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ONTOGENY AND TISSUE DISTRIBUTION OF ALPHA-1-ANTITRYPSIN OF THE MOUSE

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α_1-Antitrypsin is the second most abundant proteinase inhibitor in plasma. The fact that it is a globular glycoprotein of relatively small size (M_r 53 500) allows it access to a wide variety of fluids and tissue sites. α_1-Antitrypsin has been purified from mouse plasma by affinity chromatography and ion exchange. The purified protein exhibits homogeneity on polyacrylamide electrophoresis, but electrophoretic heterogeneity on crossed immunoelectrophoresis. Mouse and rat α_1-antitrypsin show strong crossreactivity and the half-life for mouse α_1-antitrypsin is 15.5 h. Fetal levels are 15% of adult and it requires 25–30 days before adult levels are reached in the neonate. Maternal levels remain unchanged throughout pregnancy and at parturition. The inhibitor is present in a number of body fluids including serum, breast milk, gastrointestinal washings, lung washings and bile. The source of α_1-antitrypsin for all of these fluids appears to be the liver.

Introduction

The inflammatory response is a complex process mediated by both cellular and humoral mechanisms. The humoral events include the pathways of complement activation, kinin generation and coagulation and fibrinolysis, all of which are interconnected, as products and active fragments of one pathway can activate the others.

Control of the pathways of inflammation is exerted through the plasma proteinase inhibitors: α_2-macroglobulin, α_1-antitrypsin, C_1-esterase inhibitor, antithrombin III, α_1-antichymotrypsin and antiplasmin. α_2-Macroglobulin is present in plasma in the highest concentration and has wide specificity, but being a large molecule, it is restricted in access to some sites of inflammation. α_1-Antitrypsin also termed α_1-proteinase inhibitor [1] is the next most abundant inhibitor in plasma and being much smaller (M_r human – 58 000) has a broader access to inflammatory sites. α_1-Antitrypsin has been the subject of several recent comprehensive reviews and has been shown to inhibit a variety of proteolytic enzymes including trypsin, elastase, plasmin, thrombin, and the neutral proteases of granulocytic leukocytes [2,3].

We have isolated α_1-antitrypsin from mouse plasma and prepared a monospecific antiserum to the inhibitor. We have recently used this antiserum to demonstrate the presence of the inhibitor in the hepatocyte of normal mouse liver as well as in the islet cell of the pancreas [4]. We now report on the ontogeny and quantitative tissue localization of α_1-antitrypsin in normal mouse.

Materials and Methods

Materials. DEAE-Sephadex A-50, Sepharose 4B, Sephadex G-25 and Con A-Sepharose were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden); heparin, *N*-benzoyl-DL-arginine ethyl ester (BAEE), *N*-benzoyl-DL-arginine *p*-nitroanilide (HCl) (BAPNA), and porcine pancreatic trypsin (T-0134) were pur-

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chased from Sigma Chemical Co., St. Louis, MO, U.S.A.; Cibacron Blue 3G-A was a gift from Ciba-Geigy, Toronto, Ontario, Canada: $Na^{125}I$ was purchased from New England Nuclear, Boston, MA, U.S.A.; Chloramine T was purchased from Eastman Organic Chemicals, Rochester, NY, U.S.A.; agarose Sea Kem (ME) was obtained from Marine Colloids Inc., Rockland, ME, U.S.A. *p*-Nitrophenyl guanidinobenzoate was purchased from ICN, Cleveland, OH, U.S.A.

Purification of α_1-antitrypsin. Fresh pooled citrated plasma from adult CBA/J mice was used as starting material. The isolated method was similar to that described for the purification of rabbit α_1-antitrypsin [5] and was based on the method described earlier by Pannell et al. [6] for human α_1-antitrypsin.

