Chemical degeneration of intestinal nerves

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FRANTZIDES, CONSTANTINE T., JOHN C. GARANCIS, BASIL T. DOUMAS, AND ROBERT E. CONDON. *Chemical degeneration of intestinal nerves.* Am. J. Physiol. 258 (Gastrointest. Liver Physiol. 21): G848–G855, 1990.—In 15 dogs, cobalt chloride solutions were infused close intra-arterially to perfuse a short segment of the jejunum. In an additional four dogs, the jejunum was perfused with the aqueous vehicle (perfusion control). All animals were killed after 1 mo and tissue samples from cobalt-treated and from nonperfused intestine (tissue comparison control) were obtained for electron microscopic and immunohistochemical studies. Segments infused with 0.25 g/dl cobalt solution showed minimal changes; the most striking feature was an increase of vasoactive intestinal polypeptide (VIP)- and substance P-containing neurosecretory granules. Cobalt chloride at higher concentrations (0.75–1.5 g/dl) induced degeneration of ganglion cells and axons in both the myenteric and submucosal plexi. In contrast, the smooth muscle and the mucosal cells of the cobalt-perfused intestine showed no histological abnormalities. Immunohistochemical staining of tissues treated with 0.75–1.5 g/dl cobalt solutions revealed absence of substance P, Met-enkephalin, and VIP immunoreactivity in all sections studied; control segments showed the presence of all three peptides. Cobalt chloride in concentrations of 0.75–1.5 g/dl causes degeneration of intestinal intramural nerves and provides an experimental model suitable for studying the role of these nerves in small intestinal function.

neurotoxin; cobalt; intestine; nerves; electron microscopy; immunohistochemistry

THE ENTERIC NERVOUS SYSTEM plays a major role in the control of gastrointestinal motility in both the fasting and fed states (2, 6, 7, 15). The precise role, however, of the intestinal intramural innervation in bowel functions has not yet been determined because of the inability to selectively and chronically block or destroy these nerves. Attempts to selectively ablate enteric neurons have been made repeatedly during the past quarter century (8–10, 12–14). The techniques used in these studies resulted in only partial denervation, or caused damage to the intestinal smooth muscle. The aim of the present study was to induce chronic selective degeneration of the intestinal intramural nerves by local close intra-arterial perfusion of the neurotoxin cobalt chloride.

METHODS

Studies were performed on 19 conditioned male dogs (30–40 kg). In 15 dogs, cobaltous chloride hexahydrate in an aqueous solution of 0.068 mol/l (20 g/l) of trisodium citrate dihydrate was infused close intra-arterially in order to perfuse a short segment of jejunum. Various concentrations of cobalt chloride were used (0.25 g/dl, $n = 4$; 0.75 g/dl, $n = 4$; 1.5 g/dl, $n = 5$; and 3 g/dl, $n = 2$) in a volume of 200 ml. In a control group of four dogs, the segment of jejunum was perfused with the aqueous vehicle.

A midline incision was made under intravenous anesthesia (pentobarbital sodium, 25 mg/kg) using standard aseptic surgical techniques. A jejunal mesenteric artery and vein, supplying a 15- to 20-cm segment of intestine, was isolated from the adjacent tissue. Special care was taken not to damage or transect the perivascular nerves. The artery and the vein were then clamped proximally with atraumatic microclamps and cannulated. The anastomotic branches in the mesentery were also clamped to temporarily isolate the segment of intestine from the systemic circulation. These measures were taken to avoid escape of cobalt into the systemic circulation, since cobalt is toxic to the brain and all neural tissues. Cobalt chloride solution was infused through the arterial catheter over 20 min (at a rate of 10 ml/min) and drained out through the corresponding cannulated vein. The borders of the perfused segment were marked with nylon sutures for later identification. After completion of the cobalt infusion, the vessels were flushed with 50 ml saline, the catheters were withdrawn, and the punctured vessels were reconstructed using 9-0 silk suture. The microclamps were then released and the circulation reestablished. The total ischemia time was <30 min. The same procedures were performed in the control group, except that saline was perfused.

