

Alpha-1-antitrypsin is present in the specific granules of human eosinophilic granulocytes

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Summary

Background Eosinophils may be found at sites of inflammation, for example in asthma, allergy and helminthic infestation, but their role in human inflammatory disease is unclear.

Objective In the present study, we investigated the presence of alpha-1-antitrypsin (AAT), a serine proteinase inhibitor, in human eosinophils.

Methods and results When lysates of highly purified eosinophils were subjected to Western blotting, with a chemiluminescent substrate, immunoreactive bands were seen. An ELISA was developed to measure the AAT content, which was found to be about 100 ng/ 5×10^6 eosinophils, about 50 ng/ 5×10^6 neutrophils, and about 25 ng/ 5×10^6 monocytes. Immunoelectron microscopy showed localization of AAT to the specific granules of eosinophils. During prolonged incubation of eosinophils, no significant increase in the total amount of AAT could be detected by ELISA. However, there was an increased level of AAT in the medium, in parallel with a decrease in the intracellular AAT content, suggesting release of preformed AAT. Apparent complex formation between iodinated elastase and AAT in eosinophil lysates provided evidence that the AAT is functionally active.

Conclusion On the basis of these findings it is suggested that by releasing AAT, eosinophils may, in a microenvironment, play a role in counteracting the tissue damage caused by serine proteinases released by neutrophils in inflammatory conditions.

Keywords: eosinophils, alpha-1-antitrypsin, inflammation, granules, serpins, asthma

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Introduction

Eosinophils are bone marrow-derived granulocytes that are related to neutrophils and monocytes and are recruited to sites of inflammation in conditions such as asthma, allergy and helminthic infestation [1]. They are capable of several diverse functional responses, including cytotoxicity, antigen presentation and the production of cytokines, growth factors and leukotrienes [2]. Eosinophils have a characteristic content of highly cationic proteins, including major basic protein (MBP), eosinophil peroxidase (EPO) and the

ribonucleases eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN). These are stored in the specific granules of the cytoplasm and can be released after specific stimuli [2].

In allergic inflammation both neutrophils and eosinophils are present. It is known that the cytoplasmic granules of neutrophils contain, among other proteins, preformed serine proteases, of which elastase is the most important [3]. When released at sites of inflammation, these proteases are known to play an important role in the degradation of the connective tissue matrix and to trigger pathophysiological mechanisms involved in the development of lung injury. This is seen in diseases such as cystic fibrosis, adult respiratory distress syndrome and pulmonary emphysema

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in longstanding asthma [4]. Protease inhibitors are responsible for the control of these enzymes, a function that is extremely important for the integrity of host tissues. Maintenance of a balance between the proteases and protease inhibitors of the body is therefore critical during the acute phase response.

Serine proteases are regulated by members of the superfamily of serine protease inhibitors (serpins) [5], of which α -1-antitrypsin (AAT) is the prototypic member. The primary function of AAT is to inhibit neutrophil elastase and proteinase-3 [6] and it is considered to have a crucial role in the homeostasis of connective tissue turnover in inflammation. It is synthesized primarily by hepatocytes and, to a lesser extent, by monocytes/macrophages, epithelial cells and trophoblast cells [7–9]. Most of the AAT is found in the plasma, where it normally occurs at a concentration of approximately 1.3 g/L, but since AAT is an acute phase protein the plasma concentration may rise 3–4 fold during acute inflammatory reactions [10]. Local synthesis and release of AAT is thought to be of importance at sites of inflammation, particularly at locations not directly in contact with the AAT in the blood.

In the present study we show for the first time that human eosinophils contain AAT, which may contribute to anti-elastase activity in the late allergic response, i.e. after the recruitment of neutrophils, when there is typically an influx of eosinophils at sites of allergic inflammation.

Materials and methods

Special reagents

Specific polyclonal rabbit antibodies to AAT and α -1-antichymotrypsin (ACT) were purchased from DAKO-PATTS, Denmark, as was the non-immune rabbit IgG. HRPO-conjugated sheep IgG against AAT was from Cedarlane Laboratories Ltd, Ontario, Canada. Recombinant human interleukin-5 was from R & D, Oxfordshire, England. HRPO-conjugated sheep IgG against ACT was from The Binding Site Ltd, Birmingham, U.K. The highly purified AAT, ACT and antithrombin III (AT III) were generous gifts from Prof. Em. Carl-Bertil Laurell, Malmö, Sweden. The source for preparation of AAT, ACT and AT III was citrated plasma from healthy blood donors. These proteins had been purified to homogeneity, presenting a single band on agarose gel electrophoresis and a single band of appropriate molecular weight on SDS-PAGE. Immunofixation and crossed immunoelectrophoresis of purified fractions using rabbit antihuman total serum also revealed single fractions in each case.