Plasma was precipitated with saturated $(NH_4)_2SO_4$ solution to a final saturation of 40%. The precipitate was removed by centrifugation and the supernatant was dialysed against 0.15 M NaCl and passed through a Sepharose-heparin column to remove antithrombin III. Saturated $(NH_4)_2SO_4$ was added to the effluent and the precipitate collected between 55 and 85% saturation was removed by centrifugation, dissolved in a minimal amount of water and dialysed against several changes of Tris-HCl buffer (0.05 M Tris-HCl/0.05 M NaCl, pH 8.8). The protein solution was chromatographed on a DEAE-Sephadex A-50 column (2 × 30 cm) equilibrated with the Tris-HCl buffer. The column was washed with starting buffer (100 ml, 15 ml/h), and a linear gradient (200 ml starting buffer/200 ml Tris-HCl/0.2 M NaCl) was applied. The eluted fractions exhibiting antitryptic activity were pooled and loaded onto a Con A-Sepharose column [7] (2 × 20 cm) equilibrated with 0.1 M Tris-HCl, pH 7.5/0.5 M NaCl/1 mM each of $MnCl_2$, $MgCl_2$ and $CaCl_2$. The column was washed with 5 col. vol. of this buffer and the glycoproteins eluted by 1-*O*-methyl-α-D-glucopyranoside (0.1 M) in the starting buffer. The eluate was dialysed against 0.05 M Tris-HCl/0.15 M NaCl, pH 8.0 and passed through a Cibacron blue-Sepharose column (5 × 50 cm) equilibrated with starting buffer to remove albumin. The effluent was concentrated by pressure dialysis (4°C) and dialysed against 5 mM phosphate buffer/0.05 M NaCl, pH 6.5 and loaded onto a DEAE-Sephadex A-50 column (1 × 15 cm) equilibrated with the same buffer. After washing with 50 ml of this buffer, a linear gradient from 0.05 to 0.2 M NaCl in 5 mM phosphate buffer, pH 6.5, was applied (200 ml total). Fractions exhibiting most antitryptic activity were pooled and dialysed against 5 mM Tris-HCl, pH 8.0.

Preparative polyacrylamide gel electrophoresis was carried out in an apparatus as described by Padjak [8]. The acrylamide gel (7.5%) was cast in a column 2.8 × 2.8 cm with 5 mM Tris/39 mM glycine, pH 8.2 in the anode and cathode chambers. 1 ml α_1-antitrypsin (10 mg/ml) was added and electrophoresis carried out at a constant current of 20 mA for 2 h at 4°C until the Bromphenol blue tracking dye had reached the anode chamber. Fractions were collected at 3 min intervals and examined for protein and antitryptic content. Those exhibiting high specific activities were pooled, examined for homogeneity by analytical gel electrophoresis, dialysed against 5 mM Tris-HCl pH 8.0 and stored at −20°C.

Assay of antitryptic activity. Serum or plasma antitryptic activity was determined using porcine pancreatic trypsin as enzyme and either BAEE or BAPNA as substrate. Briefly, α_1-antitrypsin and pancreatic trypsin were preincubated for 5 min followed by the addition of substrate (10 min, room temperature, BAEE; 5 min, 30°C, BAPNA) with subsequent spectrophotometric measurement of product [9,10].

In the purification of α_1-antitrypsin, one unit of antitryptic activity was defined as the amount of preparation able to completely inhibit 1 μg trypsin. The commercial trypsin was corrected for activity by determination with *p*-nitrophenyl guanidinobenzoate [11].

Analytical electrophoretic methods. Analytical electrophoresis in polyacrylamide tube gels was carried out at pH 8.2 [12]. Gels were stained with 0.05% Amido black in 7% (v/v) acetic acid and destained in 7% acetic acid.

Animals. CBA/J male and female mice were purchased from The Jackson Laboratories, Bar Harbor, ME. Pregnant mice (12–18 weeks) were produced in a controlled breeding situation and the sighting of a vaginal plug was designated as day 1 of gestation.

Immunochemical techniques. The purified α_1-antitrypsin was used to immunize rabbits (New Zealand White) and sheep with 25 and 100 μg dose, respectively. The antisera were tested for specificity by double-diffusion and immunoelectrophoresis against normal mouse serum.

α_1-Antitrypsin levels were determined by Laurell rocket immunoelectrophoresis [13] nephelometry (Technicon Automated Fluoronephelometer) [14] and an enzyme-linked immunosorbent assay [20], using sheep anti-mouse α_1-antitrypsin.