All animals were killed 1 mo later, and tissue samples from the cobalt-treated segment and adjacent nonperfused intestine (5 cm proximal and distal to the cobalt-treated segment), as well as from the saline-perfused control segments, were obtained for electron microscopic and immunohistochemical studies. The intestinal segments were cut along the mesenteric border, and full-thickness (2–4 mm) specimens were taken circumferentially from each segment. Fifteen sections per specimen were prepared for electron microscopic and for immunohistochemical studies. Sections were studied without the observer knowing whether the tissue under study came from a saline-perfused or a cobalt-perfused loop of intestine.

Electron microscopy. Ten tissue blocks were processed from each of the treated and control specimens of the jejunum. Each specimen was fixed in Karnovsky's solution, postfixed in 1% osmium tetroxide, and embedded in Spur's low-viscosity epoxy resin. One-micron thick sections were cut and stained for light microscopy. Plexi

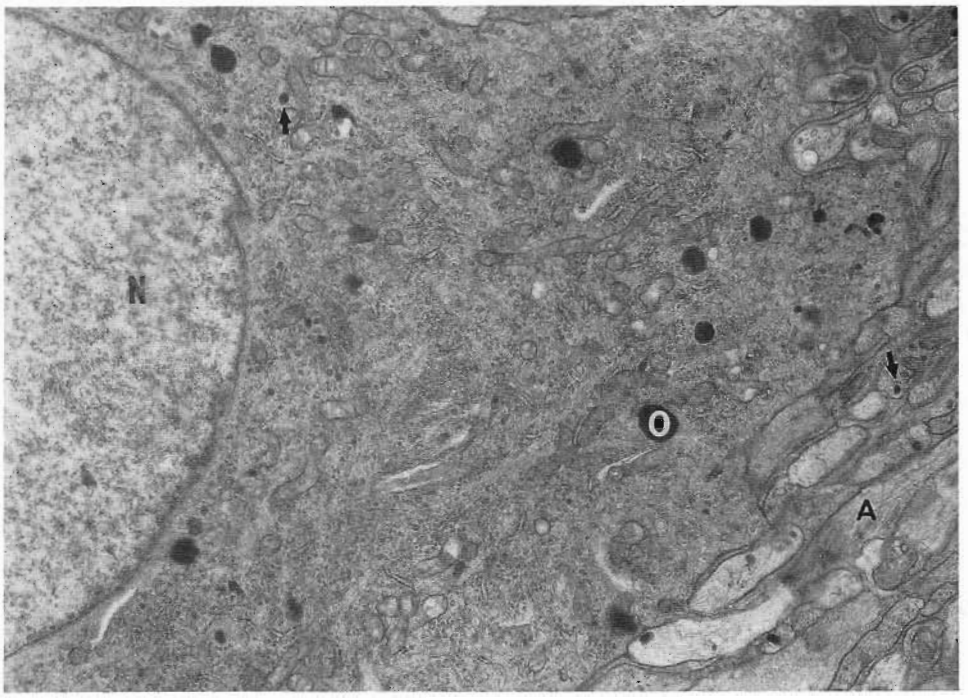

FIG. 1. Control: cross section of myenteric plexus. N, nerve cell; A, unmyelinated axon. Cytoplasm of nerve cells contains mitochondria, lysosomes (O), granular endoplasmic reticulum, and RNA particles. Few neurosecretory granules (arrows) are present in axons and nerve cells. ×9,800.

