No differences were found in staining properties for AMPS by colloidal iron among the different experimental groups. Colloidal-iron-stained areas were bluish, whereas the amyloid areas stained by methyl violet were red. As indicated in table 1, the incidence of amyloid in the spleen in control group 1 was 73% and that in the liver was 60%. In the animals given papain and CFA (groups 2 and 3) the incidence of amyloid in the spleen ranged from 23 to 50%, and that in the liver from 16 to 36%. In the animals treated with papain alone, there were no amyloid deposits and no other pathology (group 7).

Table 2 summarizes the incidence of amyloid in spleen and liver in a control group (group 5) and in the 2 papain-treated groups (groups 4 and 6) which received papain over an 8-day period. The administration of papain during the period of CFA administration decreased the incidence of splenic and hepatic amyloidosis. While the incidence of splenic amyloidosis was 73% and that of hepatic amyloidosis was 46% in control group 5, the incidence of amyloidosis in the papain-treated animals was 30 and 26%, respectively (table 2).

A high percentage, between 30 and 60%, of the papain-treated animals died. None of the mice in the CFA-treated groups (groups 1 and 5) died. The incidence of hepatic and splenic amyloidosis was lower in the papain-treated than in the control animals, but this difference was not statistically significant (by the χ^2 test).

Discussion. It has previously been shown that amyloid-laden organs contain AMPS in higher concentrations than normal organs[1,2]. There are many contradictions regarding the presence and/or role of AMPS in amyloid deposits. Glenner et al. related the presence of AMPS in the amyloid deposits to the AMPS which are normally found in the ground substance in which the amyloid is deposited[5]. On the other hand, the presence of AMPS in amyloid deposits may indicate that the AMPS play a role in the formation of amyloid fibres. It is possible that the formation of amyloid fibres is analogous to the formation of collagen fibres[4]. Pras et al. discussed the role of AMPS in the regulation of amyloid fibre formation and rigidity[9]. The fact that the decrease in AMPS synthesis in diabetes parallels a decrease in the incidence of amyloidosis also suggests that AMPS may play a role in amyloid fibre formation[6].

The AMPS consist of a linear carbohydrate chain, carrying negatively charged carboxy and sulfate groups, covalently linked to a protein core[10]. Papain is a potent proteolytic enzyme which can liberate the polysaccharides from the protein core. In papain-treated mice, the AMPS normally found in the liver cell membrane were no longer present[11]. We therefore used papain to digest the AMPS and to study the influence of the lysed polysaccharides on experimental amyloidosis. No statistically significant differences were found in the incidence of amyloidosis among the papain-treated and control groups. This can be explained by the fact that, although papain released the polysaccharides from the protein polysaccharide complex, the released polysaccharides were immediately bound to the amyloid fibres by electrostatic forces. As has been shown by Pras et al., amyloid fibre subunits are highly charged molecules containing both positively and negatively charged groups[12]. The AMPS with their negatively charged groups are linked by electrostatic forces to the amyloid fibres, and in this way stabilize and confer rigidity on the amyloid fibres, but do not interfere with amyloidogenesis. These findings support the opinion of researchers that AMPS present in the amyloid originate in the ground substance and become bound to the amyloid fibres.

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Neutral proteases in the guinea-pig lymphocytes[1]

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Summary. Partial purification of neutral proteolytic enzymes in guinea-pig lymphocytes yielded 2 enzymes. Both enzymes were heat-labile and inhibited by thiol reagents. The molecular weights were more than 200,000 and 150,000–200,000, and optimal pH around 9 and 8, respectively.

The whole question of mediators of delayed hypersensitivity reaction (DHR) and the delineation of a molecular basis for cellular immune responses is an active area of investigation. While lymphokines are thought to participate in the expression of DHR[2,3], the exact role of the lymphokines in the pathogenesis of DHR is still unclear.

The present communication describes attempts to isolate, partially purify and characterize the neutral proteases of lymphocytes (NPLs), and discuss the role of NPLs in inflammatory changes.

Materials and methods. Hartley guinea-pigs, 300–500 g, were sensitized by injecting into 4 footpads 10 µg of bovine γ-globulin (Armour, Kankakee, USA) emulsified in complete Freund's adjuvant. 9 days later, lymph node cells were teased out in Hanks solution from the regional lymph nodes of the animals. More than 90% of the cells were lymphocytes by Giemsa stain and viability (85–90%) was determined by trypan blue dye exclusion. The cells were homogenized in 0.34 M sucrose and extracted over night with 67 mM phosphate buffer, pH 7.4. Proteolytic activity

was measured utilizing ^{3}H-acetylated hemoglobin as a substrate[4].

Partial purification was performed by column chromatography using DEAE-cellulose (DE-52, Whatman, Kent, England), and Sephadex G-100 and G-200 (Pharmacia, Uppsala, Sweden), in this order.

