. This model also provides a way to analyze neurotrophic phenomena in the sympathetic nervous system, separating the contributions of cell contact from those of impulse activity. The unanticipated permanence of the antibody-mediated lesion represents an important subject for further investigation in its own right. Finally, this experimental autoimmune disorder has potential relevance to a variety of acquired dysautonomias of man.

We thank Pamela Hammond and Jon Charlesworth for technical assistance. We were supported by grants from the National Institute of Neurological and Communicative Disorders and Stroke (NS 18170 and NS 15057) and the William K. Warren Foundation.

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