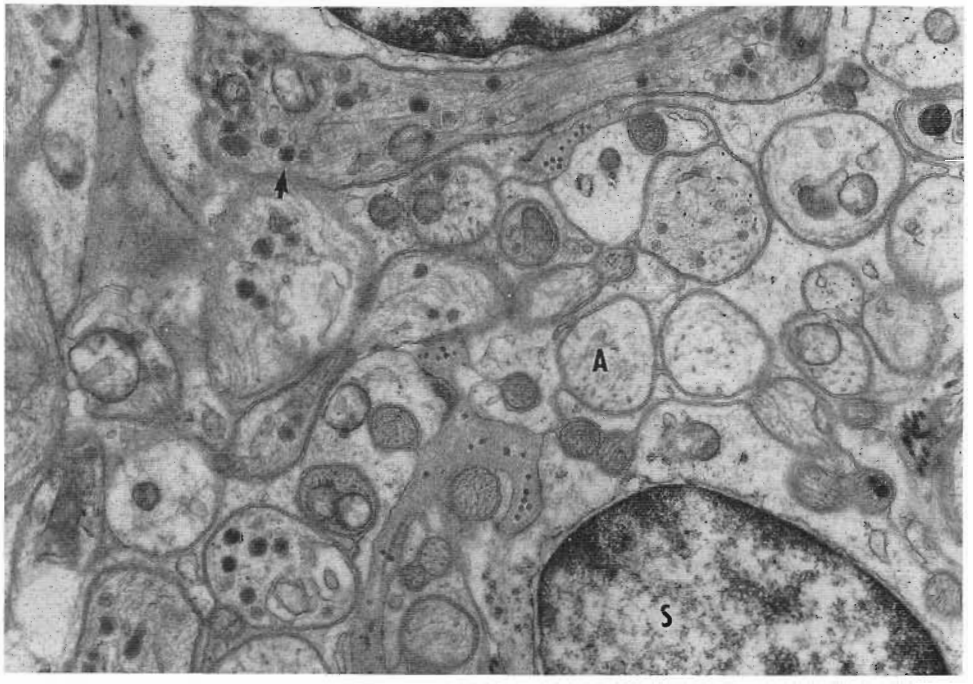

FIG. 2. Control: myenteric plexus cross and longitudinal sections of axons (A). Arrow, neurosecretory granule; S, Schwann cell. ×21,000.

TABLE 1. *Results of electron microscopic studies*

Solutions of $CoCl_2$, g/dl	Edema and Disappearance of Microtubules and Neurofilaments	Vacuolar Degeneration of Cytoplasm and Axons	Focal Necrosis of Axons and Ganglion Cells	Degenerative Smooth Muscle Changes
0.25	++	0	0	0
0.75	++++	++++	+++	0
1.5	++++	++++	++++	0
3	++++	++++	++++	++

0, No changes compared with control; ++, 25–50%; +++, 51–75%; and ++++, 76–100% of axons and ganglion cells or muscle fibers examined exhibited structural changes.

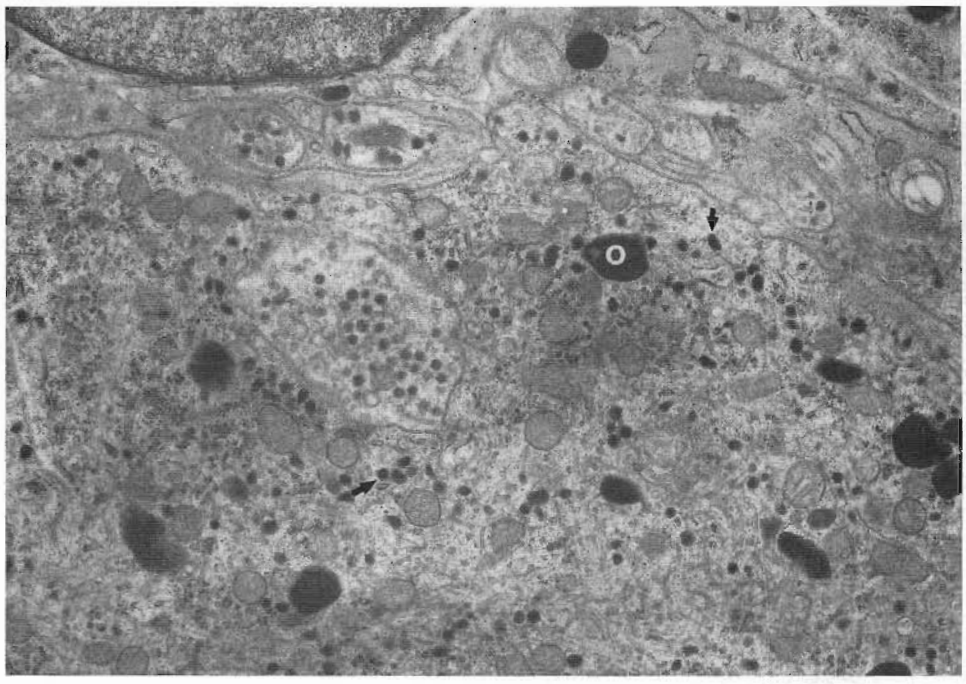

FIG. 3. Jejunal tissue treated with 0.25 g/dl $CoCl_2$ solution. There is an apparent increase of neurosecretory granules (arrows) and lysosomes (O) in the cytoplasm of nerve cells. ×16,000.