Crossed immunoelectrophoresis. α_1-Antitrypsin was characterized by crossed immunoelectrophoresis [15] against sheep anti-mouse α_1-antitrypsin. Serum samples were electrophoresed in the first dimension at 50 mA, 12 V/cm at 4°C for 4 h and 25 mA, 7 V/cm for 18 h at 4°C for the second dimension.

Radioactive labelling. Pure α_1-antitrypsin and porcine pancreatic trypsin were iodinated by the Chloramine T method [16].

Half-life of mouse α_1-antitrypsin. CBA/J female mice (approx. 12-week-old) were used as recipients. Each animal was injected intravenously (tail vein) with 200 µl ^{125}I-labelled α_1-antitrypsin in saline ($1 \cdot 10^6$ cpm). After an initial period of 30 min allowed for equilibration, $T(o)$, a serum sample was collected from the retroorbital plexus. Animals were then bled out, by cardiac puncture, at selected times, $T(n)$, over a period of 60 h, to determine the in vivo α_1-antitrypsin clearance rate. Radioactivity of the plasma samples was determined and protein-bound iodine was assessed by precipitation with 10% trichloroacetic acid.

Collection of body fluids. A section of small intestine (20 cm in length), consisting of the jejunum and ileum, was removed, filled with 2 ml saline and clamped at both ends. After gentle mixing, the fluid was removed, centrifuged and 0.1% sodium azide was added. Breast milk was obtained from nursing mothers by manual manipulation after stimulation by oxytocin (1 i.u.). The milk was centrifuged and the supernatant stored with 0.1% sodium azide. Bile was removed by suction from the gall bladder and pooled before concentration and dialysis against phosphate-buffered saline with 0.1% sodium azide. Bronchio-lavage fluid was obtained by infusion of 10-times 1 ml portions of phosphate-buffered saline via cannulation of the trachea [17]. Fetuses were removed from pregnant CBA/J mice at various times of gestation. A sample of the amniotic fluid was also taken. The fetuses were washed in phosphate-buffered saline and blood samples collected by decapitation.

Results

Isolation of α_1-antitrypsin. The trypsin inhibitory capacity of pooled CBA/J mouse plasma was 1.42 mg trypsin inhibited/ml plasma (51 mg protein) and the specific activity of the final preparation was 336 µg trypsin/mg α_1-antitrypsin, representing a purification factor of 12 (Table I).

The α_1-antitrypsin recovered from the final DEAE-Sephadex column represented 28% of the plasma antitryptic activity, however. other proteins present in whole plasma, e.g., antithrombin III, also inhibit the esterolytic activity of trypsin and the recovery was probably slightly higher. The purified protein showed a single homogeneous band on analytical polyacrylamide gel electrophoresis (Fig. 1) and has a molecular weight of 53 500 ± 2000 in SDS-polyacrylamide gel electrophoresis [29].

Immunochemical characterization. Antisera to mouse α_1-antitrypsin reacted with a single precipitin line in double-difussion against normal mouse serum showing identity with that developed against purified inhibitor. The antiserum did not show any cross-reactivity against sheep, goat, rabbit or guinea pig but cross-reacted strongly with rat α_1-antitrypsin (Fig. 2).

On crossed immunoelectrophoresis against normal mouse serum, two apparently crossing precipitin peaks are seen (Fig. 3), purportedly indicating the presence of two antigenically distinct entities as had been previously shown [18].

We have demonstrated that both electrophoretic variants bind ^{125}I-labelled trypsin as demonstrated by autoradiography after separation by crossed immuno-electrophoresis (data not shown). The ratio of the peak areas (slow peak: fast peak 2.7 : 1 in CBA/J mice) indicates the electrophoretic variants are present in substantial quantities. The same two peaks, in similar relative concentrations, are shown to be present in fetal circulation, gut washings, breast milk, purified mouse α_1-antitrypsin as well as in supernatants of cultures of isolated hepatocytes. There is, however, strain variation in the ratio of the peaks as shown in Table II. Absorption of the antiserum with purified mouse albumin does not alter the relative proportions. These two forms are found by us and others (Lee, D. and Janoff, A., personal communication) in rat [21] and rabbit plasma [5] though the forms in rabbit exhibit immunological identity.