Results and discussion. 2 pH optima of the proteolytic activities were obtained at acidity and neutrality in the lymphocyte extract (figure 1). It was of interest to note that the proteolytic activity in extracts of lymph node cells from sensitized animals was twice as high as that of extracts from normal animals at both pH optima. The DEAE-cellulose chromatography of the extract with a linear gradient of 0–0.5 M NaCl in 20 mM phosphate buffer, pH 7.4, gave 5 protein peaks. Major NPL activity was recovered in fraction III (ionic strength corresponding to 100 mM NaCl in 20 mM phosphate buffer) with acid protease activity. The pooled fraction III was concentrated and was subjected to Sephadex G-100 filtration. NPL activities eluted in the void volume and were well separated from acid protease activities. The NPL fractions of Sephadex G-100 were pooled, concentrated, and subjected to Sephadex G-200 filtration (figure 2). The NPL activities eluted as biphasic peak; one eluted at the void volume (NPL-I) and the other retarded (NPL-II). The mol. wt was thus estimated to be more than 200,000 (NPL-I) and between 150,000 and 200,000 (NPL-II).

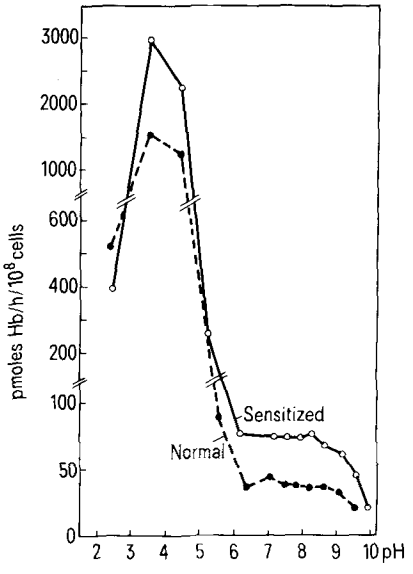

Fig. 1. pH curve of the proteolytic activities in the lymphocyte extracts of sensitized and normal guinea-pigs. The proteolytic activities at various pH were assayed in mixtures containing 100 µl of ^{3}H-hemoglobin (HB), 100 µl buffer, and 200 µl sample. After 60 min incubation at 37 °C in a shaker, 200 µl of cold 2.5% HB were added, followed by 100 µl of 50% trichloroacetic acid. The radioactivity of 200 µl acid soluble material was determined by a Packard Tri-Carb scintillation spectrometer (Model 3385, Packard, New York, USA). Appropriate blanks consisting of 0.15 M NaCl or heated enzyme preparation (100 °C for 10 min) were incubated simultaneously. All assays were conducted in duplicate, and activity is expressed as pmoles HB degraded per h per 10^8 cells.

The NPL-I had optimal activity around pH 9 and NPL-II around pH 8. As demonstrated in the table, both enzymes were heat-labile. Thiol specific reagents, p-chloromercuribenzoic acid and p-chloromercuriphenylsulfonic acid, were inhibitory to both enzymes. Trasylol also suppressed NPL-I activity. These observations suggest both NPL-I and NPL-II fractions contain mainly thiol protease.

As preliminary, intradermal injection of 0.2 ml aliquot of NPL-I or NPL-II fraction into normal guinea-pigs induced a mixed mononuclear-polymorphonuclear infiltrate in the deep dermis with a peak around 3 h after injection, suggesting their participation in cellular events in the

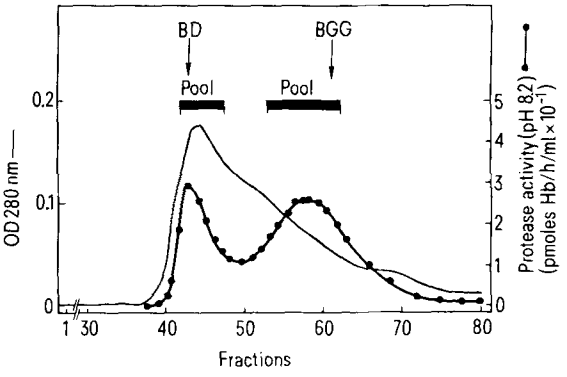

Fig. 2. Sephadex G-200 chromatography of NPLs. Extract was chromatographed on DEAE-cellulose and most active fractions were pooled, concentrated, and eluted on Sephadex G-100. Neutral protease from Sephadex G-100 was concentrated by ultrafiltration and eluted in Sephadex G-200 equilibrated with 20 mM phosphate-buffered 0.15 M NaCl. Fractions (2 ml) were collected and assayed for absorbance at 280 nm (——) and pH 8.2 protease activities (●——●). BD, blue dextran (mol. wt 2,000,000, Pharmacia); bovine γ-globulin (mol. wt 150,000, Sigma, St. Louis, USA).

Properties of neutral proteases of sensitized guinea-pig lymphocytes. The Sephadex G-200 eluate of the enzymes was incubated with the indicated compounds at the final concentration designated for 20 min at room temperature (20 °C) before the substrate was added. Data are given as relative activity in duplicate assays.