Isolation of cells

Blood was obtained from healthy volunteers after informed consent and eosinophils were isolated essentially as previously described [11]. Mononuclear cells were removed by centrifugation over Ficoll-Paque (Pharmacia Biotech, Sweden). After isotonic lysis of the erythrocytes with ammonium chloride, the granulocytes were washed in HBSS without calcium and magnesium. Immunomagnetic beads coated with antibodies to CD16 were used to retrieve the neutrophils in a magnetic column, allowing the isolation of highly purified eosinophils (viability was > 98% as judged by trypan blue exclusion and purity > 96%; contaminating cells were mainly lymphocytes and neutrophils, as judged by May-Grünwald-Giemsa staining). For experiments where neutrophils were used for prolonged incubation, cells were obtained from the granulocyte fraction after centrifugation over Ficoll-Paque (viability was > 98% as judged by trypan blue exclusion and purity > 92%; contaminating cells were mainly eosinophils and, to a lesser extent, lymphocytes as judged by May-Grünwald-Giemsa staining). In addition, highly purified neutrophils were recovered to make lysates. The CD16-positive neutrophils trapped in the column during negative immunoselection of eosinophils were washed out after removal from the magnetic field (purity > 98%, contaminating cells were mainly eosinophils and lymphocytes, as judged by May-Grünwald-Giemsa staining).

'Untouched' monocytes of high purity were obtained using a commercially available monocyte isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany). The procedure was performed as recommended by the manufacturer. In brief, mononuclear cells were obtained by centrifugation over Ficoll-Paque. Thereafter, non-monocytic cells were removed by retention in a magnetic column, using immunomagnetic beads labelled with CD3, CD7, CD19, CD45RA, CD56, and IgE antibodies. The purity of the monocytes was determined in a flow cytometer (Becton Dickinson) after labelling with PE-conjugated CD14 and FITC-conjugated CD45 antibodies (DAKO). The purity of monocytes was > 96% and contaminating cells were mainly lymphocytes and neutrophils.

To obtain cell lysates, the cells were treated with 1% (v/v.) Triton X-100 and 10 mM benzamidine in PBS for 20 min on ice. The supernatant was collected after centrifugation at $18\,000 \times g$ for 10 min and stored at -80°C .

Western blot

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of eosinophil lysates was performed using a Mini PROTEAN

3 electrophoresis cell (Bio-Rad) on 7.5% homogeneous gels, with a discontinuous buffer system containing 25 mM Tris and 19.2 mM glycine, pH 8.3, as recommended by the manufacturer. The electrophoretically separated proteins were transferred to a polyvinylidene difluoride membrane (PVDF-PLUS; Micron Separations Inc., Westborough, MA.) in 50 mM Tris, 40 mM glycine, 0.04% SDS and 20% (v/v) methanol, pH 9.2, using a semidry electrophoretic transfer system (Hoefer Semiphor, Amersham Pharmacia Biotech, Uppsala, Sweden). After transfer, the membranes were blocked with 3% (w/v) non-fat dry milk (Bio-Rad Laboratories, Hercules, Ca.) in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20). AAT was visualized using peroxidase-conjugated antibodies against AAT followed by incubation in a chemiluminescent substrate (Supersignal Ultra; Pierce, Rockford, Illinois) for 5 min and subsequent autoradiography. The same method was also used in a further attempt to demonstrate the presence of alpha-1-antichymotrypsin (ACT).