TABLE I

PURIFICATION OF MOUSE α_1-ANTITRYPSIN

Purification step	Volume (ml)	Protein content (mg/ml)	Antitryptic activity (units/ml)	Specific activity (units/ mg protein)	Purification (-fold)	Recovery of inhibition activity (%)
1 Plasma	20	51.0	1420	27.8	1.00	100
2 Sepharose-Heparin $(NH_4)_2SO_4$ 55–85%	25.0	23.0	920	40.0	1.44	83
3 DEAE 8.8	73.0	3.9	225	57.7	2.07	59
4 Con A-Sepharose	10.0	4.5	1270	282.2	10.15	52
5 C.B.-Sepharose	17.0	2.5	758	303.2	10.90	46
6 DEAE 6.5	3.8	6.1	2050	336.0	12.08	28

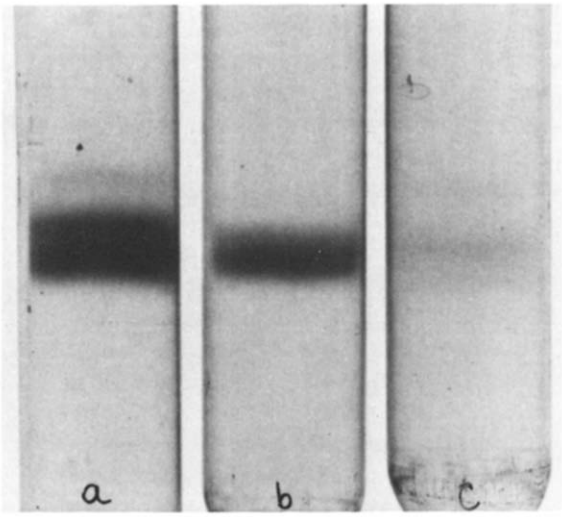

Fig. 1. Purification of mouse α_1-antitrypsin. Polyacrylamide gel electrophoresis. a, DEAE, pH 6.5 active fraction; b, final product; c, and contaminant after preparative polyacrylamide gel electrophoresis.

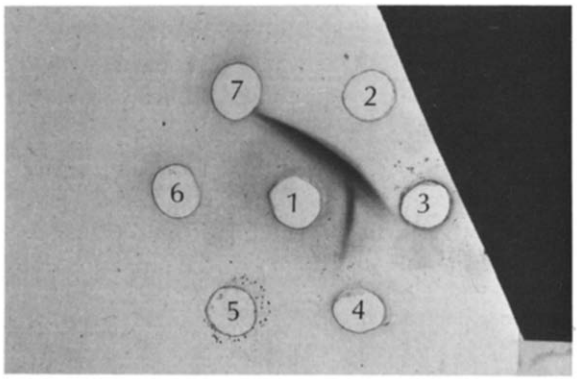

Fig. 2. Specificity of α_1-antitrypsin antiserum. Well 1 contains sheep anti-mouse α_1-antitrypsin; well 2, mouse serum; 3, rat serum; 4, guinea pig serum; 5, rabbit serum; 6, goat serum and 7, sheep serum.

In separate experiments (Murray, R., Gauldie, J. and Lamontagne, L., unpublished data) we have shown that our antiserum precipitates a single precursor polypeptide from mRNA extracts of heptocytes from all three species in a reticulocyte cell free system.

Ontogeny. As shown in Fig. 4, the serum levels in the fetus and at birth represent less than 15% of the normal adult level. Over the next 25 days, there is a gradual increase to adult levels. The maternal level during pregnancy and at parturition falls within the

TABLE II

STRAIN DIFFERENCES IN ELECTROPHORETIC HETEROGENEITY OF α_1-ANTITRYPSIN IN THE MOUSE

Strain	Ratio slow : fast variants
CBA/J	2.7
RF/C57B1	2.6
Balb/C	2.3
DBA	2.3
RJ/J	2.1
nu/nu	2.1
C3H	2.1
A/J	1.9
C57B1	1.6