Treatment of enzyme	Final concentration (mM)	Relative activity (%) NPL-I	NPL-II
None		100	100
Diisopropylfluorophosphate	1	72	116
	10	56	98
Soybean trypsin inhibitor	10^{-2}	100	108
	10^{-1}	65	91
p-Chloromercuribenzoic acid	1	40	0
p-Chloromercuriphenylsulfonic acid	1	29	5
Disodium ethylenediaminetetraacetate	1	95	102
	10	85	67
Trasylol	1,600 (units/ml)	20	75
Heparin	5 (units/ml)	88	107
	50 (units/ml)	68	109
Heating, 56 °C for 30 min		23	13
Heating, 100 °C for 10 min		0	1

inflammation. Whether the inflammatory activity of NPLs might be due to production of a macrophage-chemotactic factor which we have recently isolated and purified from the delayed hypersensitivity skin sites in guinea-pigs[5], needs further investigation.

The presence of proteolytic enzymes acting in acid media in lymphocytes has been described occasionally by several authors[6-12]. However, scant attention has been paid to the neutral protease in the lymphocytes[7,8,12,13]. We have previously shown that rabbit lymph node cells had a neutral protease and inflammatory activity to the normal rabbit skin[14,15]. Recently, Fräki et al.[16] and Grazel et al.[17] suggest that rat lymph node lymphocytes[16] and human peripheral blood lymphocytes[17] contain a number of proteases. However, no isolation or purification has been performed, and it is difficult to compare these enzymes to the present NPLs.

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Effects of the antiprotease Trasylol® on peripheral blood leucocytes*

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Summary. Binding of the antiprotease Trasylol® to human peripheral blood lymphocytes and polymorphonuclear leucocytes (PMNs) was demonstrated at the ultrastructural level using an indirect immunoperoxidase technique. This also revealed endocytosis of membrane bound Trasylol by PMNs. Trasylol inhibited PHA- and ConA-induced lymphocyte stimulation, and was cytotoxic to unstimulated cells.

Trasylol® (aprotinin) is a low-mol-wt polypeptide obtained from bovine lung. It is a broad-spectrum antiprotease which inhibits the enzymes trypsin, chymotrypsin and plasma kallikrein[1]. Trasylol binds to sialyl residues and has been used to demonstrate their presence on the surfaces of a variety of animal cells[2] including human lymphocytes[3].

In this study we have sought to confirm at the ultrastructural level, the interaction of Trasylol with human blood leucocytes. In addition, we hoped to use Trasylol to test the hypothesis that proteolytic enzyme activity may be involved in lymphocyte stimulation[4-7] but as we show in this paper Trasylol exhibited a cytotoxic effect on cultured leucocytes.

Materials and methods. Trasylol® was supplied as a solution (1.5 mg/ml; Bayer Pharmaceuticals Ltd, Haywards Heath, Sussex, England) and contained 10,000 kallikrein inactivator units per ml. Antiserum was raised by i.m. injection of a rabbit with 1 mg freeze-dried Trasylol emulsified with complete Freund's adjuvant (Difco). 1 month later the animal was boosted by i.v. (1 ml) and i.m. (2 ml) injection of Trasylol solution. After 8 days the animal was bled and on testing the serum against Trasylol using a double immunodiffusion technique a single strong precipitin line was obtained.

Human leucocytes were obtained from heparinized venous blood by density gradient centrifugation[8], then washed and suspended in Eagle's MEM (Wellcome Reagents Ltd). Binding of Trasylol was investigated by adding 0.5 ml of a 1:5 dilution of Trasylol to 1 ml leucocyte suspension (4×10^6 cells/ml). The cells were incubated for 2 h at 37 °C, then washed in phosphate buffered saline prior to fixation in 2% glutaraldehyde. The fixed cells were then incubated first in the presence of rabbit anti-Trasylol antibody and then, following several rinses with phosphate buffer, with peroxidase conjugated goat anti-rabbit antibody (Dakopatts A/S, Denmark). The sites of cell-bound peroxidase conjugated antibody were revealed by the cytochemical procedure of Graham and Karnovsky[9] and the cells were then post fixed for 1 h at 4 °C in 1% w/v osmium tetroxide followed by dehydration in graded ethanols and embedding in TAAB epoxy resin. Ultrathin sections were stained in uranyl acetate[10] and lead citrate[11] prior to examination, in an AEI EM6B transmission electron microscope.

To investigate its effects on viability and mitogen responsiveness Trasylol was added to microcultures (2×10^5 cells in Eagle's MEM supplemented with 10% heat-inactivated human AB serum) at the start of the culture period and immediately before addition of either phytohaemagglutinin (PHA-P, Wellcome) or concanavalin A (grade IV, Sigma, London). For estimation of DNA synthesis (methyl-^{3}H)-thymidine (0.2 µCi; specific radioactivity 5 Ci/mmol was added to each culture at 48 h and the cells harvested, at 72 h, onto glass fibre discs (Gelman type A) using a multiple harvester (Skatron AS, Norway).

Results. Using the indirect immunoperoxidase staining procedure our ultrastructural studies revealed an electron dense precipitate on the surfaces of peripheral blood leucocytes which had been incubated with Trasylol but which was absent from the surfaces of 'control' cells which had not been incubated with Trasylol (figures 1-4). In the case of lymphocytes the precipitate was localized entirely on the