ELISA

A sandwich ELISA for AAT was developed using 96-well plates (Maxisorp; Nunc, Roskilde, Denmark). The plates were coated for 1 hour at 37 °C with antibodies diluted 1 : 1000 in carbonate/bicarbonate buffer (50 mM Na₂CO₃/NaHCO₃, pH 9.6). The remaining binding sites were blocked by incubation for 1 hour at 37 °C with 200 µL/well of blocking buffer (PBS with 0.5% bovine serum albumin). Samples and standards were then added for 2 hours, diluted in sample buffer (PBS, 0.1% Tween, 0.1% Triton X-100, 0.2% bovine serum albumin and 20 mM EDTA, pH 7.4). Peroxidase-conjugated goat anti-AAT (Cedarlane) diluted in blocking buffer at 30 ng/mL was used as the reporting antibody, with 30 minutes' incubation. Between each step the plates were washed four times using a microplate washer. Finally 100 µL of substrate (DAKO TMB One-step Substrate System, Dako Corp., Carpinteria CA, USA) was added, the reaction stopped after 10 min with 1 M H₂SO₄, and absorbance measured in an ELISA reader at 450 nm. All assays were performed in duplicate and the results given in mean values. The ELISA was used in a linear range of 1–25 ng/mL.

Immunoelectron microscopy

Immunoelectron microscopy was performed as previously described [12,13], with slight modifications. Leukocytes were isolated from heparinized blood by dextran sedimentation. The leucocyte-rich supernatant was collected and fixed in 1% (v/v.) glutaraldehyde plus 3% (v/v.) formaldehyde. Dehydration was carried out in graded acetone, followed by embedding in Epon (Polarbed 812;

Polaron Equipment Ltd, Watford, England). Post-fixation osmification was omitted. Ultrathin sections (50 nm) were incubated with 0.5% (w./v.) bovine serum albumin to block non-specific binding, followed by incubation with specific antibodies against AAT or ACT diluted 1 : 1000. After rinsing, sections were incubated with protein A-gold complex with a gold particle size of 10 nm (Bio Cell, Cardiff, Wales) followed by rinsing with distilled water, and staining with 2% (w./v.) uranyl acetate and lead citrate. For viewing, a JEOL EM 200 electron microscope operating at 80 kV was used.

Prolonged incubation

Eosinophils and neutrophils were incubated in 96-well plates (Nunc, Roskilde, Denmark) at 2×10^6 cells/mL, 200 µL per well, in RPMI 1640 (Gibco, Life Technologies, Täby, Sweden) supplemented with 2% synthetic serum (Ultrosor G; Gibco) and gentamycin at 50 µg/mL. After incubation for 16 or 24 h at 37 °C in a 5% CO₂ atmosphere, the cells were pelleted by centrifugation. The cell viability was > 98% initially, > 98% after 16 h of incubation, and > 96% after 24 h of incubation, even in the absence of IL-5, as judged by Trypan Blue exclusion. The cell-free supernatants were collected from each well and thereafter 1% (v./v.) Triton X-100 and 10 mM benzamidine in PBS was added to each well to lyse the remaining cells. The lysis of cells was for 20 min on ice. The supernatants and cell lysates were stored at –80 °C until assayed for their AAT content by ELISA. All experiments were performed in duplicate.

Elastase-binding capacity of eosinophil lysates

Human neutrophil elastase (Calbiochem, San Diego, California) was labelled with ¹²⁵I using Iodo-beads (Boule, Huddinge, Sweden), as described elsewhere [13]. Eosinophil lysate (5 µL, corresponding to 25×10^3 eosinophils) or purified AAT (1.5 µg) was mixed with human neutrophil ¹²⁵I-elastase (1, 2.5, and 5 µL) and incubated for 15 min at room temperature. The reaction was stopped by adding SDS-PAGE sample buffer and heating at 90 °C for 5 min. Samples were then separated by 10% SDS-PAGE under reducing conditions. For autoradiography, gels were exposed to X-ray film (BioMax, Kodak) for 24–48 h at –80 °C.

Results

Western blot

Lysates from purified eosinophils were subjected to SDS-PAGE and subsequent Western blotting. A chemiluminescent