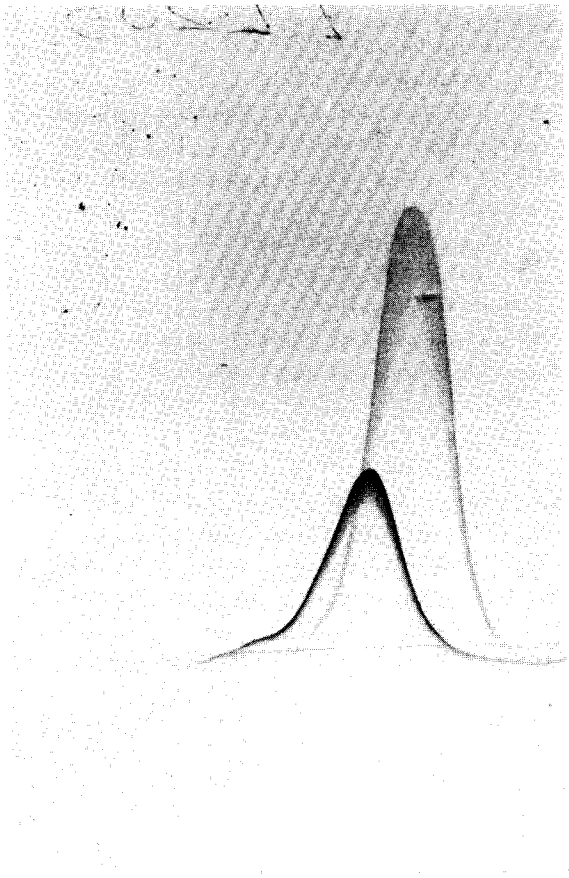

Fig. 3. α_1-Antitrypsin variants. Mouse α_1-antitrypsin was electrophoresed in the first dimension (cathode at right) in 1% agarose and electrophoresed in the second dimension (cathode at bottom) against 3.2% sheep anti-mouse α_1-antitrypsin.

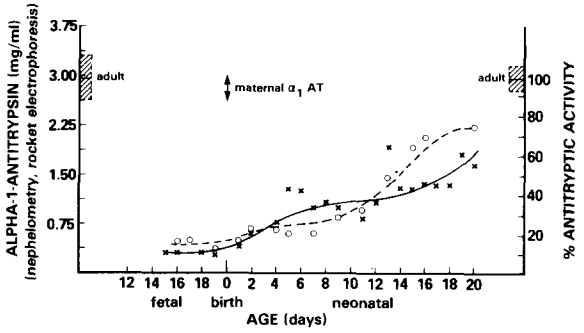

Fig. 4. Ontogeny of α_1-antitrypsin in CBA/J mice. Each data point represents a pool of sera from seven mice. α_1-Antitrypsin levels (X———X) were determined using sheep anti-mouse α_1-antitrypsin and antitryptic activity (o - - - - - - o) using BAPNA as substrate.

TABLE III

PRESENCE OF α_1-ANTITRYPSIN IN VARIOUS BODY FLUIDS (CBA/J MICE)

N, assayed by nephelometry; E, assayed by ELISA.

Fluid	α_1-Antitrypsin concentration	
Adult serum	3 mg/ml	N
Fetal serum	0.4 mg/ml	N
Amniotic fluid	28 μg/ml	E
Bile	19 μg/ml	E
Breast milk	2 mg/ml	N
Gastrointestinal washings	7.2 μg/20 cm gut	E
Bronchiolavage fluid	65 μg/lung	E

normal adult range. The antitryptic activity correlates with the immunoquantitation data.

Normal adult levels showed variation when pools of plasma of several strains of mice were compared by nephelometry (CBA/J, 3.0 ± 0.7 mg/ml; C3H 3.5 ± 0.6 mg/ml; C3D2, 4.6 ± 1.0 mg/ml n = 10).

Tissue distribution. α_1-Antitrypsin was found in all samples examined though the amounts varied greatly and never exceeded plasma levels (Table III).

Half-life ($T_{1/2}$) of mouse α_1-antitrypsin. Using purified α_1-antitrypsin, the half-life for the plasma clearance of the proteinase inhibitor in CBA/J mice was 15.5 h.

