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

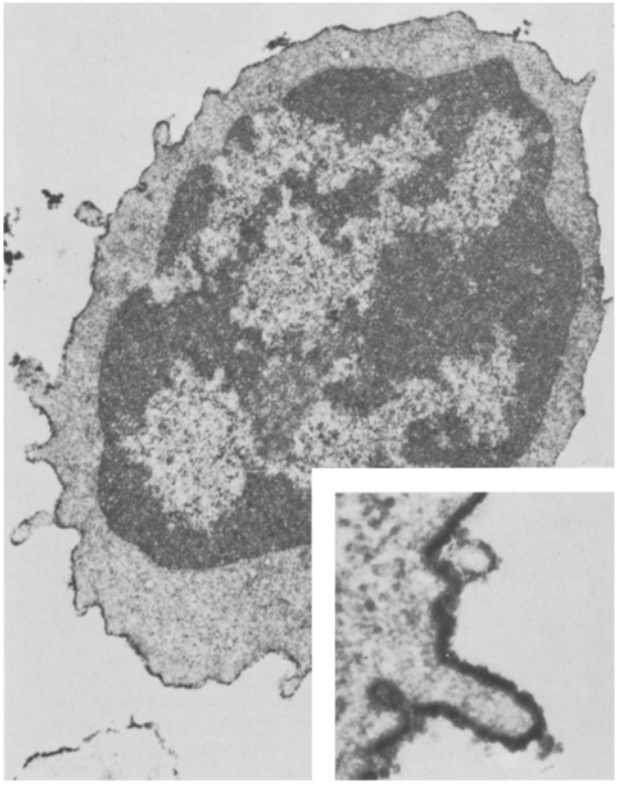

Fig. 1. A lymphocyte showing binding of Trasylol to the plasma membrane as revealed by the electron dense peroxidase reaction product. × 10,000. Inset: Detail of plasma membrane, showing binding of the immunoperoxidase product to the outer osmiophilic leaflet of the plasma membrane. × 40,000.

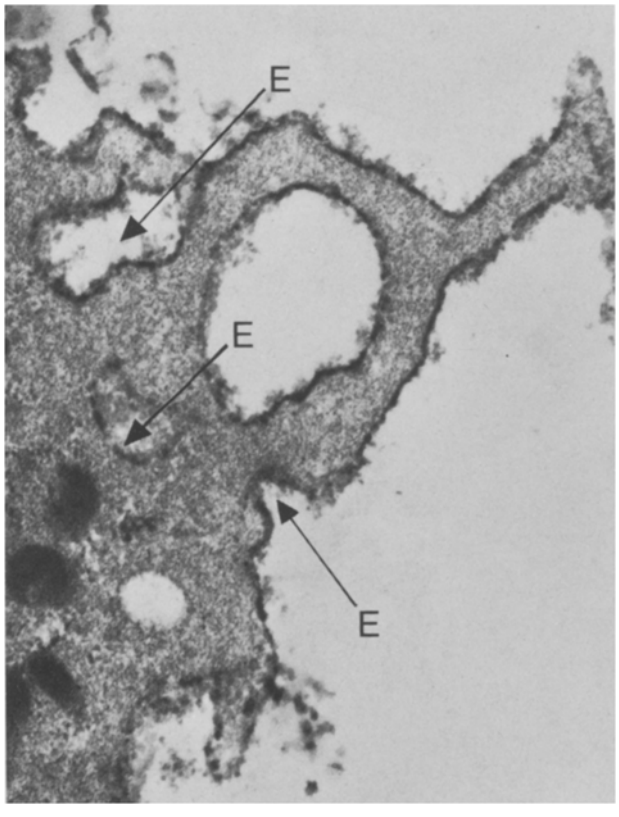

Fig. 3. Endocytosis (E) of membrane bound Trasylol by a polymorphonuclear leucocyte. × 30,000.