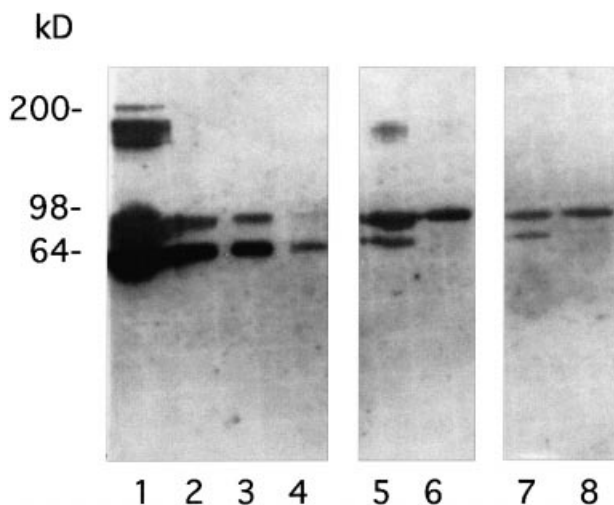


Fig. 1. Western blot showing immunoreactive bands corresponding to AAT in eosinophil (lanes 5 and 7) and neutrophil (lanes 6 and 8) lysates from two individuals. Ten microlitres of lysate corresponding to 50 000 cells was used in each lane. Highly purified AAT was used as control (lanes 1–4, containing 100, 10, 5 and 1 ng of AAT, respectively).

substrate was used for improved sensitivity and distinct immunoreactive bands corresponding to AAT were seen (Fig. 1). A double band was seen both with the highly purified AAT used as control and with the eosinophils. The double band consisted of one band at approximately 56 kDa, corresponding to native AAT, and one band at about 86 kDa corresponding to the expected position of an AAT-elastase complex. The most likely source of elastase in the eosinophil preparations is the presence of a few per cent of neutrophils. At higher concentrations of the purified AAT (100 ng sample), larger bands were observed, probably representing polymerization of AAT. Blotting in the same way with antibodies against neutrophil elastase revealed an immunoreactive band at about 86 kDa (not shown), indicating that there was some elastase contaminating the purified AAT. The polymerization of AAT has also been described [14,15]. Alpha-1-antichymotrypsin, another member of the serpin family, could not be detected in eosinophil lysates by Western blotting (not shown).

Quantification of AAT in cells

According to ELISA measurements the AAT content in the eosinophil lysates was approximately 100 ng/ 5×10^6 cells (range 65–140, $n = 5$), which may be compared with approximately 50 ng/ 5×10^6 cells (range 34–70, $n = 5$) in the neutrophil lysates, and approximately 25 ng/ 5×10^6 cells (range 17–49, $n = 4$) in lysates of monocytes. In

lymphocytes, AAT was not detectable. The ELISA showed no cross-reactivity with ACT or AT III, proteins that are homologous to AAT.

Immunoelectron microscopy

The subcellular localization of AAT was investigated by immunoelectron microscopy on ultrathin sections of cells. AAT, as detected by specific rabbit antibodies against human AAT and visualized by protein A-gold complex, was present in the crystalloid-containing specific granules of eosinophils (Fig. 2). AAT was seen over the crystalloid as well as in the matrix of the granules. Weak background staining was observed as sparse gold particles randomly distributed in the space between the granules and over the nucleus. ACT could not be detected (not shown).

Detection of AAT in medium and cells during prolonged incubation

To determine if there was a net increase in AAT during prolonged incubation, indicating *de novo* synthesis of the protein, eosinophils were incubated for up to 24 h (Fig. 3). Eosinophils were incubated in medium alone and in the presence of interleukin (IL)-5 at 0.1 nM. No significant change of the total AAT content could be detected by ELISA (Fig. 3a). During incubation, the intracellular content of AAT decreased (Fig. 3b) and the medium showed a corresponding increase (Fig. 3c), suggesting release of pre-formed AAT from intracellular stores. The presence of IL-5 did not significantly affect the AAT levels.

Complex formation with elastase

Incubation of ^{125} I-labelled elastase with eosinophil lysates or purified AAT resulted in both cases in the appearance of an 86-kDa band, which strongly suggests complex formation between human neutrophil elastase and AAT present in eosinophil lysates, and thus the presence of functionally active AAT in eosinophils (Fig. 4).

Discussion

This study shows that human eosinophils store AAT in the specific granules of their cytoplasm and complex formation with neutrophil elastase could be demonstrated in lysates of purified eosinophils, indicating the presence of functionally active AAT.

