MATERIALS AND METHODS

Mesenteric preparations

28 albino rats of both sexes weighing 280 ± 9 g were used in our experiments. All rats had fasted 1 day before surgery. During this time, water was made available ad libitum. The animals were anesthetized with sodium pentobarbital (30 mg/kg body weight, i. m.), and a 4-cm long midline abdominal incision was made through the skin, underlying the fascia with a heat cautery. After intravenous heparin infusion (500 I.E./kg), a polyethylene cannule was inserted into the main postnodal intestinal lymph duct. Lymph flow velocity was determined by measuring the rate of meniscus movement in 50- to 200 μl calibrated micropipets arranged at the level of the heart,[12] and a sample of the lymph was taken.

At the end of the experiment, each rat's vascular system was washed out with a cooled (6°C) 154 mM NaCl solution and mesenteric lymph nodes were removed, placed in a cooled (6°C) phosphate buffered solution containing 150 mM NaCl, 5 mM Na_2HPO_4 (pH 7.4), and homogenized in a Polytron type homogenizer. The lymph nodes homogenates mixed with plasma at a ratio 1 : 1 and samples of lymph with or without white blood cells were used for proteolysis level assay.

Protein concentration and proteolytic activity analysis

The lymph samples were divided into two tubes, one of which was centrifuged at 800 g for 10 min for white blood cells sedimentation. In the samples of non-centrifugated lymph, the total amount of leukocytes was determined with a "Picoscale" cytometer (Medicor, Hungary), and in the samples of centrifuged lymph, the total protein concentration was measured with a biuret reaction.[13] All white blood cells in mesenteric lymph were considered as lymphocytes because it has been established that the lymph normally contains two morphological cell types: both lymphocytes and mononuclear phagocytic cells - although the latter are present in afferent lymph only.[14]

The determination of total proteolytic activity level (including activities of proteases and peptidases) was carried out according to the next developmental assay. As an index of the level of proteolytic activity we used the increase in the content of free amino acids and peptides having NH_2-groups in samples stored for 4 h at 37°C. As the substrate for the assay of the total proteolytic activity of lymph node homogenates, the blood plasma was used at a ratio 1 : 1. The substrate for the proteolytic activity determination of lymph samples was their own lymphatic proteins. At the end of the incubation period the reaction was terminated by adding a double volume of ethyl alcohol, and 10 min later the mixture was centrifuged at 800 g 10 min. The level of the total proteolytic activity of the lymph node homogenates with plasma (1 : 1) and lymph without an incubation at 37°C for 4 h served as the control.

Following this, 0.5 ml of a mixture containing 0.2 M of ninhydrin (Lachema, Brno) along with 0.1 M of sodium metabisulfite (Sigma) in 0.2 M acetate buffer having pH 5.5 were added to 0.2 ml of a supernatant, and then - 1 ml of ethyl alcohol solution with 0.3 mg of stannous chloride (Serva) was also added. The obtained mixture was incubated for 20 min at 70°C. The intensity of the proteolysis was determined on the basis of the solution stained on the spectrophotometer Specol-211 ("Carl Zeiss", Gemany) at 570 nm.

Glycine (Reanal, Hungary) solutions of 30, 40, 50, 100, 200, 300 and 400 μg/ml were used for standards in this analysis. The data obtained was expressed in μg of glycine (Gly) per 1 g of a tissue (or 1 ml of lymph) per 1 hour of incubation. The minimal detectable level of proteolytic activity using this test is typically < 40 μg Gly/ml $\times$ 1 hour of incubation with SE typically < 0.14. A typical standard curve using this test can be seen in Fig. 1.

In the first series of experiments ($n = 12$), we studied lymph flow velocity and the level of total proteolytic activity of mesenteric lymph and lymph node homogenates in the control rats. On the basis of a difference between total proteolytic activity values of both intact and centrifuged lymph (with and without lymphocytes, respectively) the total activity of proteolytic enzymes incorporated into the lymphocyte membranes was estimated and their activity was calculated by 10^6 cells.