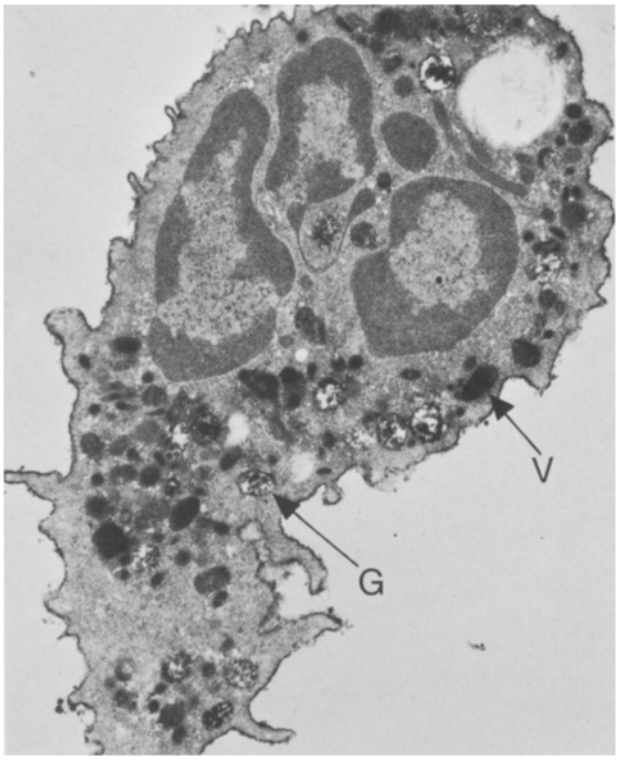

Fig. 2. A polymorphonuclear leucocyte. Electron dense deposits are seen both on the plasma membrane and in cytoplasmic vesicles (V) and granules (G). × 10,000.

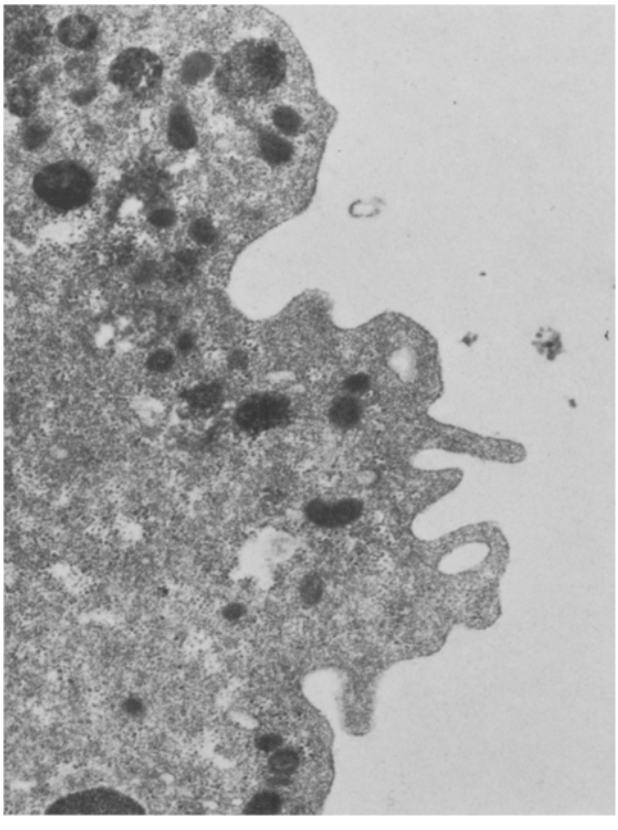

Fig. 4. A polymorphonuclear leucocyte, treated by the indirect immunoperoxidase procedure but without prior exposure to Trasylol. Note absence of staining of plasma membrane. Electron dense cytoplasmic granules represent lysosomes containing endogenous myeloperoxidase. × 20,000.

