Pediat. Res. 9: 123–126 (1975)

Neuraminidase
serum α_1-antitrypsin
sialic acid

Analysis of Homozygous Serum α_1-Antitrypsins: Effects of Neuraminidase

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Extract

The molecular basis for the complex electrophoretic banding patterns of serum α_1-antitrypsins of different protease inhibitor (PI) types is unknown. In order to demonstrate the role of sialic acid residues in this polymorphism, serums of homozygous PI, types FF, MM, and ZZ, were studied by acid starch and crossed electrophoresis, before and after treatment with neuraminidase. Increasing enzyme concentrations, temperatures, and times of incubation led to greater release of sialic acid and progressive slowing of PI bands. Exhaustive incubation of the three serum types with neuraminidase produced a single broad cathodal peak of α_1-antitrypsin.

Isoelectric focusing in gel with a pH 4–6 gradient, followed by immunoprecipitation of α_1-antitrypsin bands, resulted in complex banding patterns with the expected differences in isoelectric points among the three serum types. After exhaustive neuraminidase treatment, each of the banding patterns remained complex, but shifted toward a more basic pH. Although sialic acid residues are one of the major determinants of the net charge and electrophoretic mobility of different PI types, differences remain after removal of sialic acids which may represent other genetically determined differences in primary structure.

Speculation

Differences in isoelectric point of the banding patterns of PI, FF, MM, and ZZ α_1-antitrypsins, remaining after removal of their terminal sialic acid residues, suggest that other primary molecular differences exist among these inherited variants. By analogy with other well known protein polymorphisms, these differences are likely to consist of variations in primary amino acid sequence. Detailed analysis of purified PI system variants will be required to establish this possibility.

α_1-Antitrypsin, the major serum protease inhibitor in man, is inherited through a system of more than 20 codominant alleles which determine the amount of circulating α_1-antitrypsin as well as its electrophoretic mobility (6). Each allele of this PI (protease inhibitor) system produces up to eight protein bands, which can be demonstrated in starch gels at acid pH or on crossed antigen-antibody electrophresis. The biochemical reason for this structural polymorphism is not clear at the present time. The major hypotheses which have been suggested include: (*1*) inherited differences in carbohydrate side chain moieties, especially the sialic acid groups (1, 2) and (*2*) differences in primary amino acid structure, such as those found among the various hemoglobin types (3, 6, 7).

We have studied the effect of enzymatic removal of sialic acid from serum α_1-antitrypsins of homozygous PI types MM, FF, and ZZ. Removal of sialic acids alters the mobility of PI bands on acid starch gel electrophoresis and crossed antigen-antibody electrophoresis. Isoelectric focusing in gel, combined with immunofixation of PI bands, has demonstrated a shift of these bands to characteristic new isoelectric points after enzymatic removal of sialic acids. However, a complex banding pattern still remains, suggesting that genetically determined primary structural differences still exist between PI types.

METHODS AND MATERIALS

Human serum was obtained from freshly drawn, clotted venous blood specimens and stored at -70°. α_1-Antitrypsin function was determined by the method of Erlanger *et al.* (4), and α_1-antitrypsin concentration was measured by electroimmunoassay (8). PI typing was performed by the method of Fagerhol (5), using acid starch gel electrophoresis and crossed antigen-antibody electrophoresis. Serum samples used in this study included the homozygous PI types MM, FF (kindly provided by Dr. M. K. Fagerhol), and ZZ. Highly purified, functionally active PI MM α_1-antitrypsin was kindly provided by Dr. S. K. Chan, of the University of Kentucky.

Clostridium perfringens neuraminidase (with activity of 0.50 units/mg) was obtained from Worthington Biochemical Corp. The enzyme was diluted in water to a concentration of 0.02 mg/ml. Neuraminidase concentrations (Table 2) and time of incubation (Fig. 1) were varied. In another set of reactions 0.1 ml (0.001 units) of enzyme was incubated with 0.2-ml aliquots of PI MM serum, over a 1-hr period, at temperatures of 4°, 15°, 25°, 37°, and 46°. Reactions were stopped with 0.1 N sodium hydroxide, bringing the pH to 8. The sialic acid released in each of these studies was measured using the assay of Warren (14), and PI typing was performed on each sample by the method of Fagerhol (5).

In a separate set of experiments, 0.2 ml of serum of PI types FF, MM, or ZZ, or purified PI MM α_1-antitrypsin was incubated with 0.15 ml (0.0015 units) of neuraminidase at 37° for 24 hr. The reactions were stopped with sodium hydroxide and PI typing was done (5).