The double band seen on the Western blot may be explained by complex formation between AAT and elastase. In monocytes, for example, AAT is found not

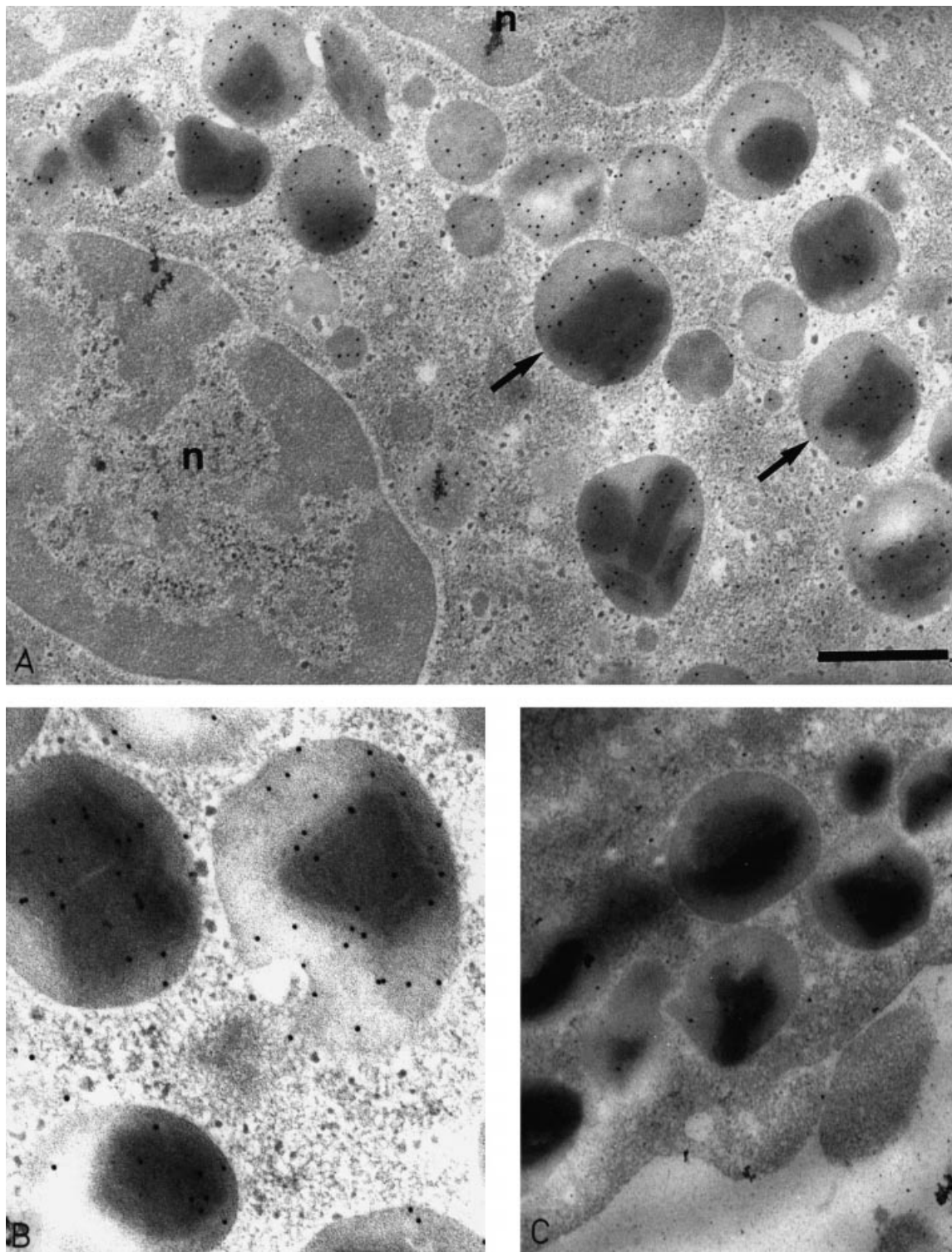


Fig. 2. Immunoelectron microscopy showing subcellular localization of AAT to the specific cytoplasmic granules (arrows) of a mature eosinophil. Specific polyclonal rabbit antibodies against human AAT were detected using protein A-gold complex with a particle size of 10 nm. A few gold particles are seen over the nucleus (*n*) and in the cytoplasm between granules, and represent background staining. Original magnification $\times 15\,000$. Bar = 500 nm.