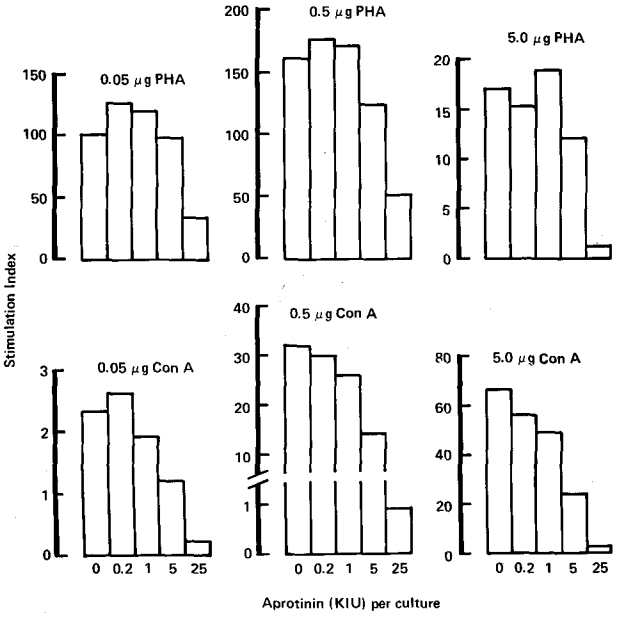

Fig. 5. Phytomitogen transformation of human peripheral blood lymphocytes cultured in the presence of various doses of Trasylol. Results were obtained from replicate cultures of 5 healthy adults. Stimulation index represents mean cpm from treated cells divided by mean cpm from untreated cultures.

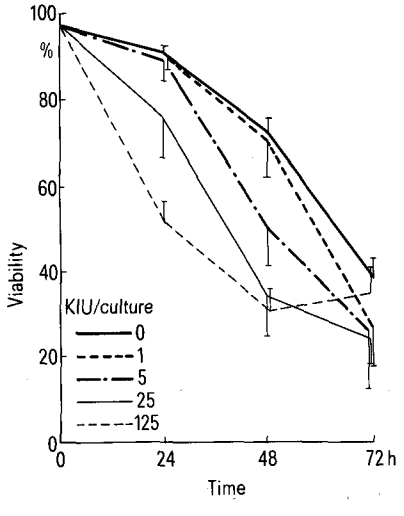

Fig. 6. Viability assessed by trypan blue dye exclusion of peripheral blood leucocytes cultured in the presence of Trasylol for various periods. Results are means of 6 cultures±S.D.

plasma membrane and little or no intracellular staining was observed. In contrast, electron dense deposits were observed both on the plasma membrane and in cytoplasmic vesicles and granules within polymorphonuclear leucocytes (PMNs). The densely stained cytoplasmic granules, which were also observed within 'control' PMNs, correspond to lysosomes which contain endogenous myeloperoxidase[12] whereas the densely stained vesicles represent membrane bound endosomes and canaliculi containing endocytosed Trasylol.

Trasylol caused a dose dependent inhibition of the mitogenic response to both PHA and Con A, marked reduction in stimulation index being found with 25 KIU Trasylol at each dose of mitogen (figure 5). Examination of the viability of cells cultured in the presence of Trasylol showed however that this effect could be explained by the dose dependent cytotoxicity of the drug (figure 6). No cell agglutination was observed in any of these cultures.

Discussion. Our ultrastructural studies have shown that Trasylol is bound by peripheral blood leucocytes and is endocytosed by PMNs. It is likely therefore that lysosomal neutral proteases are inactivated within secondary lysosomes subsequent to fusion of primary lysosomes with the Trasylol-containing endosomes; in addition any neutral proteases released extracellularly from the PMNs and which potentiate lymphocyte stimulation[12] will be inactivated by the Trasylol within the ambient culture medium. Further, the antiprotease activity of Trasylol could also be exerted against proteases present at the lymphocyte plasma membrane[13].

The dose-dependent cytotoxic effect of Trasylol evidenced by the dye exclusion data may be attributable to one or both of the following events. First, Trasylol may be cytotoxic for mononuclear phagocytes in vitro, with the result that they fail to elaborate the necessary 'growth supporting factors'[14,15] for continued survival of lymphocytes. Alternatively, Trasylol may be cytotoxic for cultured blood leucocytes due to inhibition within these cells of a protease dependent metabolic pathway. Inhibition of phytomitogen responsiveness by Trasylol may be accounted for by cytotoxicity and/or inhibition of leucocyte neutral proteases which are themselves mitogenic for lymphocytes[6].

Our results apparently conflict with those of Hirschhorn et al.[4] who observed no inhibitory effect of Trasylol on PHA transformation of human lymphocytes. The latter authors attributed the lack of effect of Trasylol to an alleged failure of the antiprotease to enter the cell. However, in the presence of phytomitogens the uptake of Trasylol by lymphocytes may be considerable since mitogen transformed lymphocytes display enhanced endocytotic activity[16]. In such instances the endocytosed Trasylol would inactivate neutral proteases within the lysosomes present within mitogen transformed lymphocytes[16,17] subsequent to the fusion of lysosomes with Trasylol-laden endosomes.

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