Discussion

Isolation of immunologically pure but electrophoretically heterogeneous mouse α_1-antitrypsin has been achieved by a combination of ion exchange and affinity chromatography. The specific activity (336 units/mg protein) of the purified molecule approaches the theoretical optimum (446 units/mg) and the purification factor we obtained (12.1) represents a significant improvement over that reported previously (purification factor 6.9 for mouse) [18].

The α_1-antitrypsin is homogeneous in analytical polyacrylamide gel electrophoresis (Fig. 1) but demonstrates heterogeneity on crossed immunoelectrophoresis against a monospecific sheep antibody to α_1-antitrypsin (Fig. 2).

Rat α_1-antitrypsin has recently been purified to homogeneity on analytical polyacrylamide gel elec-

trophoresis. Lee and Janoff (personal communication) have shown the rat to exhibit similar electrophoretic heterogeneity with apparent immunological non-identity. Takahara et al. [21], with a purification method similar to ours have not examined the product by crossed immunoelectrophoresis but showed two major bands in the purified protein by isoelectric focusing. These results, taken with the findings that both forms bind trypsin, are synthesized by isolated hepatocyte cultures and only a single polypeptide precursor is identified in translated hepatic mRNA extracts, would argue that the electrophoretic heterogeneity arises as a result of post-transcriptional events and the non-identity in rodent species is probably false-spur formation in crossed immunoelectrophoresis [19]. Clarification of this must await separation of the forms and comparative sequence analysis.

The short half-life of α_1-antitrypsin in the mouse, ($T_{1/2}$ of 15.5 h) may be compared with the values reported for rat α_1-antitrypsin of 25 h [24], for rabbit of 53.6 h [25], for dog of 72 h [26] and for human of 5–6 days [27].

The ontogeny of α_1-antitrypsin in the mouse differs from that in the human. Fig. 4 demonstrates that fetal and newborn levels are 15% of the adult and there is a slow steady rise to approx. 70% of normal adult values by day 20. In addition, the maternal level does not vary from the adult normal range. At birth the low levels of α_1-antitrypsin and the coincident normal levels in the mother contrast with the findings in the human [22,23] in which the newborn exhibits a level close to the normal adult while the maternal level can rise up to 2-fold over the normal adult levels. The reasons for this contrast are not clear and remain as a question for further investigation.

The low fetal levels probably represent fetal synthesis rather than transfer across the placenta though it is not totally precluded as a contributing mechanism.

The presence of α_1-antitrypsin in the various body fluids (Table III) indicates the ubiquitous nature of this inhibitor. While we expected to find α_1-antitrypsin in serum and bronchiolar fluid, it was surprising to find such a quantity in breast milk. The possible role of the inhibitor in this material is open to speculation.

More surprising was the quantity found in gut washings and bile. Subsequent to our previous report on the immunohistochemical localization of α_1-antitrypsin in hepatocytes [4] we also noted the inhibitor in the epithelial cells at the tips of the villi in the gut (unpublished data). Tomasi and Hauptman found a significant quantity of α_1-antitrypsin in human colostrum and gastrointestinal fluids [28] and speculated on a protective role for α_1-antitrypsin on the stability of IgA since these two molecules share avidity for each other. Preliminary experiments with cultures of lung, pancreas and gut tissue failed to demonstrate synthesis of α_1-antitrypsin and would suggest that α_1-antitrypsin is synthesized primarily in the liver and transported in bile to the gut where it is then presumably taken up by the epithelial cells either in the native form, or complexed with a proteinase.

We have isolated mouse α_1-antitrypsin and characterized the antiserum resulting from immunization with the purified inhibitor. Mouse α_1-antitrypsin exhibits electrophoretic heterogeneity and is found widely distributed in tissue and fluids throughout the body, which may reflect the important role played by this inhibitor in the control of inflammation. The plasma clearance of α_1-antitrypsin in the mouse is consistent with rates in other species and these baseline studies allow us to examine alterations in tissue distribution that we have shown to occur during an inflammatory response (Gauldie, J., Lamontagne, L. and Befus, A.D., unpublished data).

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