formation of its densest and strongest form at the late stage do not depend much on the implant composition. The key factor is the time required for the formation of a healthy region of native bone replacing the implant volume.

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Morphological Analysis of Protease Treatment of Damaged Nerves

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Intravitam phase-contrast microscopy of individual isolated myelin fibers of rabbit tibial nerves shows that 40-min treatment with 0.2% pronase solution is harmless to intact myelin fibers. In injured nerves such treatment causes sharply expressed proteolysis and a rapid process of wallerian degeneration with ruptures of the axial cylinders and the formation of degeneration ovoids. The findings prove the feasibility and desirability of early primary protease treatment of nerve wounds.

Key Words: *degeneration of nerve fibers; protease treatment of nerves; regeneration*

The problem of hastening nerve regeneration is not a new one. Attempts at modifying the nerve suture, the use of natural and artificial tubular structures as growth guides, and the use of growth factors and other chemical and physical stimulants [3-7] have not yielded the expected results, evidently because of insufficient knowledge about the general biological processes developing during nerve damage. At present we are trying to develop a method for speeding up the regeneration of an injured nerve after protease treatment. Protease treatment of nerves is believed to have a number of advantages: it promotes faster resorption of degradation products, reduces the number of nerve membrane phagocytes capable of migrating to the site of injury, and exerts neurotropic, vasotropic, and antibacterial effects. The aim of the present study was to assess the possible speeding up of autolysis and resorption of degenerating nerve fibers and to analyze the conditions under which secondary protease injury to intact fibers is precluded.

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MATERIALS AND METHODS

Two series of experiments were carried out. In group 1 (8 rabbits) both tibial nerves were cut at

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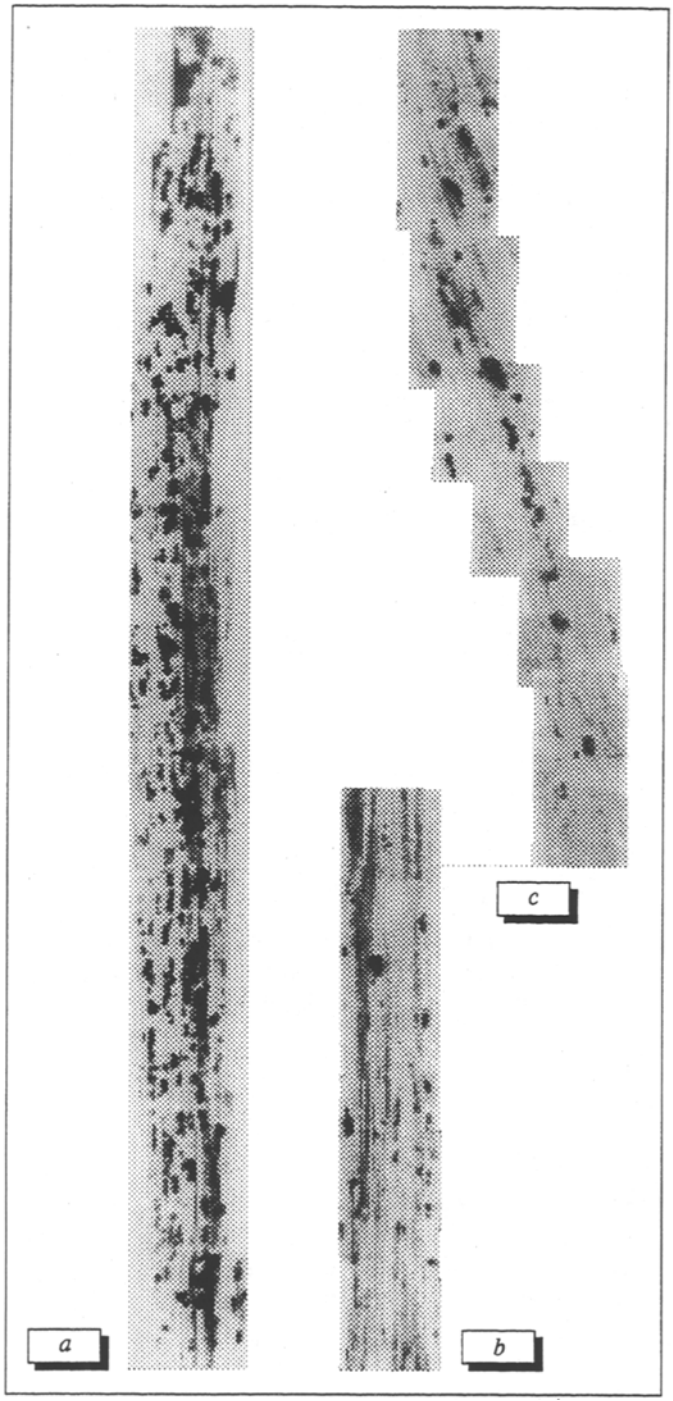

