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Material Isolated from Normal and Variant Human Liver that Immunologically Crossreacts with Alpha$_1$-Antitrypsin

(pulmonary emphysema/liver cirrhosis/trypsin inhibition/antitrypsin deficiency)

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ABSTRACT **A material that strongly reacts with antibodies against alpha$_1$-antitrypsin, but has little trypsin inhibitory capacity, has been isolated and purified to homogeneity from human liver. The molecular weight of the crossreacting material is about 18,000, which is significantly lower than that of serum alpha$_1$-antitrypsin. The material isolated from the liver of a homozygous variant subject (*ZZ*) with alpha$_1$-antitrypsin deficiency is readily distinguished by electrophoresis from the material extracted from a normal (*MM*) subject. The tissues from a heterozygous variant subject (*MZ*) contain the two components. The immunologically crossreacting material is presumably synthesized by the gene that codes for alpha$_1$-antitrypsin.**

An inherited deficiency of alpha$_1$-antitrypsin (A$_1$AT) in human serum is associated with pulmonary emphysema (1–3) and liver cirrhosis (4). Autosomal inheritance of the gene for A$_1$AT has been demonstrated in man (5, 6). Of the genetic variants of A$_1$AT thus far reported, homozygous *ZZ* has severe serum A$_1$AT deficiency, possessing only 10–15% of the activity of the normal type *MM* subject (5). Since the specific antitryptic activities of *M* type A$_1$AT and *Z* type A$_1$AT are essentially identical, the lower activity of *ZZ* sera is attributed to its lower concentration of A$_1$AT (7).

It has been observed, by staining the tissues with fluorescent antibody, that individuals who are either homozygous variant *ZZ* or heterozygous variant *MZ* store material in their liver that immunologically reacts with anti-A$_1$AT serum, while normal liver does not have such material (8, 9). From these findings, it has been suggested that the A$_1$AT deficiency in the variant sera occurs because the variant *Z* type protein remains in the liver and is not efficiently released into the sera. The mechanism of release or accumulation of A$_1$AT and the nature of the immunologically crossreacting material (CRM) in the variant liver are not known.

In contradiction to these histological observations, CRM was extracted and purified to homogeneity from both normal and variant livers. The properties of normal and variant CRM are reported in this paper.

MATERIALS AND METHODS

Autopsy liver samples from normal *MM*, heterozygous variant *MZ*, and homozygous variant *ZZ* subjects were used. The phenotypes of these subjects were identified by measurement of serum trypsin inhibitory capacity and by electrophoresis of the serum on acid starch gel as previously described (1). Liver samples from all three sources were stored frozen at −15° before use. Fresh normal autopsy liver was also used for a preparation. Trypsin inhibitory capacity was assayed by Eriksson's method (11) with crystalline salt-free trypsin (Worthington Biochemical Corp.) and benzoyl-D,L-arginine-*p*-nitroaniline hydrochloride (Nutritional Biochemical Corp.) as substrate.

Normal A$_1$AT was purified from normal human serum by a combination of the method described by Crawford (12) and that described by Murthy and Hercz (13) using concanavalin A-Sepharose 4B. Ultracentrifugal analysis was performed in a Spinco model E centrifuge. The sample was dissolved in 0.05 M sodium phosphate, pH 7.0, containing 0.1 M NaCl.

DEAE-cellulose (DE32) from Whatman; concanavalin A-Sepharose 4B, QAE-Sephadex, Sephadex G-200 from Pharmacia Fine Chemicals; and rabbit antiserum to human A$_1$AT from Behring Diagnostics were used.

RESULTS

Isolation of CRM from Normal and Variant Liver. Liver (wet weight about 600 g) was homogenized with an equal volume of 0.2 M sodium phosphate, pH 7.6, filtered through gauze, and centrifuged. The supernatant was purified by the following six steps. At each step the protein content (absorbancy at 280 nm) and immunoprecipitation capacity against anti-human A$_1$AT serum of the effluents from the column were assayed. After each chromatography step the active effluent was dialyzed against water, lyophilized, and subjected to the next step of purification: (*1*) 50–100% saturated ammonium sulfate precipitation. (*2*) DEAE-cellulose treatment. The dialyzed precipitate (500 ml) was mixed with DEAE-cellulose (50 g) and adjusted to pH 7.6. The cellulose was washed with 10 mM sodium phosphate, pH 7.6, and the CRM was eluted with 0.2 M of the same buffer (500 ml). (*3*) DEAE-cellulose column (4.3 × 60 cm) chromatography on which the elution was accomplished by a linear gradient from 10 mM to 0.2 M sodium phosphate, pH 7.6. The gradient was produced by adding 2500 ml of 0.2 M buffer into a mixing chamber that contained 2500 ml of 10 mM buffer. CRM was eluted between 1000 and 1500 ml of the effluent. (*4*) QAE-Sephadex A-50 column (4.3 × 60 cm) chromatography on which CRM was eluted by a linear gradient of NaCl concentration from 0.1 to 0.5 M in 50 mM Tris·HCl, pH 7.6.