In order to clarify whether lymphatics affect the level of lymph proteolytic activity, in the second series of experiments ($n = 11$) the sections of intestinal lymph ducts, weighing an average of 2.4 ± 0.1 mg, were added to some lymph samples. In the special third series of experiments ($n = 9$), we studied the degree of participation of the lymphatics' own enzymes in a lymph proteolysis, adding the sections of lymphatic vessels (either intact or heated at 90°C for 20 min) into the samples of centrifuged lymph.

To verify the suggestion that denaturated proteins are catabolized in the first place, in the fourth series ($n = 9$) we compared the total proteolytic activity of native lymph and a mixture (1 : 1) of native lymph

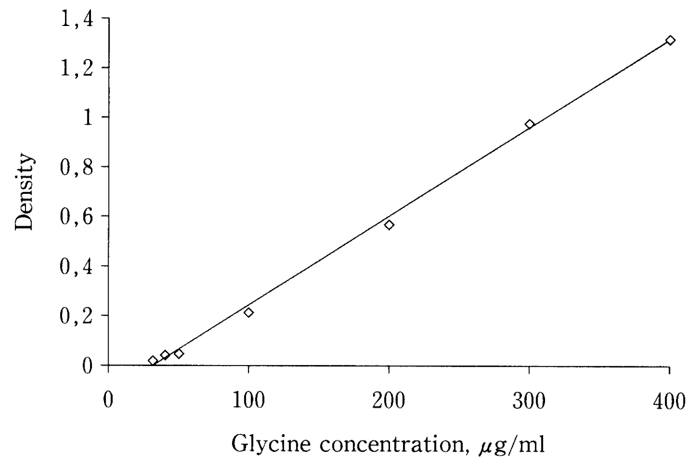

Fig. 1. A typical standard curve for total proteolytic activity assay used in this study. Glycine was used for standards.

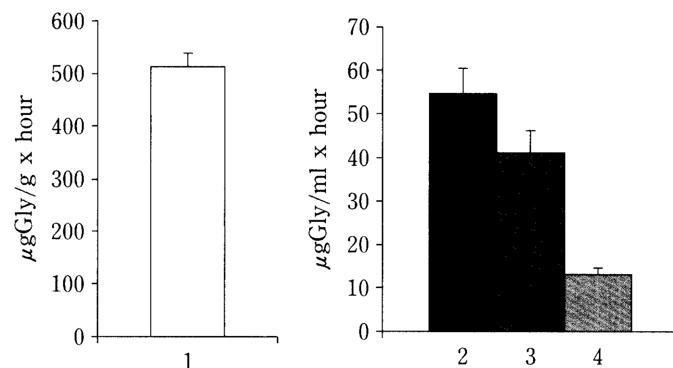

Fig. 2. The level of total proteolytic activity of rat mesenteric lymph node homogenates (1), intestinal lymph with (2) or without lymphocytes (3), and of lymphocytes themselves (4).

and lymph heated at 55-60°C for 45 min, as well as the total proteolytic activity of lymph node homogenates mixed with native plasma or that heated at 55-60°C for 45 min.

Variables are expressed as means±SEM. The significance of the differences was assessed by an unpaired Student's t-test.

RESULTS

Experiments of the first series have shown that the average postnodal intestinal lymph flow velocity in rats was 3.59 ± 0.28 μl/min×100 g of body weight; in addition, the lymph protein and lymphocytes contents were 2.30 ± 0.15 g/dl and $(3.77\pm0.26)\times10^6$/ml, respectively. The level of total proteolytic activity of the homogenates of mesenteric lymph nodes was equal on average to 512.12 ± 27.24 μgGly/g×hour (Fig. 2). The total activity of proteolytic enzymes in intestinal lymph with lymphocytes averaged around 54.22 ± 4.87 μgGly/ml×hour. The removal from the intestinal lymph of lymphocytes by centrifugation resulted in a statistically significant decrease in the value of total proteolytic activity, which became equal to 40.98 ± 3.42 μgGly/ml×hour. The calculations have shown that the level of total activity of proteolytic enzymes of lymphocytes equals an average of 13.24 ± 1.19