Fig. 1. Different concentrations of myelin degradation products and different extents of ascending degeneration zone in a control nerve bundle (*a*) and in pronase-treated nerve bundles (*b*, *c*). Treatment with 2.5% $K_2Cr_2O_7$ solution with 1% OsO_4. ×250.

the level of the upper third of the shin. The right nerve was treated for 40 min with 0.2% pronase solution (protease complex, Sigma, molecular weight 20,000 D) in Ringer's solution. For this purpose, the central stump of the nerve was sucked into a specially devised pipette with a reservoir. The left cut nerve was the control. After 30 days the central stump was fixed in 2.5% potassium bichromate solution with 1% osmium tetroxide in a 4:1 ratio. Quantitative assessment of the preparations was carried out using a television interactive scanning measurement system (IRIS-B) hookeded up to a microscope. The percent ratio of the total area of osmiophilic corpuscles to the area of the nerve bundle section was assessed in μ^2. The results were statistically processed after Student.

The second series of experiments studied the effects of proteolytic enzymes on isolated live intact and damaged myelin fibers of frog sciatic nerve (31 experiments). Phase-contrast microscopy with serial microphotographs was carried out (period of observation 3 h at 25°C). The fibers were placed in a microchamber with a flow system.

RESULTS

The first series of experiments revealed a much higher content of myelin degradation products at the site of dissection of a nerve which was not treated with pronase. This was true for both the concentration of osmiophilic corpuscles and the extent of the ascending degeneration zone (Fig. 1). Besides degeneration of fibers 30 days after nerve dissection, regeneration, myelinization of fibers, and repeat degeneration of fine myelinizing fibers at the site of regeneration were observed in control preparations. Far-advanced reactive changes were observed in many fibers, presenting as separation of the node myelin, stratification of Schmidt-Lantermann clefts, and varicose deformation of the axial cylinder. Stratification of myelin from the clefts and nodes developed along the fibers. The axial cylinder retained its continuity even in the case of almost total destruction of myelin, although it was greatly thinned. This resembled the picture of changes of myelin fibers in experimental allergic neuritis [1].

In this connection a possible development of local autoimmune processes in the stump of the dissected and regenerating nerve may be hypothesized.

After pronase treatment of the proximal stump of the dissected nerve, large areas with a sharply reduced (in comparison with the control) content of myelin degradation products were seen. This was true, primarily, for the terminal portion of the stump and for the surface sites of the nerve, that were evidently more readily accessible to the pronase during treatment. Some nerve bundles did not contain degradation products at all. It should be emphasized that the diameter of regenerating myelin fibers in such bundles was 1.5 to 2 times