Isoelectric focusing was performed on the last group of samples by the method of Righetti and Drysdale (11), using a pH gradient of 4–6 in polyacrylamide gels. After electrofocusing to equilibrium, the α_1-antitrypsin bands were precipitated by overnight incubation in specific rabbit antiserum to the α_1-antitrypsin (15). The monospecificity of this antiserum for α_1-antitrypsin was confirmed by electroimmunoassay (8) and immunoelectrophoresis (12). The gels were then washed for several days in saline and stained for the precipitated immune

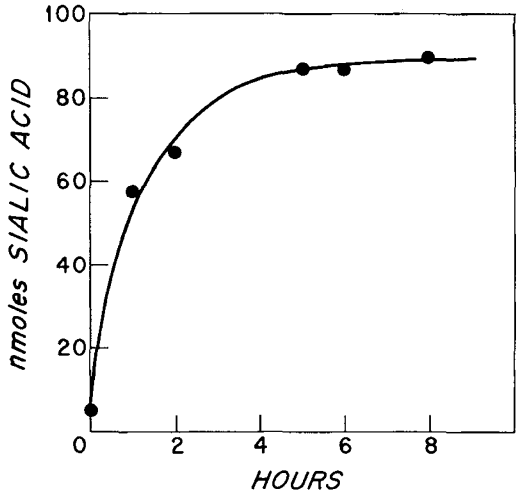

Fig. 1. Sialic acid release by neuraminidase from human plasma. While reacting 0.1 ml (0.0010 units) of enzyme solution with 0.2 ml protease inhibitor MM serum at 37°, the time of the reaction was varied from 1 to 8 hr.

complexes by Coomassie blue. (Duplicate control gels, incubated with nonimmune rabbit serum and washed free of protein, were blank when stained with Coomassie blue.)

Another commercially available enzyme, *Vibrio cholerae* neuraminidase (16), activity 500 units/ml, was incubated as above with PI MM serum for 24 hr, follwed by PI typing (5). In our laboratory, both Behring neuraminidase and a neuraminidase prepared by affinity chromatography remove sialic acid residues from erythrocyte surfaces, leaving the cells intact, and have thus been judged to be essentially free of proteolytic activity; Worthington neuraminidase does cause cell lysis and thus may contain some proteolytic enzyme.

RESULTS

SIALIC ACID RELEASE

α_1-Antitrypsin function and concentration for the serums of PI types MM, FF, and ZZ used in this study are shown in Table 1.

The release of sialic acid by increasing amounts of neuraminidase over a fivefold range of enzyme concentrations at 25° is presented in Table 2. When incubations were carried out for 5 hr, approximately twice the amount of sialic acid was released as in the 1-hr incubations. Incubation for 3 hr at 37° (not shown) produced a release of sialic acid approximately double that seen after 5 hr at 25°.

At 37°, 0.1 ml (0.001 units) of neuraminidase solution produced a plateau of release of sialic acid by 5 hr, which was maintained at 8 hr (Fig. 1).

Equal amounts of sialic acid were released by prolonged neuraminidase treatment or incubation of serum with 0.1 N sulfuric acid for 4 hr at 80°.

The serum trypsin inhibitory capacity was unchanged after exhaustive desialylation.

CHANGES OF PI BAND MOBILITY

The progressive release of sialic acid from serum with increasing concentrations of neuraminidase was accompanied by a change in the pattern of PI bands in PI type MM serum. There was a progressive decrease in the height of bands in the PI M zones, and an increase in zones of slower electrophoretic mobility, where the PI S and PI Z α_1-antitrypsin bands usually migrate (Fig. 2).

With time as a variable, increasing release of sialic acid was also accompanied by an increasingly slower migration of PI bands, and patterns resembling PI types SZ and ZZ resulted by 24 hr of incubation (Fig. 2). Incubation for 55 hr led to a disappearance of PI bands in the usual M, S, or Z regions, and the appearance of a single, broad α_1-antitrypsin peak which could be found cathodal to the sample origin.

Incubation of PI type MM serum with neuraminidase for 24 hr at either 4° or 25° produced essentially no change in the original pattern of PI bands, whereas incubation at 37° produced a pattern resembling PI type ZZ. Incubation for 48 hr at 4° produced some lowering of the PI M bands and the appearance of some slower bands; at 25° a "ZZ" pattern was produced, while at 37° the entire pattern of PI bands was observed in a more cathodal position.

Thus, with increasing concentrations of neuraminidase, increasing times of incubation, and increasing temperatures, alterations in the PI MM banding pattern were produced which resulted in the appearance of new, progressively slower bands.

