Structural heterogeneity of faecal $\alpha_1$ antitrypsin shown by immunoblot analysis in patients with Crohn's disease

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Abstract
Faecal $\alpha_1$ antitrypsin was determined in 34 patients with Crohn's disease and in 19 healthy subjects by immune nephelometry. A structural analysis of faecal $\alpha_1$ antitrypsin was carried out using immunoblot analysis under non-reducing conditions. Native serum $\alpha_1$ antitrypsin migrated with an apparent molecular weight of 45 kDa. Proteolytic $\alpha_1$ antitrypsin fragments (5–42 kDa) were specifically immunostained in 13/19 and 22/34 stool samples from control subjects and from patients with Crohn's disease respectively. There was a weak correlation ($r=0.47\, ,\ p<0.02$) between the molecular weight of fragmented $\alpha_1$ antitrypsin and the faecal concentration in both groups, indicating that $\alpha_1$ antitrypsin inhibits its own proteolysis by intestinal proteases in a dose dependent way. The incidence of polymeric forms (>45 kDa) was similar in patients (10/34) and control subjects (5/19). In only one case in each group was the native serum form of $\alpha_1$ antitrypsin found in faeces. We conclude that faecal $\alpha_1$ antitrypsin differs structurally from the native serum form. Immunochemical measurements, therefore, reflect rather than represent faecal concentrations of $\alpha_1$ antitrypsin. The controversial results in published reports may be partly explained by these findings. The molecular heterogeneity of faecal $\alpha_1$ antitrypsin is not specifically associated with Crohn's disease.

The use of faecal $\alpha_1$ antitrypsin excretion as a measure of enteric protein loss and the activity of Crohn's disease remains controversial. In a number of studies excellent correlations between the activity of Crohn's disease and faecal excretion or clearance of $\alpha_1$ antitrypsin have been described whereas we and others have found little or no interdependence between these parameters. It has been suggested that differences in patient selection may contribute to the differences found in published reports. Another possible explanation was offered by Buffone and Shulman. They found two forms of $\alpha_1$ antitrypsin in stool samples from Crohn's disease patients, one showing a higher molecular weight than the native serum form. They claimed that by using standard radial immunodiffusion these smaller $\alpha_1$ antitrypsin forms are underestimated by almost 20% compared with the native serum form.

Recently, we described a simplified procedure for determining faecal $\alpha_1$ antitrypsin, which is suitable for routine clinical purposes. In this paper we report on our investigation of whether structural alterations of $\alpha_1$ antitrypsin in the intestine interfere with the measurement carried out by this method in a predictive way. Given the contradictory findings regarding molecular heterogeneity of faecal $\alpha_1$ antitrypsin, our interest was also focused on a possible association between certain forms of faecal $\alpha_1$ antitrypsin and Crohn's disease.

Methods

Quantitative and Qualitative Analysis of Faecal $\alpha_1$ Antitrypsin
Aqueous extracts of stool specimens from 34 patients with Crohn's disease (21 men, 13 women, aged 20–50 years, median 28 years) and 19 healthy subjects (nine men, 10 women, aged 21–68 years, median 30 years) were prepared and the faecal $\alpha_1$ antitrypsin concentration was determined by immune nephelometry as previously described.

Aliquots (1 ml) of stool extracts were subjected to trichloroacetic acid (7–2%) precipitation for 30 minutes at 4°C. Protein precipitates were centrifuged (10 000 g, 20 minutes, 4°C) and washed by two cycles of resuspension in 120 mM Glycine, 20 mM Tris-HCl, pH 8.3 containing 20% methanol (transfer buffer), followed by a further centrifugation. The final pellets were suspended in 0.1 ml of 125 mM Tris-HCl, 5% sodium dodecyl sulphate, 0.01% bromophenol blue, 10% glycerol, pH 6.8, and incubated at room temperature for 2 hours with 1% SDS and the solution was electrophoresed by centrifugation (10 000 g, 15 minutes, room temperature).

Aliquots (50 μl) of the supernatant were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions, using a Mighty-Small slab gel unit (Hoefer Scientific, USA). Gradient gels (8×8 cm) contained 5–25% acrylamide and 0.08% bisacrylamide. Electrophoresis was carried out at a constant voltage (150 volts) and temperature (4°C) for two hours, using a discontinuous buffer system. After equilibrating gels for 20 minutes with transfer buffer, proteins were electrophoretically transferred (200 mA for two hours) onto 8×8 cm nitrocellulose sheets, using a Mighty-Small Trans-
phor tank-blotting unit (Hoefer Scientific, USA).

Immunostaining of nitrocellulose-adsorbed α-antitrypsin was carried out in phosphate buffered saline with gentle shaking at room temperature. Non-specific protein binding sites of the nitrocellulose were first blocked by incubation for 10 minutes with phosphate buffered saline containing 2% non-fat dry milk powder (Toepfer, FRG), followed by admixture of 0.1% of the antibody-horseradish peroxidase conjugate and further incubation for one hour. Unbound conjugate was then removed by two washing cycles (five minutes each) and the staining was developed for 2–10 minutes with 0.1% diaminobenzidine and 0.01% H₂O₂ in 100 mM imidazole buffer, pH 8.0, containing 2 mM CoCl₂. The molecular size of the stained protein bands was judged by comparing the migration distance with that of prestained marker proteins, which were included in each run.