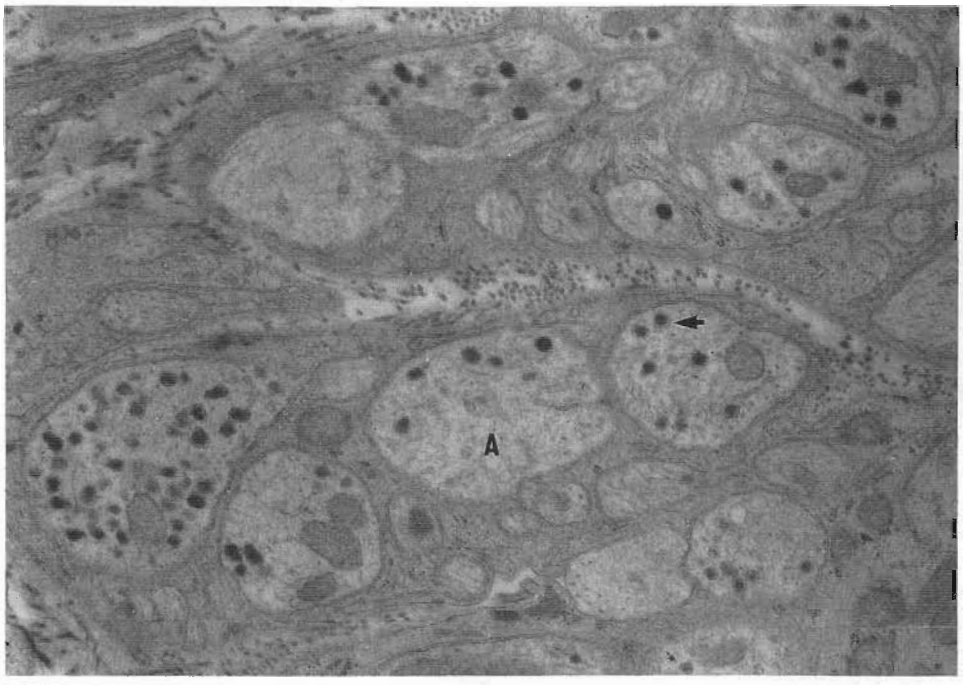

FIG. 4. Jejunal tissue treated with 0.25 g/dl $CoCl_2$. A cross section of axons (A) shows an apparent increase of neurosecretory granules (arrow). ×20,000.

were randomly selected for ultrathin sectioning. Ultrathin sections were stained with lead citrate and uranyl acetate and then examined with a Zeiss (EM 10C) electron microscope operated at 60 kV.

Immunohistochemical studies. From both the treated and control specimens of the jejunum, the entire circumference of the bowel wall, divided into three segments, was fixed in Bouin's solution and embedded in paraffin (1). The peroxidase anti-peroxidase method was used to demonstrate the presence of substance P, Met-enkephalin, and vasoactive intestinal polypeptide (VIP) in the small intestine. Samples taken from experimental (cobalt-treated) and from the perfusion and comparison control segments of dog jejunum were fixed in Bouin's solution or in Formalin and embedded in paraffin. After deparaffinization, the tissue sections were treated with 0.3% hydrogen peroxide to block endogenous peroxidase. Three sets of slides of control and experimental tissues each were stained using immunoperoxidase kits: anti-substance P and anti-VIP (Biogenex Laboratories, Dublin, CA) and anti-Met-enkephalin (Accurate Chemical and Scientific, Westbury, NY). The tissue sections were stained according to the instructions provided with each staining kit. Specimens from two dogs were processed as frozen sections for comparison.