Abbreviations: A$_1$AT, alpha$_1$-antitrypsin; CRM, material that immunologically crossreacts with alpha$_1$-antitrypsin; CRM-M and CRM-Z are isolated from subjects of genotype *MM* and *ZZ*, respectively.

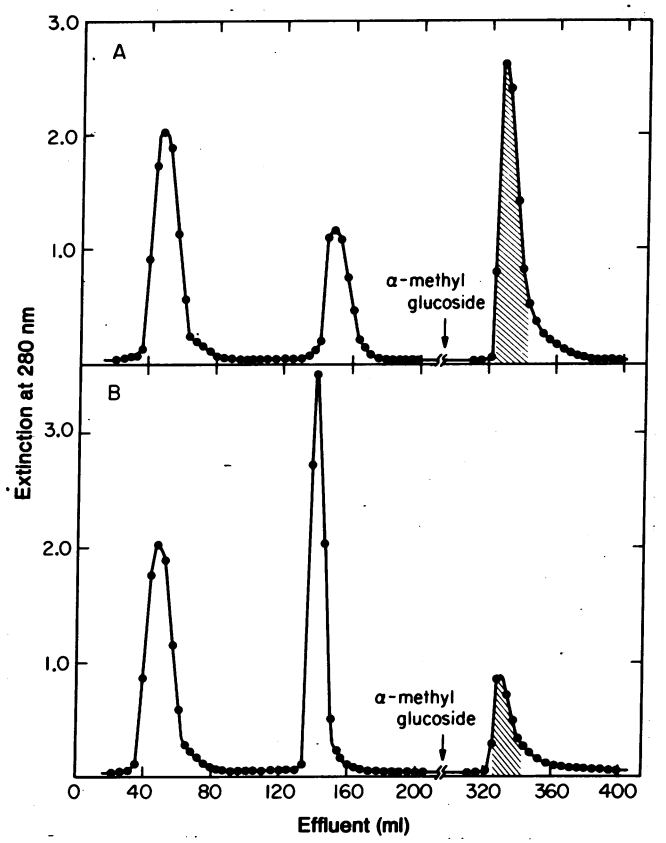

FIG. 1. Elution profile of partially purified CRM of normal liver (*A*) and that of homozygous variant liver (*B*) from a concanavalin A-Sepharose column. About 200 mg of protein, previously purified through Sephadex G-200 gel filtration, was applied to a 2 × 30-cm column. The column was washed with 10 mM sodium acetate, pH 5.8, containing 0.9% NaCl, and at the point indicated by an *arrow* elution was started with 50 mM α-methyl-D(+)-glucoside dissolved in the same buffer. The fractions indicated by the shaded areas, which had immunologic cross-reacting capacity, were collected.

The gradient was produced by adding 1500 ml of the buffer containing 0.5 M NaCl into a mixing chamber that contained 1500 ml of the buffer containing 0.1 M NaCl. (*5*) Sephadex G-200 gel filtration (2.2 × 100-cm column) in 20 mM sodium phosphate, pH 7.6. (*6*) Concanavalin A-Sephadex 4B column (2 × 30 cm) chromatography, on which CRM was eluted with 50 mM α-methyl-D(+)-glucoside dissolved in 10 mM sodium acetate, pH 5.8, containing 0.9% NaCl (Fig. 1). CRM was finally dialyzed against water and lyophilized. The yield of purified CRM was 42 mg from *MM* liver, 30.5 mg from *MZ* liver, and 18 mg from *ZZ* liver (about 600 g starting material each).