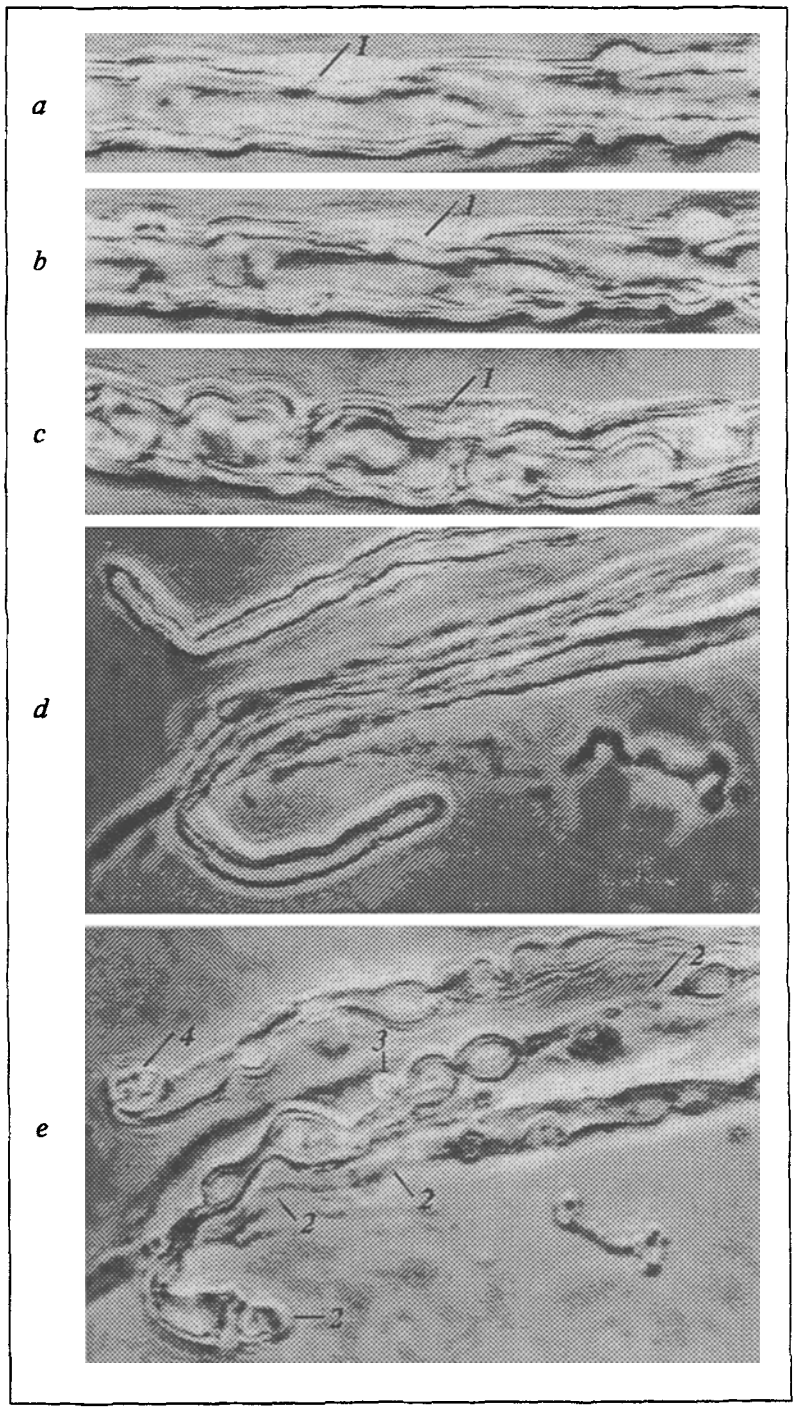

Fig. 2. Time course of structural changes in intact (*a*–*c*) and damaged (*d*, *e*) isolated living myelin nerve fibers of a frog after 40–min pronase treatment. *1*) neurolemmocyte perikaryon; *2*) ruptures of degenerating myelin nerve fibers; *3*) detached contrasting spherical myelin fragment; *4*) retraction of end portions of ruptured myelin fibers. Vital microscopy. Phase contrast. ×400.

greater than that of the fibers in the bundles filled with degradation products. These data indicate a more rapid course of regeneration and myelinization in enzyme-treated nerve bundles free of degradation products. The above-mentioned reactive changes were almost absent. Automated morphometric analysis of the material revealed a reliable difference between the levels of osmiophilic products of degeneration in the control and the experiment. For example, whereas in the control the total area of osmiophilic corpuscles expressed in percent to the area of the bundle section was 12.9±6.5, in the experiment it was only 3.5±2.0%.