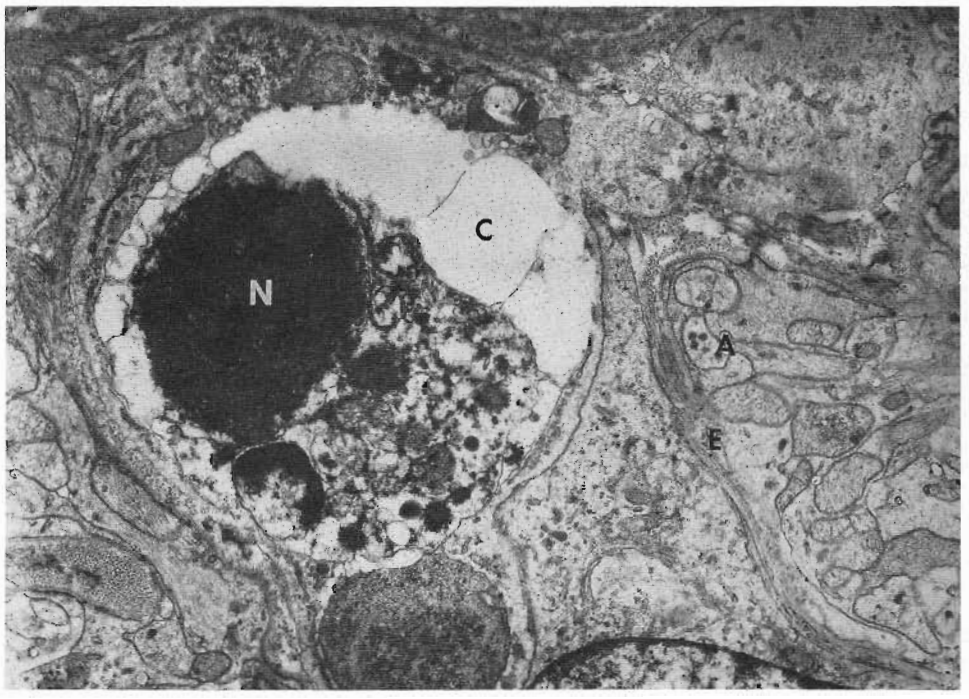

FIG. 7. Tissue perfused with 1.5 g/dl $CoCl_2$ solution. Nerve cell shows extensive cytoplasmic necrosis (C) and condensation of nuclear chromatin (N). A, axons; E, endoneurium. ×13,000.