Properties of CRM Isolated from Normal and Variant Liver. The purified CRM from *MM* liver (CRM-M) and that from *ZZ* liver (CRM-Z) and A$_1$AT from normal serum each indicated a single protein band on polyacrylamide gel electrophoresis using a Tris–glycine buffer system at pH 9.3 (14) (Fig. 2). CRM-M moved faster toward the anode than CRM-Z, and CRM from the heterozygous variant *MZ* liver indicated two protein bands, one corresponding to CRM-M and the other corresponding to CRM-Z (Fig. 2). A$_1$AT from normal serum moved more slowly than the CRM from liver.

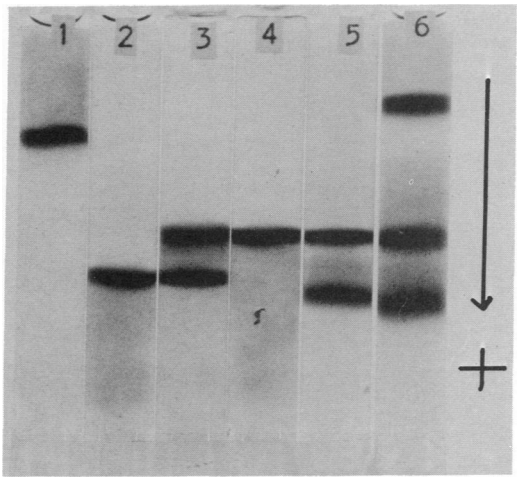

FIG. 2. Polyacrylamide gel electrophoresis of purified CRM and serum A$_1$AT. Electrophoresis was carried out in 7.5% acrylamide gel, with Tris–glycine, pH 9.3. (*1*) A$_1$AT from normal serum; (*2*) CRM from normal liver; (*3*) CRM from heterozygous variant liver; (*4*) CRM from homozygous variant liver; (*5*) mixture of 2,3, and 4; (*6*) mixture of 1,2,3, and 4.

Homogeneity of the purified CRM-M and CRM-Z was also demonstrated by ultracentrifugal analysis. Schlieren patterns of the sample solution showed single sedimentation boundaries. The sedimentation constant ($s_{20,w}$) of both CRMs was estimated as 2.57 ± 0.02 S.

For determination of the molecular weight of CRM and the serum A$_1$AT, the lyophilized samples were dissolved in 1% sodium dodecyl sulfate containing 0.01 M 2-mercaptoethanol and incubated at 37° for 1 hour. Analytical sodium dodecyl sulfate disc electrophoresis in various concentrations of polyacrylamide gel (ranging from 5 to 15%) was carried out as previously described (15). Bovine serum albumin, trypsin, beef heart lactate dehydrogenase, and alpha chain of human hemoglobin were used as markers.

CRM from both normal and variant liver indicated a single protein band on the sodium dodecyl sulfate gel and the molecular weight was estimated as 18,000. The molecular weight of serum A$_1$AT was estimated as about 55,000, confirming the previous value (MW 50,000–54,000), which was estimated by sodium dodecyl sulfate polyacrylamide electrophoresis (12, 16) and by the sedimentation equilibrium centrifugation method (12). Correspondence between the molecular weight determined by sodium dodecyl sulfate disc electrophoresis and the sedimentation constant estimated by ultracentrifugation suggests a single chain structure for the CRM.

The immunoprecipitation capacity of CRM and A$_1$AT was semi-quantitatively determined by double gel diffusion test on micro slides at pH 5.6 (0.05 M sodium acetate) and at pH 8.2 (0.05 M sodium Veronal–Tris), using rabbit antiserum to human A$_1$AT and the antigens at various concentrations. The purified CRM had much higher immunoprecipitation capacity against rabbit antiserum to human A$_1$AT than A$_1$AT itself. The minimum concentration of the antigens required to produce a discernible precipitin line in the double diffusion test was 1–2 μg/ml for CRM (at pH 8.2 and pH 5.6). For A$_1$AT the values were 8 μg/ml (at pH 8.2) and 120 μg/ml (at pH 5.6). At pH 8.2, a continuous precipitin line was observed and no spur was visible between the antiserum and A$_1$AT and