REAGENTS

Chemicals for SDS-PAGE were obtained from Roth, FRG. Nitrocellulose, 0.2 μm pore size, was purchased from Schleicher und Schüll, FRG. Human α-antitrypsin and prestained molecular weight marker proteins were from Sigma, Deisenhofen. Horseradish peroxidase-labelled rabbit anti-human α-antitrypsin-antiserum was bought from Organon Technika, USA. All other reagents were of the highest degree of purity commercially available.

Results

To avoid the dissociation of protein complexes and polymers, disulphide bond cleavage was omitted from SDS-PAGE. Under non-reducing conditions native serum α-antitrypsin migrated with an apparent molecular weight of 45 kDa (Figs 1 and 2, serum sample) while under reducing conditions a molecular weight of 54 kDa was found (data not shown). In both cases the antibody also recognised minor amounts of a proteolytic degradation product with a molecular weight of ~20 kDa (Figs 1 and 2, serum sample).

The comparative qualitative and quantitative analysis of α-antitrypsin in the faeces of 34 patients with Crohn’s disease at various stages of disease activity (Fig 1) and of 19 healthy subjects (Fig 2) is presented in decreasing order of faecal

Figure 1: Faecal α-antitrypsin in aqueous stool extracts of patients with Crohn’s disease. Comparison of results obtained by immunoblot analysis and immune nephelometry.

Figure 2: Faecal α-antitrypsin in aqueous stool extracts of healthy subjects. Comparison of results obtained by immunoblot analysis and immune nephelometry.
concentration. In 13 of the control samples (Fig 2, lanes from top: 3, 5–6, 9–11, 13–19) and in 22 of the patient samples (Fig 1, lanes from top: 1–5, 9, 12–16, 21, 23, 25, 27–34) proteolytic α, antitrypsin fragments of various sizes (5–42 kDa) were specifically immunostained. We plotted in both groups the observed molecular weight of the α, antitrypsin fragments against the respective faecal concentration as determined by immune nephelometry (Fig 3) and found a weak positive correlation (Spearman’s correlation coefficient r=0.467, p<0.025), indicating that α, antitrypsin inhibits its own proteolysis by intestinal proteases in a dose dependent way and that in most samples inhibition is incomplete.

In 10 samples from Crohn’s disease patients (Fig 1, lanes from top: 6, 8, 10, 13, 15–17, 19–20, 25) and in five normal samples (Fig 2, lanes from top: 1–2, 5, 7–8) protein bands with a higher molecular weight (>45 kDa) than that of the native serum form were immunostained. These bands consisted of either heteropolymers or partially degraded homopolymers of α, antitrypsin, because they were too small (46–50 kDa) to be intact di/polymers.

As summarised in Figure 4 the incidence of proteolytic degradation products of α, antitrypsin was 67% and 68% and the incidence of complexes larger than the native serum form of α, antitrypsin was 30% and 27% in Crohn’s disease patients and control subjects respectively. The native serum form occurred only in one sample from each collective (Fig 1, lane 11; Fig 2, lane 4).

**Discussion**

The choice of α, antitrypsin as a faecal marker for enteric protein loss is in part based on the assumption that due to its protease inhibitory properties α, antitrypsin is not modified by digestive enzymes in the intestinal tract. Given recent findings and the data presented here, however, this assumption is invalid. The formation of stable complexes with digestive enzymes and intestinal proteolytic degradation result in a pronounced structural heterogeneity of faecal α, antitrypsin. Obviously, the predominant enteral modification of α, antitrypsin is proteolysis, which was found in samples from patients and control subjects with a similar frequency (Fig 4).

It is apparent from our data that with increasing intestinal concentations α, antitrypsin becomes more efficient in inhibiting its own proteolysis. In most of the patient samples (26/34) faecal α, antitrypsin concentrations were higher (>0.49 mg/g) than in normal samples. Consequently, these samples showed higher molecular weight fragments of α, antitrypsin compared with normal samples. Molecular weight patterns of faecal α, antitrypsin, however, were similar in patient and normal samples when both were in the same concentration range (<0.5 mg/g). The structural pattern of faecal α, antitrypsin is mainly determined by its concentration in the faeces, and there seems to be no principal difference between healthy subjects and patients suffering from chronic inflammatory bowel disease with regard to this phenomenon. Contrary to the findings of Mizon et al, we could not find an association between certain molecular forms of faecal α, antitrypsin and Crohn’s disease. We therefore think that morphological differences in faecal α, antitrypsin can hardly be used as a diagnostic tool.

In the light of these data and the findings of others it remains uncertain whether faecal α, antitrypsin can be correctly measured by immunochemical methods, which make use of antisera raised against the native serum form and which are calibrated with serum standards. Given the pronounced structural heterogeneity of faecal α, antitrypsin, it also seems difficult to define a common antigen structure, which could be the basis of a better immunochemical measurement. From a practical point of view, the immunochemical approach still seems to be the most suitable to differentiate between high and low concentrations of faecal α, antitrypsin, although it yields semiquantitative data, which reflect rather than represent the faecal concentration of α, antitrypsin. Various systems of measurement, such as nephelometry and radial diffusion, and sample preparation methods are probably affected to some extent by molecular antigen alterations. This can, in part, explain the controversial results in published reports on the use of faecal α, antitrypsin in estimating the inflammatory activity of inflammatory bowel diseases.
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