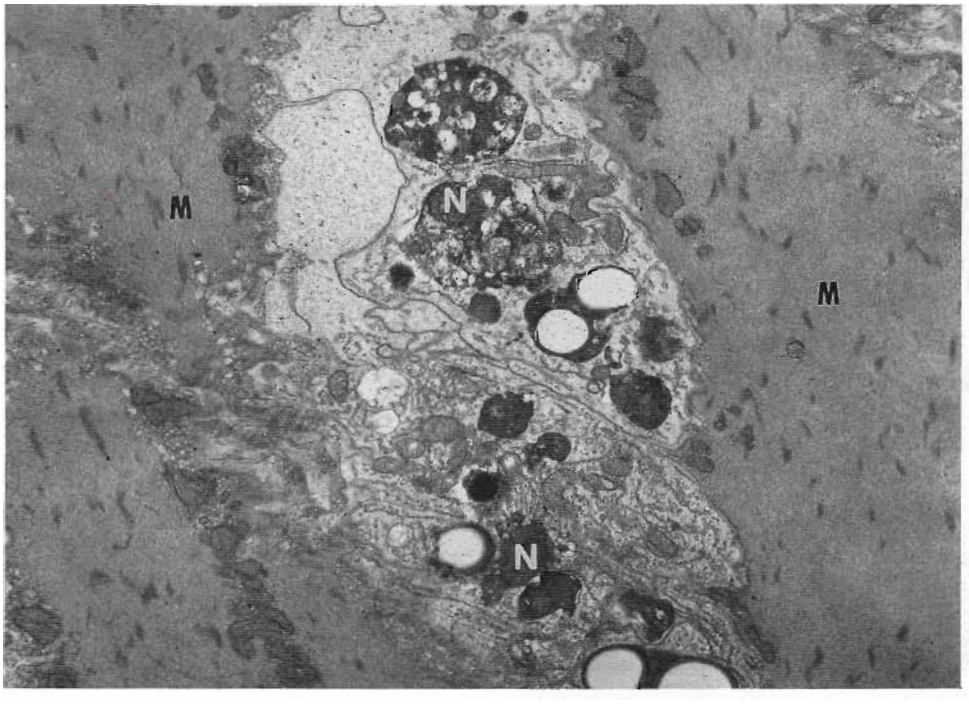

FIG. 8. Tissue perfused with 1.5 g/dl $CoCl_2$ solution. There is focal cytoplasmic necrosis with formation of autophagic vacuoles in nerve and satellite cells (N). Smooth muscle cells (M) are not affected. ×10,000.

rosecretory granules (Fig. 4). Some axons, however, exhibited alterations noticeably affecting the axoplasm and its components. Edema of axoplasm was accompanied by the loss of microtubules and neurofilaments. In certain mitochondria, the cristae were absent and the matrix appeared swollen. The smooth muscle cells and blood vessels were not affected.

Tissues treated with 0.75 g/dl cobalt chloride solution had degenerative changes involving the nerve cells and axons, but the smooth muscle cells and blood vessels were not affected. The nerve cell showed a marked increase of lysosomes and focal cytoplasmic vacuolization (Fig. 5). The nature and extent of these cytoplasmic changes varied to a moderate degree from one to another nerve cell. Neurosecretory granules were considerably decreased or absent. Axonal changes were common. In swollen axons, the axoplasm was disorganized. Microtubules, neurofilaments, neurosecretory granules, and mitochondria were partly or completely lost (Fig. 6). Focal cystic degeneration of the axoplasm was sometimes seen.

The 1.5 g/dl cobalt chloride solution produced severe degenerative changes in the nerve cells and axons of the myenteric and submucosal plexi. The nerve cells showed focal or diffuse cytoplasmic necrosis and condensation of nuclear chromatin (Fig. 7). Axonal changes consisted of severe edema and or focal necrosis. The smooth muscle