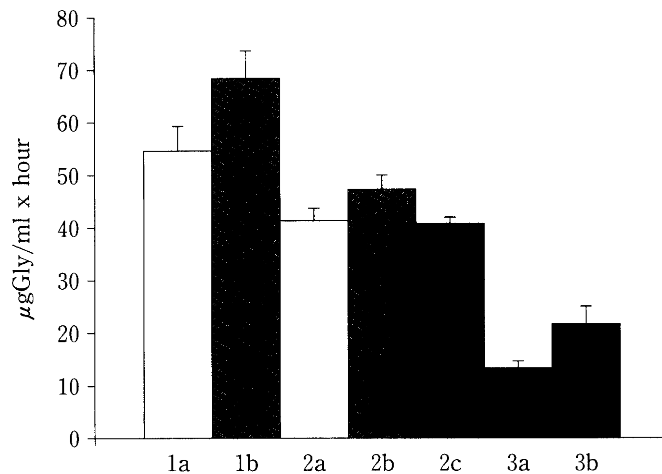

Fig. 3. The level of the total proteolytic activity of intestinal lymph with lymphocytes (1), without lymphocytes (2), and of lymphocytes themselves (3) without a lymphatic vessel section added (a) and with intact (b) and heated (c) lymphatic vessel sections added.

μgGly/ml×hour (Fig. 2), and that by recalculating on the lymph total lymphocyte amount gives us a value of 3.51±0.37 μgGly/10^6×hour.

In the second series of experiments, when we incubated the samples of mesenteric lymph with the sections of intestinal lymph vessels, we discovered that the lymphatics themselves change the intensity of the lymph proteolysis. In the initial lymph samples without lymphocytes, the level of total proteolytic activity was 40.98±3.42 μgGly/ml×hour. When a section of intact lymphatic vessel was aded, the activity was increased (though not significantly) up to 46.92±3.80 μgGly/ml×hour (Fig. 3). The addition of a section of lymphatic vessel in lymph samples with lymphocytes, having a reference level of total proteolytic activity 54.22±4.67 μgGly/ml×hour, led to an insignificant increase in the proteolytic enzymes activity in such a sample of up to 68.16±5.22 μgGly/ml×hour. Thus, the placing of lymphatic sections in the samples of uncentrifuged lymph resulted in an insignificant increase in the total proteolytic activity of its lymphocytes from 13.24±1.19 up to 21.24±4.44 μgGly/ml×hour (Fig. 3).

For verification of our research regarding the contribution of the proteases of lymphatic vessels themselves to the common proteolytic activity of a lymph incubated with a lymphatic vessel, special experiments of the third series were conducted. The elimination of the activity of the lymphatics' own proteases by heating resulted in the level of total proteolytic activity of a lymph without lymphocytes insignificantly reducing by nearly 14.5% (from 46.92±3.80 μgGly/ml×hour in the control test with an intact lymphatic vessel, up to 40.12±1.34 μgGly/ml×hour - with heated lymphatics; Fig. 3).

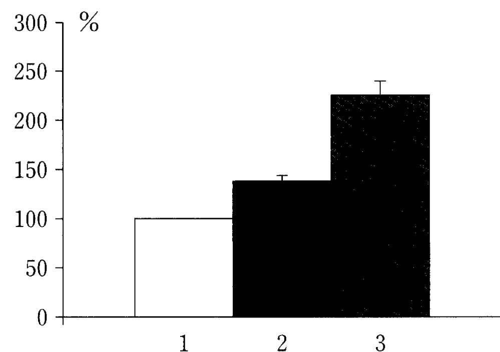

Fig. 4. The effect of lymph and plasma sample heating on the level of the total proteolytic activity of rat intestinal lymph (2) and mesenteric lymph nodes (3) when compared with control values (1) taken as 100%.

In the fourth series of experiments we investigated the effect of lymph and plasma protein denaturation on the total proteolytic activity level of lymph without lymphocytes and lymph node homogenates. The data obtained showed that the total proteolytic activity of native lymph was 39.88±1.05 μgGly/ml×hour.