In the second series of experiments control intact frog myelin nerve fibers placed in flowing Ringer-Locke solution did not change for a long time. When the Ringer-Locke solution was replaced by pronase solution, the intact nerve fibers underwent barely discernible and easily reversed reactive changes [1]. These changes slowly augmented with

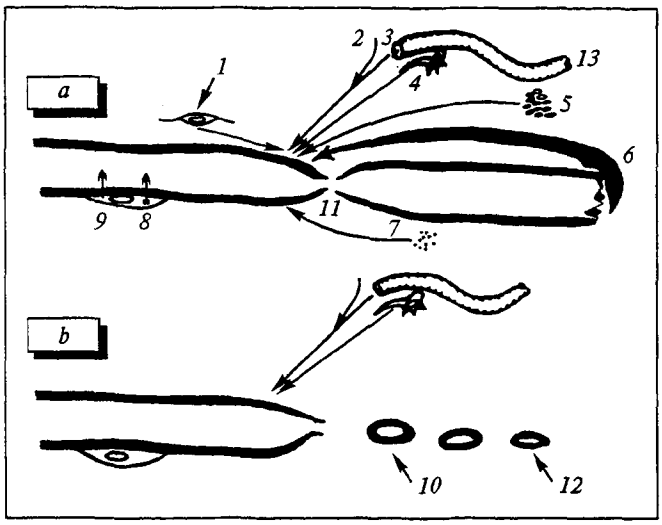

Fig. 3. Scheme of proteolysis components in a damaged nerve in the control (*a*) and after early primary treatment with proteases (*b*). *1*–*7*) extracellular proteolysis caused by proteases secreted by local fibrocytes of nerve membranes (*1*), intercellular fluid proteases activated in the wound (*2*), plasma proteases (*3*), blood cell proteases released during injury (*5*), enzymes secreted by the cells migrating from vessels (*4*), protease activation in the myelin and destroyed nerve cells (*6*), and bacterial factor (*7*); *8*, *9*) lysosomal and nonlysosomal intracellular proteolysis associated with altered neurolemmocytes and characteristic mainly of the peripheral stump; *10*) proteolysis caused by pronase treatment of the wound; *11*) Ranvier's node; *12*) degeneration ovoids; *13*) nerve microvessel.

time, but were reversible even after 3-hour exposure to pronase. An increase in the volume of neurolemmocyte perikaryon sometimes occurred after 40 min (Fig. 2, *a*-*c*), as well as a hardly discernible narrowing of the axial cylinder near the clefts, and a negligible increase of the node slit.

Typical signs of wallerian degeneration with destruction of myelin nerve fibers developed after 40-min pronase treatment of initially damaged myelin fibers (Fig. 2, *d*, *e*). Thinning and ruptures of the axial cylinder near Schmidt-Lantermann clefts were observed, as well as the formation of ovoids in the cylindroconical segment between the clefts. Complete demyelinization and destruction of Ranvier's nodes were observed. In some cases contrasting spherical fragments of myelin or proteolysis products became pinched off in the form of weakly contrasting spherical droplets in such damaged fibers.

Hence, intravitam observation of the effects of pronase on myelin nerve fibers helped reveal several important features. First, initially intact myelin fibers are resistant to proteases, whereas damaged fibers are easily destroyed after 40-min exposure to pronase at 25°C. Hence, a 40-min treatment with 0.2% pronase solution is harmless to intact myelin nerve fibers and may be used for primary treatment of a nerve wound. Second, the protease-effected hastening of structural processes resembling wallerian degeneration is worthy of note. The process, which commonly lasts for days or weeks, is shortened to several tens of minutes under the action of exogenous enzymes. Acceleration of processes is known to be a most important characteristic of proteolytic enzymes as catalysts of biological degradation processes [8].

On the other hand, this acceleration might be explained by the fact that under natural conditions the process of wallerian degeneration is also caused by endogenous proteases [2], whose concentration and, hence, activity are much lower than those of proteases used experimentally. During the usual type of nerve damage, lysosomal proteases of dead or injured cells and myelin, enzymes secreted by fibrocytes of nerve and blood cell membranes migrating from the vessels, proteases of intercellular fluid and blood plasma, and bacterial enzymes may be the factors increasing proteolytic activity in a wound (Fig. 3, *a*). After early primary protease treatment of a damaged nerve, the activity of proteolysis connected with dying cells and destroyed myelin, with blood cells in the wound, with membrane fibrocytes migrating into the wound, and with bacterial contamination falls off sharply (Fig. 3, *b*).

Hence, pronase treatment of nerves does not impair intact nerve fibers and at the same time, by speeding up the autolysis of nonviable fibers and the resorption of degradation products, it may facilitate the course of regeneration.

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