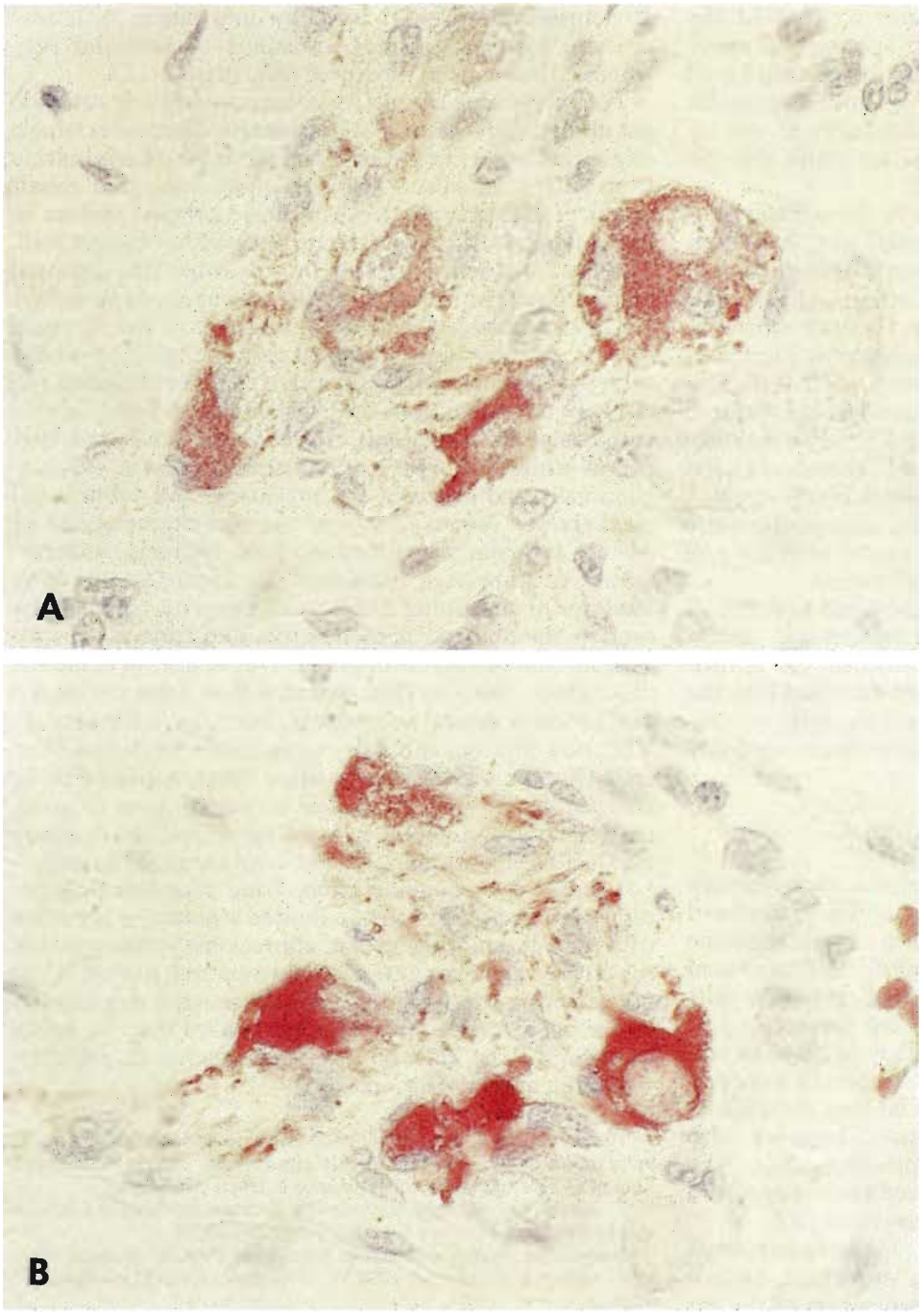

FIG. 9. *A*: control jejunum; VIP stain of myenteric plexus demonstrates immunoreactivity of the neurons and nerve fibers. ×2,400. *B*: jejunum treated with 0.25 g/dl $CoCl_2$ solution. VIP stain of myenteric plexus is more intense than in control specimen. ×2,400.

TABLE 2. *Results of immunohistochemical studies*

$CoCl_2$ Solutions, g/dl	VIP Immunoreactivity	Substance P Immunoreactivity	Met-enkephalin Immunoreactivity
0.25	↑↑↑	↑↑	0
0.75	↓↓↓	↓↓↓	↓↓↓↓
1.5	↓↓↓↓	↓↓↓↓	↓↓↓↓
3	↓↓↓↓	↓↓↓↓	↓↓↓↓

0, No change compared with control; ↑↑, 25–50% and ↑↑↑, 51–75% increase of immunoreactivity; ↓↓↓, 51–75% and ↓↓↓↓, 76–100% decrease of immunoreactivity.

cells and blood vessels were not damaged (Fig. 8).

Tissue treated with 3 g/dl cobalt chloride solution developed necrosis and subsequent fibrosis, affecting nerve cells as well as smooth muscle cells.

Immunohistochemistry. Immunoreactivity for substance P, VIP, and Met-enkephalin was observed in the myenteric and submucosal plexi of control tissues regardless of the fixation method, although 10% buffered Formalin fixation resulted in lower staining intensity compared with Bouin's solution. However, tissue fixed with Bouin's or Zamboni's solution yielded the same staining quality. We used Bouin's solution because it is widely accepted as a fixative for immunohistochemistry in surgical pathology (1). The staining quality of paraf-

fin-embedded specimens was superior to that of the frozen sections. The myenteric and submucosal nerve cells exhibited focal granular cytoplasmic staining for all three peptides; immunoreactivity was most intense for VIP, less for Met-enkephalin, and substance P was intermediate (Fig. 9*A*). Changes induced by cobalt chloride perfusion are summarized in Table 2.