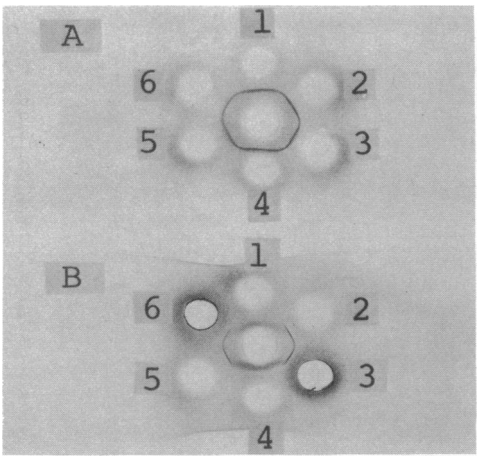

FIG. 3. Double gel diffusion on agar. (*A*) at pH 8.2 and (*B*) at pH 5.6. *Center well,* rabbit anti-A_1AT. *Outer wells, 1* and *4:* purified serum A_1AT; *2* and *5:* purified CRM-M; *3* and *6:* purified CRM-Z.

between the antiserum and CRM-M or CRM-Z, indicating immunologic homology of A_1AT and CRM (Fig. 3*A*). However, at pH 5.6, the precipitin line between the antiserum and A_1AT did not completely fuse with the precipitin between the antiserum and CRM (Fig. 3*B*). Since the immunoprecipitation capacity of A_1AT is lower at pH 5.6 than at pH 8.2, structural modification of A_1AT occurred at low pH, presumably producing the diffused precipitin line on double gel diffusion.

The purified CRM from all sources had very low trypsin inhibitory capacity. The trypsin inhibitory capacity expressed as milligrams of trypsin inhibited by one milligram of inhibitor protein are: CRM-M = 0.010; CRM from *MZ* liver = 0.006; CRM-Z = 0.001. Although it was not detected in polyacrylamide gel electrophoresis (Fig. 2), the low trypsin inhibitory capacity of the purified CRM could be due to small contamination (less than a few %) of A_1AT in these samples. The trypsin inhibitory capacity of pure A_1AT has been estimated as about 0.56. (12).

The above characterization was carried out on the samples prepared from frozen normal and variant livers. CRM purified from a fresh autopsy normal liver was identical to CRM purified from a frozen normal liver with respect to electrophoretic mobility and immunoprecipitation capacity.

DISCUSSION

This work has demonstrated that both normal and variant liver contain CRM that immunologically crossreacts with anti-A_1AT serum but has little trypsin inhibitory capacity. The yield of CRM from the normal, *MM*, liver was higher than that from the variant, *ZZ*, liver. Previous histological observations (8, 9) that show the existence of CRM in *ZZ* and *MZ* variant tissues, but not in normal tissues, are not in agreement with the present results.

Chemically CRM is a single chain glycoprotein with a molecular weight of about 18,000. Sialic acid content of CRM was estimated to be about six residues per molecule (unpublished observation).

CRM is not a product produced by intermolecular association of A_1AT with trypsin or other proteins, since the molecular weight of CRM is lower than that of A_1AT.

It is most likely that CRM is synthesized by the gene that controls A_1AT synthesis, because (*a*) the normal subject (*MM*) produces one type of CRM (CRM-M), (*b*) the homozygous variant (*ZZ*) produces another type of CRM (CRM-Z), and (c) the heterozygous variant subject (*MZ*) produces both CRM-M and CRM-Z. It should be mentioned that CRM-M is more acidic than CRM-Z, and similarly, A_1AT from the normal *MM* sera is more acidic than A_1AT from the variant *ZZ* sera (10).

Alternatively, a putative transferase, which transfers an acidic residue (or residues) such as sialic acid to both A_1AT and CRM, existing in the normal tissues, but not in the variant tissues, or a putative hydrolylase, which removes an acidic residue (or residues) from both A_1AT and CRM, existing in the variant tissues but not in the normal tissues, might induce the parallel secondary modification in A_1AT and CRM molecules. If this was the case, such enzymes should be dominantly expressed in heterozygous variant tissues, and the existence of two distinctive forms of CRM in the heterozygous variant tissues is not readily reconciled with this hypothesis.

The exact biological and structural relationships between CRM and A_1AT are not yet clear. CRM may be part of a precursor for A_1AT and, after activation and completion of the molecule, be released from tissues into sera. Or A_1AT may be trapped in tissues after partial degradation and inactivation resulting in the accumulation of CRM in the liver. These and other possibilities can be examined by comparison of the structure of A_1AT, CRM-M, and CRM-Z, and by *in vitro* activation of CRM.

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