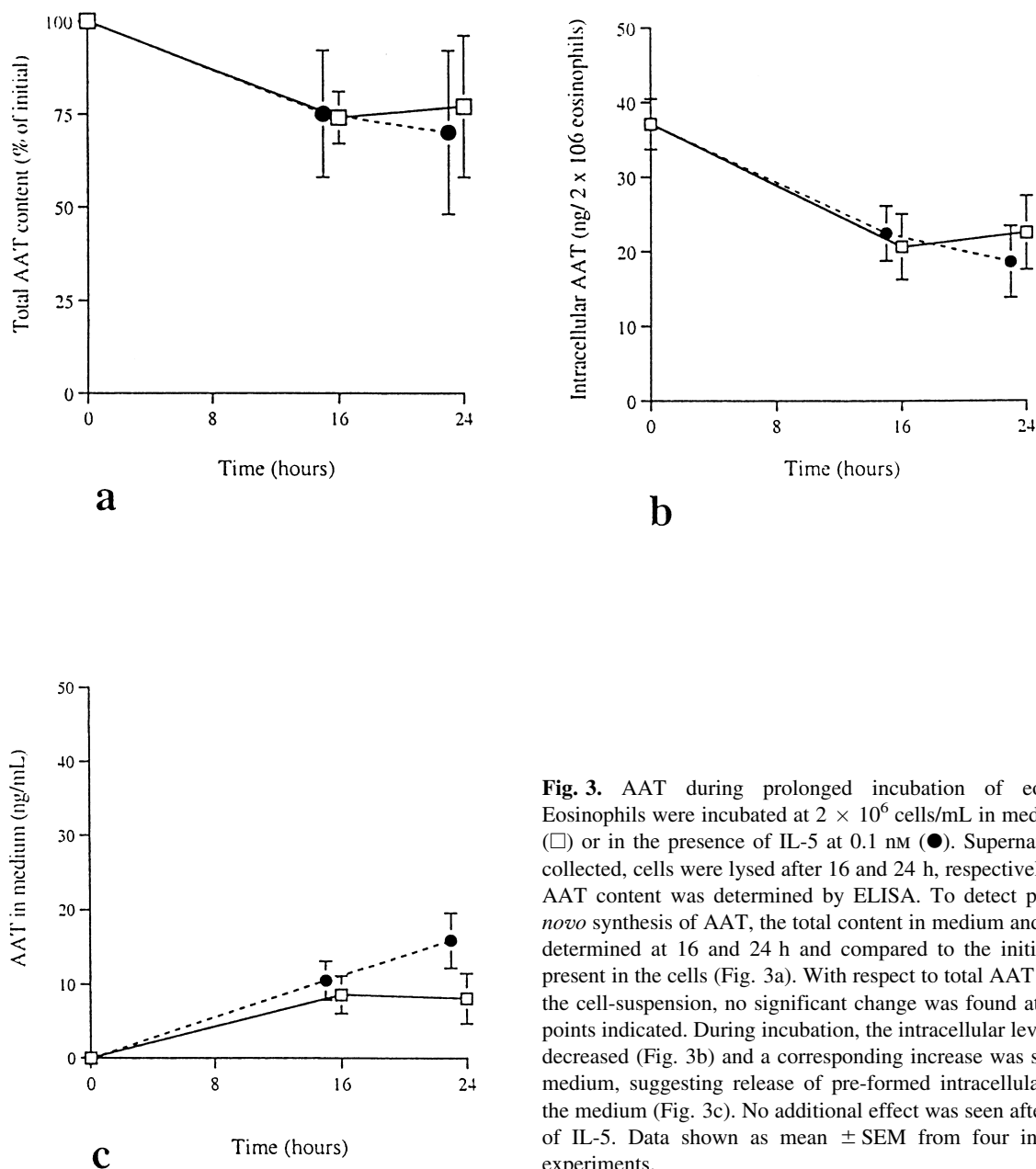


Fig. 3. AAT during prolonged incubation of eosinophils. Eosinophils were incubated at 2×10^6 cells/mL in medium alone (□) or in the presence of IL-5 at 0.1 nM (●). Supernatants were collected, cells were lysed after 16 and 24 h, respectively, and the AAT content was determined by ELISA. To detect possible *de novo* synthesis of AAT, the total content in medium and cells was determined at 16 and 24 h and compared to the initial amount present in the cells (Fig. 3a). With respect to total AAT content in the cell-suspension, no significant change was found at the time-points indicated. During incubation, the intracellular level of AAT decreased (Fig. 3b) and a corresponding increase was seen in the medium, suggesting release of pre-formed intracellular AAT to the medium (Fig. 3c). No additional effect was seen after addition of IL-5. Data shown as mean $\pm$ SEM from four independent experiments.

only in its native 56-kDa native form, but also in 66- and 75-kDa forms, in complex with serine proteinase [16]. A likely source for elastase in the eosinophil preparations, may be contaminating neutrophils.