Immunoreactivity of the nerve cells for substance P and VIP was more intense in tissues that had been perfused with 0.25 g/dl cobalt chloride (Fig. 9*B*) than in control specimens, but it was not enhanced for Met-enkephalin. The cytoplasmic staining for both substance P and VIP consisted of coarse granules with focal confluence in some cells. Tissues treated with 0.75 g/dl cobalt chloride solution showed considerably reduced immunoreactivity to substance P and VIP. The majority of the nerve cells of the myenteric and submucosal plexi were nonreactive; only a few cells showed faintly staining cytoplasmic granules. Stains for Met-enkephalin were negative. The nerve cells in tissues treated with 1.5 g/dl cobalt chloride were nonreactive to all stains.

The intestinal wall of specimens perfused with 3 g/dl cobalt chloride solution showed transmural active chronic inflammation and fibrosis. Mucosal ulcers usually were shallow, but sometimes they extended into the submucosa. Smooth muscle layers were partially or complete fibrotic. These specimens had no immunoreactivity to any of the peptides examined.

DISCUSSION

Attempts have been made in the past to selectively ablate enteric neurons. Hukuhara et al. (8, 9) perfused the vascular supply of a loop of canine small intestine for 4 h with unoxygenated Tyrode solution. This caused degenerative changes in the intramural ganglion cells and abolition of enteric reflexes. Okamoto et al. (12) added 0.002% mercuric chloride to Tyrode solution and used Hukuhara's technique to induce intrinsic denervation of a segment of canine colon. The long duration of ischemia induced by these techniques, however, also caused damage to the intestinal smooth muscle. The changes of intestinal motility observed could have been due to both neural and muscular alterations (10).

Sato et al. (14) and Sakata et al. (13) have suggested that serosal application of a cationic surfactant, benzalkonium chloride, to the colon and anorectum of the rat destroys the enteric neurons. Similarly, Fox et al. (5) used various concentrations of cationic, anionic, and nonionic surfactants to ablate the enteric neurons of the rat jejunum. These techniques, however, resulted in incomplete intrinsic denervation, since degenerative changes in the submucosal plexi were not observed.

Our study used an alternative approach to produce selective degeneration of intestinal intramural nerves. Close intra-arterial injection of cobalt chloride, which is taken up by the neurons and transported or diffused along cell processes, induces their degeneration (3). Cobalt ions are known to compete with calcium, which is essential for many neuronal functions (3). It has been shown that calcium ions are necessary for transport of neurosecretory substances centrifugally from the soma of neurons (4). Cobalt ions, by interfering with this process, may prevent the transport of essential substances, thus causing neuronal destruction (11).

The morphological methods employed in our study do not allow differentiation between nerve fibers of extrinsic origin that enter the gut wall and nerve fibers originating from enteric ganglia. Thus we cannot claim that cobalt produces damage only to the enteric nervous system to the exclusion of extrinsic nerve fibers within the gut wall. The first organelles affected by cobalt are the neuronal microtubules, which are thought to function as pathways for neurotransmitters. The significance of the increase of VIP and substance P secretory granules that we observed in specimens treated with 0.25 g/dl solution is not clear. In concentrations of 0.75–1.5 g/dl, cobalt chloride had a selective neurotoxic effect in that it affected only nerves while sparing intestinal smooth muscle. All ganglion cells and axons of the myenteric and submucosal plexi showed severe structural changes characterized by edema, vacuolar degeneration, focal necrosis, and formation of autophagic lysosomes. Structures known to be vital for maintaining functional integrity of neurons, such as the nucleus, mitochondria, and endoplasmic reticulum, showed severe degenerative changes or complete destruction. We also demonstrated that these nerves did not produce neurotransmitters, such as substance P, VIP, and Met-enkephaline, after cobalt perfusion. The smooth muscle cells, on the other hand, appeared to be structurally normal. At higher concentrations (3 g/dl), the selectivity of cobalt-induced intestinal nerve injury was lost as smooth muscle cells were damaged as well.

Thus we have demonstrated, using morphological parameters, that perfusion of a limited segment of intestine with cobalt chloride at an appreciable concentration selectively damages intestinal intramural nerves while not affecting the intestinal smooth muscle. Application of our observations in functional studies may be useful in defining the role of intestinal nerves in motility, secretion, and absorption.

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