Incubation of PI MM, FF, or ZZ serums or of purified PI MM α_1-antitrypsin for 24 hr with a larger amount of neuraminidase, 0.15 ml (0.0015 units), led to the disappearance of the banding pattern from the regions of PI M, S, and Z, and the appearance of a broad single peak of α_1-antitrypsin, cathodal to the sample origin (Fig. 2). The peak derived from each of these serums had a similar degree of cathodal migration.

The above results were all obtained using *C. perfringens* neuraminidase (Worthington). However, an identical, single, broad, cathodal α_1-antitrypsin peak was found on PI typing of a PI MM serum incubated for 24 hr with *V. cholerae* neuraminidase (Behring).

ISOELECTRIC FOCUSING

Serums of PI types MM, FF, and ZZ demonstrated complex banding patterns with different isoelectric points, when separated by isoelectric focusing in gels, and precipitated with antiserum to α_1-antitrypsin (Fig. 3). Each serum demonstrated two major bands and several minor bands. The isoelectric points of the PI type FF bands were more acid than that of the PI type MM serum, and the isoelectric points of the PI

Table 1. *Serums used in the study*[1]

PI type	Trypsin inhibitory capacity, mg/ml serum	α_1-Antitrypsin concentration, mg/ml
MM	0.86	1.50
FF	0.72	1.40
ZZ	0.30	0.70

[1] PI: Protease inhibitor.

Table 2. *Release of sialic acid in protease inhibitor (PI) MM serum by neuraminidase*[1]

Amount of enzyme, ml	Sialic acid released, nmol		
	Units	1 Hr	5 Hr
None	None	5.0	5.0
0.02	0.0002	8.2	16.1
0.04	0.0004	12.0	27.3
0.06	0.0006	18.0	33.7
0.08	0.0008	22.5	40.1
0.10	0.0010	25.3	42.3

[1] A PI MM serum (0.2 ml) was incubated with varying amounts of neuraminidase, from 0.02 ml (0.0002 units) to 0.10 ml (0.0010 units) of enzyme solution in 0.1 M ammonium acetate buffer (pH 5.0) for 1 or 5 hr in a total reaction volume of 0.5 ml, at 25°.