The mixing (1 : 1) of native and heated lymph led to the level of proteolytic activity greatly increasing up to 54.70 ± 1.92 μgGly/ml×hour ($p < 0.001$: Fig. 4). The incubation of lymph node homogenates with heated plasma increased the level of proteolytic activity to 1250.02 ± 44.58 μgGly/g×hour from 556.50 ± 33.13 μgGly/g×hour ($p < 0.001$) in the case when native plasma was added to lymph node homogenates (Fig. 4).

DISCUSSION

The intestinal collecting vessel lymph is a mixture of fluid derived from all portions of the lymphatic drainage proximal to the collection site. Thus, in our case, the intestinal lymph protein content was determined by four processes: 1) the transcapillary fluid and macromolecule exchange in the intestine; 2) water and nutrient absorption from the gut lumen during digestion; 3) lymph concentrating during its transportation via lymphatic vessels, and[4] the effect of mesenteric lymph nodes.

The values we collected concerning lymph flow velocity, lymph protein, and lymphocyte content under resting, and the physiological condition obtained in our experiments, are similar to that of other investigators.[8,15]

Our data showed that the intestinal lymph and mesenteric lymph nodes have properties to catabolize transported proteins. The low velocity of lymph flow can be considered an important factor of proteolysis because it leads to an increase in protein and proteolytic enzyme interaction time.

The total proteolytical activity of lymph includes the activity of enzymes which were transported by lymph, incorporated into the lymphocyte membranes, and embedded into the lymphatic vessel wall. Calculations show that the level of proteolytic activity of the enzymes in lymph is 3.5 times as much as the activity of enzymes on the lymphocyte.

The calculations demonstrate that when lymphatic sections are added into the incubation medium, the proteolytic activity of noncentrifugated lymph is increased by 25.7%, together with those of the lymph itself, which is only 14.1%, while the proteolytic activity of lymphocytes is increased by 66.5%. These data indicate that an increase in the total proteolytic activity of the lymph samples when the section of the lymphatic vessel is added to them is conditioned not only by proteases embedded into the lymphatic wall, but also by a definite modulate effect of the lymphatic wall-produced factors upon the activity of the proteases of a lymph, and in particular those of lymphocytes.

It is known that the purification of an organism from denatured proteins is implemented in many organs and systems with different levels of activity of proteolytic enzymes and their inhibitors.[15-17] It has been shown that proteins, conformationally changed by some agents (for example by oxidation), are more susceptible to proteolysis and consequently appear to be a signal for protein degradation.[18] In our experiments we showed that the presence of denatured proteins in the incubation medium leads to an elevation of the total proteolytic activity in lymph by nearly 37.16%, and in the lymph node by 124.62% ($p < 0.001$ in both cases). These data are proof that the lymphatic system participates in the catabolism of proteins, especially of denatured ones, and this process takes place most actively in the lymph nodes. The lymphocytes which are transported by lymph also play a definite role in the protein catabolism. Thus, the intensity of a proteolysis is influenced also by the endothelium of the lymphatic vessel wall. However, some investigators have shown that lymph samples inactivate creatine kinase in vitro due to the inactivation of thiol groups of proteins, but not due to enzymatically mediated proteolysis.[19]

Our data suggest that lymph nodes can change the lymph protein concentration not only by the absorption of protein free fluid into the lymph from blood capillaries of lymph node, but also by the removal of denatured proteins from the lymph by their proteolysis. Moreover, when calculating the protein concentration intensity during the process of the lymph passing through regional lymphatics, it is necessary to take into account that some proteins, especially denatured ones, have been catabolized by enzymes of lymph, lymphocytes, and lymphatic endothelium.

In conclusion, the data obtained concerning the values of the proteolytic activity of lymph nodes, lymph and lymphocytes, transported by lymph, show that all parts of the lymphatic system of the intestine play a vital role in the formation of the protein content of the lymph by means of enzymatically denatured protein catabolism. Moreover, the wall of lymphatics itself (endothelium) not only can participate in the proteolysis, but also modulates this process, which is carried out by other parts of the lymphatic system.

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