Hepatocytes seem to be the major source of AAT in the human body and the level of AAT in the plasma is relatively high. However, other cells are also capable of synthesizing AAT and, interestingly, two other cells of myeloid origin express the AAT gene, namely monocytes/macrophages [17,18] and neutrophils [19,20]. The expression of the AAT gene is regulated by different promoter

regions in hepatocytes and monocytes/macrophages, respectively [21]. Possibly, in cells of myeloid origin the expression of the AAT gene is subject to a common regulation. Thus in hepatocytes, for example, the genes for both AAT and ACT are expressed, whereas in eosinophils only the gene for AAT is expressed.

The specific granules of eosinophils are formed at the promyelocytic stage of differentiation in the bone marrow [12,22]. Since the AAT is found in these granules, it is probably also synthesized in eosinophil progenitors in the bone marrow, and stored in granules ready to be

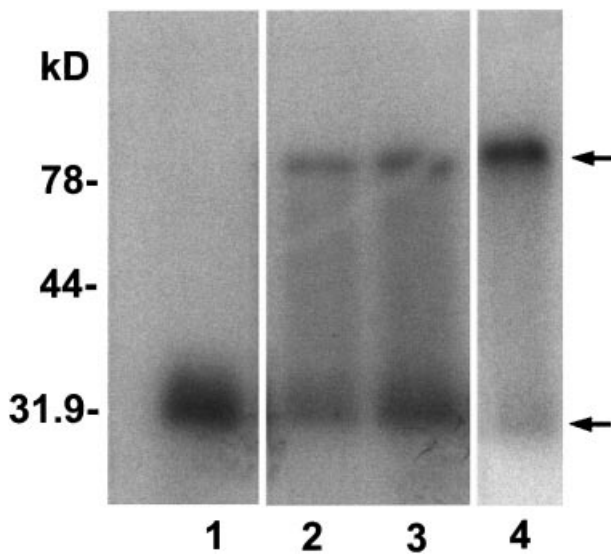


Fig. 4. Complex formation between elastase and AAT in eosinophil lysates. ^{125}I -labelled human neutrophil elastase alone (lane 1), a mixture of eosinophil lysate and ^{125}I -labelled elastase (eosinophils from two different donors are shown in lanes 2 and 3, respectively), and a mixture of purified AAT and ^{125}I -labelled elastase (lane 4), were subjected to SDS-PAGE and subsequent exposure to film. In lane 1, a band of approximately 29 kDa is seen, corresponding to elastase. In lanes 2, 3, and 4 the shift to approximately 86 kDa suggests complex formation between elastase and AAT.

released at sites of inflammation. Pre-formed proteins have an advantage over proteins synthesized *de novo* at sites of inflammation, since they are immediately available.

The release of eosinophil granule contents is subject to complex regulation. Therefore, the release of AAT probably occurs in response to specific stimuli, for example after activation by cytokines and subsequent binding to immunoglobulins and complement products via specific cell surface receptors [2].

In allergic inflammation, there is an early influx of neutrophils with a high content of potent proteases, such as elastase, which may cause injury to normal tissues [23]. Eosinophils are recruited to the site of inflammation at a later stage, in the late allergic response, which occurs 8–24 h after the initiation of inflammation [24]. Therefore it is possible that this later wave of eosinophils attenuates the effect of the proteases released by the preceding neutrophils. Although the levels of AAT found in eosinophils were relatively low, the effects in the micro-environment may be important. Eosinophils themselves contain very low amounts of pre-formed proteases, or none at all [25].

It is also possible that released AAT has immunomodulatory functions in addition to the inactivation of

serine proteases. AAT inhibits the neutrophil respiratory burst [26] and the release of AAT could therefore be one of the mechanisms participating in downregulation of the inflammatory response.

Tissue eosinophils probably have an extended life span compared to eosinophils isolated from the blood and several genes are upregulated in these cells [2]. However, in experiments where eosinophils were subjected to prolonged incubation, we were not able to detect significant *de novo* synthesis of AAT. In experiments using biosynthetic labelling of proteins being synthesized, little or no *de novo* synthesis of AAT could be detected (data not shown).

These findings indicate that eosinophils may have a role in counteracting the proteases released at sites of inflammation, protecting against the development of, for example, pulmonary emphysema in the asthmatic patient. This contrasts with the hitherto dominating belief that eosinophils only contribute to the destruction of normal tissue in inflammation [27].

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