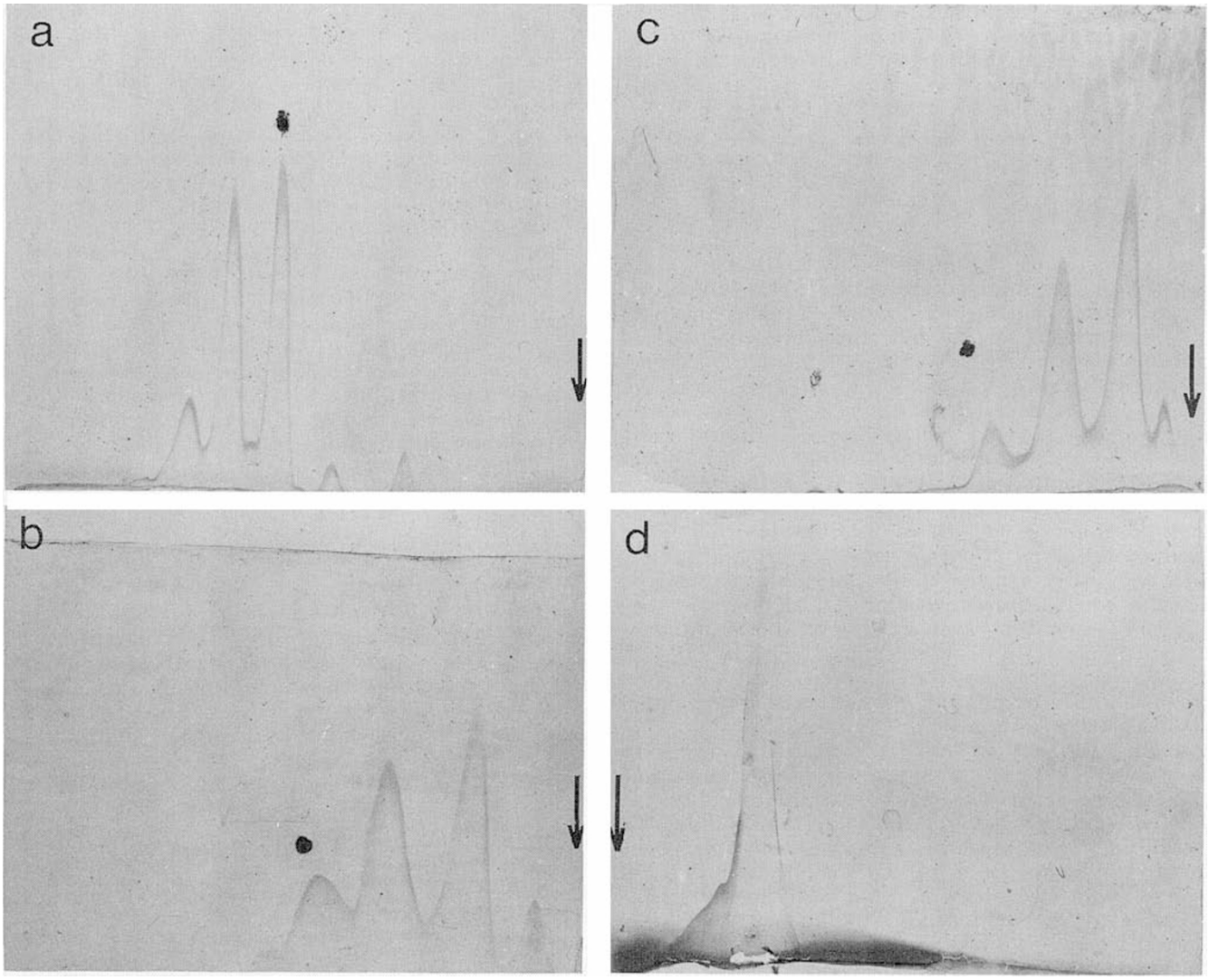

Fig. 2. Crossed electrophoretic patterns. *a:* normal protease inhibitor (PI) MM serum; *b:* PI MM serum after partial desialylation; *c:* PI ZZ serum; *d:* PI MM serum after exhaustive desialylation, showing cathodal migration (*dark spot:* location of band 6 of PI MM pattern, used as reference for *b* and *c;* ↓: location of origin of each sample on initial acid starch gel electrophoresis; anode is at the left of each pattern for initial starch electrophoresis and at the top of each pattern for crossed electrophoresis).

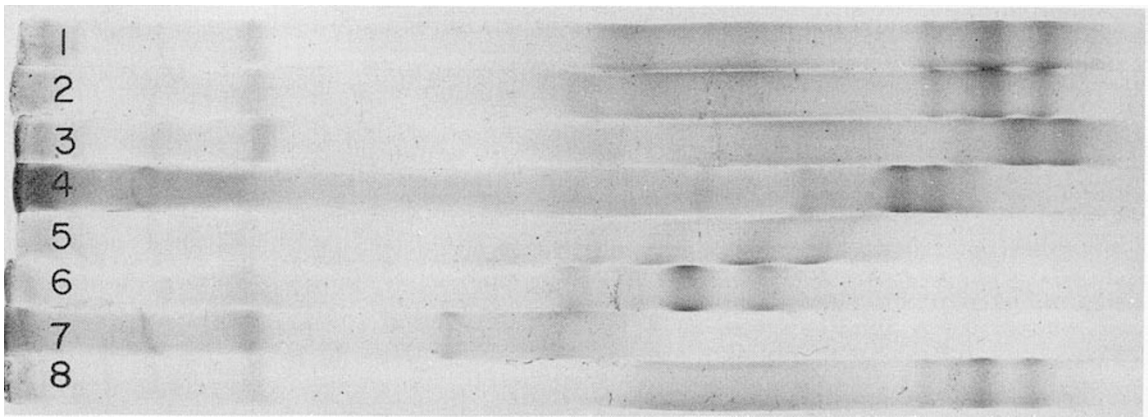

Fig. 3. Isoelectric focusing gels, developed with antiserum to α_1-antitrypsin. *1:* protease inhibitor (PI) MM serum; *2:* PI MM serum; *3:* PI FF serum; *4:* PI ZZ serum; *5:* PI MM serum, after exhaustive desialylation; *6:* PI FF serum, after exhaustive desialylation; *7:* PI ZZ serum, after exhaustive desialylation; *8:* PI MM serum.

type ZZ bands were more basic than that of PI type MM. (These findings are consistent with the relative mobility of these types on acid starch gel electrophoresis.)

After exhaustive incubation of these serums with neuraminidase, the banding pattern of each PI type was noted to shift toward a more basic pH. The pattern of the PI type FF bands was now intermediate between the desialylated MM and ZZ bands. A considerable heterogeneity of banding was still present in each of the types.

In addition to the major banding patterns which have just

been described, which shifted after incubation with neuraminidase, there appeared to be at least one distinct α_1-antitrypsin band identified by the antiserum at a rather basic pH, which did not appear to change after removal of sialic acid (Fig. 3). This was demonstrable in all three PI types studied and appeared in an identical position in the 4–6 gradient at the basic end.

DISCUSSION

In this study, it has been demonstrated that the release of sialic acid from serum α_1-antitrypsin may be correlated with a progressive slowing of the electrophoretic mobility of the complex banding pattern of the protein. Increasing concentrations of neuraminidase, increasing times of incubation and increase in temperature from 4° to 37° all result in a greater release of sialic acid and a greater slowing of PI bands. Further, α_1-antitrypsins of three different homozygous PI types have slightly different isoelectric points; these isoelectric points can be altered by removal of sialic acid but the banding patterns still remain complex, and still differ in mobility from one another. Thus, it appears that the molecular basis for the complex pattern of PI bands in each type of serum α_1-antitrypsin and the difference between types is not simply a difference in the number of sialic acids, but must depend upon other genetically determined structural differences.

Laurell (7) first demonstrated in 1965 that α_1-antitrypsins of three different electrophoretic types could be made to move more slowly on crossed electrophoresis at pH 8.6 after incubation with neuraminidase, but still retained their differences in mobility and their complex patterns.

Recently, Bell and Carrell (1) have demonstrated that the appearance of PI type ZZ α_1-antitrypsin could be duplicated by partial removal of sialic acid from α_1-antitrypsin of PI type MM. As demonstrated in the present study, these authors were able to show a progressive decrease in mobility in the α_1-antitrypsin in agar gel at pH 8.6 with increasing times of incubation with neuraminidase. It was also demonstrated in their study that the addition of 6 M urea to the acid starch gel system of Fagerhol (5) results in a single band of α_1-antitrypsin. It was concluded, therefore, that the essential differences among inherited PI types were in the carbohydrate side chain, specifically in the amount of sialic acid present. More recently, Cox (2) has confirmed the results of Bell and Carrell (1) and reiterated that the molecular abnormality in α_1-antitrypsin deficiency resides in the number of sialic acid residues.

In contrast, Crawford (3) has found that the pattern of α_1-antitrypsin in isoelectric focused gels moves as a unit to a higher pH after neuraminidase treatment. He concluded that it was likely that some form of amino acid difference was responsible for the heterogeneity in banding pattern. Laurell (9) has confirmed these results recently by electrophoresis on acid gel composed of acrylamide and agarose.

The existence of a band present in all the PI types at the more basic end of the pH 4–6 gradient suggests a molecular form of α_1-antitrypsin not affected by neuraminidase and common to all PI types. This form of α_1-antitrypsin may represent a complex of this protein with immunoglobulin A, which has been demonstrated by Tomasi and Hauptman (13) in myeloma sera, a complex with a protease, or a degraded form (10).

The exact molecular basis for the banding patterns of a single α_1-antitrypsin type and the structural differences between the various inherited PI types will await the detailed analysis of the PI bands obtained from purified α_1-antitrypsins. However, the results of this study suggest strongly that, while the sialic acid residues of different α_1-antitrypsin types are one of the major determinants of their net charge and electrophoretic mobility, other differences remain after the removal of sialic acids which may represent genetically determined differences in primary structure.

SUMMARY

In order to demonstrate the role of sialic acid residues in the polymorphism of serum α_1-antitrypsin, serums of homozygous PI types FF, MM, and ZZ were studied by acid starch and crossed electrophoresis, before and after treatment with neuraminidase. Increasing enzyme concentrations, temperatures, and times of incubation led to greater release of sialic acid and progressive slowing of PI bands. Exhaustive incubation of the three serum types with neuraminidase produced a single broad cathodal peak of α_1-antitrypsin.

Isoelectric focusing in gel, followed by immunoprecipitation of α_1-antitrypsin bands, resulted in complex banding patterns with the expected differences in isoelectric points among the three serum types (ZZ, MM, FF). After neuraminidase treatment, each of the banding patterns remained complex, but shifted toward a more basic pH. Although sialic acid residues are one of the major determinants of the net charge and electrophoretic mobility of different PI types, differences remain after removal of sialic acids which may represent other genetically determined differences in primary structure.

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17. The authors thank Ruth L. Coston for her expert technical assistance.
18. Dr. R. C. Talâmo is recipient of United States Public Health Service Research Career Development Award AI31937.
19. This work was supported by United States Public Health Service Research Grants nos. HL 14479 and CA-12389 and American Cancer Society Grant no. IM-14A.
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21. Accepted for publication November 1, 1974.

Printed in U.S.A.