Structural heterogeneity of faecal α_1 antitrypsin shown by immunoblot analysis in patients with Crohn's disease

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Abstract

Faecal α_1 antitrypsin was determined in 34 patients with Crohn's disease and in 19 healthy subjects by immune nephelometry. A structural analysis of faecal α_1 antitrypsin was carried out using immunoblot analysis under non-reducing conditions. Native serum α_1 antitrypsin migrated with an apparent molecular weight of 45 kDa. Proteolytic α_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 α_1 antitrypsin and the faecal concentration in both groups, indicating that α_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 α_1 antitrypsin found in faeces. We conclude that faecal α_1 antitrypsin differs structurally from the native serum form. Immunochemical measurements, therefore, reflect rather than represent faecal concentrations of α_1 antitrypsin. The controversial results in published reports may be partly explained by these findings. The molecular heterogeneity of faecal α_1 antitrypsin is not specifically associated with Crohn's disease.

The use of faecal α_1 antitrypsin excretion as a measure of enteric protein loss and the activity of Crohn's disease remains controversial.¹ In a number of studies excellent correlations between the activity of Crohn's disease and faecal excretion or clearance of α_1 antitrypsin have been described²⁻⁷ whereas we⁸ and others^{9,10} 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.^{7,8} Another possible explanation was offered by Buffone and Shulman.⁹ They found two forms of α_1 antitrypsin in stool samples from Crohn's disease patients, one showing a higher molecular weight than the native serum form. They suggested that stable complexes of α_1 antitrypsin and trypsin or chymotrypsin are formed in the intestine. As they showed, both digestive enzymes may interfere with the immunochemical determination of α_1 antitrypsin *in vitro*.⁹ Colombel *et al* reported finding two forms of α_1 antitrypsin in the faeces of Crohn's disease patients, both having a lower molecular weight than the native

serum form.¹¹ They claimed that by using standard radial immunodiffusion these smaller α_1 antitrypsin forms are underestimated by almost 20% compared with the native serum form.¹²

Recently, we described a simplified procedure for determining faecal α_1 antitrypsin, which is suitable for routine clinical purposes.¹³ In this paper we report on our investigation of whether structural alterations of α_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 α_1 antitrypsin, our interest was also focused on a possible association between certain forms of faecal α_1 antitrypsin and Crohn's disease.

Methods

QUANTITATIVE AND QUALITATIVE ANALYSIS OF FAECAL α_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 α_1 antitrypsin concentration was determined by immune nephelometry as previously described.¹³

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 12 hours followed by centrifugation (10 000 g, 15 minutes, room temperature).

Aliquots (50 μ l) 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 $\times$ 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.¹⁴ After equilibrating gels for 20 minutes with transfer buffer, proteins were electrophoretically transferred (200 mA for two hours) onto 8 $\times$ 8 cm nitrocellulose sheets,¹⁵ using a Mighty-Small Trans-

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phor tank-blotting unit (Hoefer Scientific, USA).

Immunostaining of nitrocellulose-adsorbed α_1 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% H_2O_2 in 100 mM imidazole buffer, pH 8.0, containing 2 mM $CoCl_2$. 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_1 antitrypsin and prestained molecular weight marker proteins were from Sigma, Deisenhofen. Horseradish peroxidase-labelled rabbit anti-human α_1 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_1 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_1 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_1 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_1 antitrypsin in aqueous stool extracts of healthy subjects. Comparison of results obtained by immunoblot analysis and immune nephelometry.

Figure 3: Relation between concentration and observed molecular weight of faecal α_1 antitrypsin in control subjects and patients with Crohn's disease. Each protein band observed in immunoblot analysis is represented by a separate point.

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_1 antitrypsin fragments of various sizes (5–42 kDa) were specifically immunostained. We plotted in both groups the observed molecular weight of the α_1 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_1 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_1 antitrypsin, because they were too small (46–50 kDa) to be intact di(poly)mers.

As summarised in Figure 4 the incidence of proteolytic degradation products of α_1 antitrypsin was 67% and 68% and the incidence of complexes larger than the native serum form of α_1 antitrypsin was 30% and 27% in Crohn's

Figure 4: Incidence of various molecular forms of α_1 antitrypsin in aqueous stool extracts of 34 patients with Crohn's disease and 19 healthy subjects.

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_1 antitrypsin as a faecal marker for enteric protein loss is in part based on the assumption that due to its protease inhibitory properties α_1 antitrypsin is not modified by digestive enzymes in the intestinal tract.²¹⁶ Given recent findings^{9 11 12} and the data presented here, however, this assumption is invalid. The formation of stable complexes with digestive enzymes⁹ and intestinal proteolytic degradation result in a pronounced structural heterogeneity of faecal α_1 antitrypsin. Obviously, the predominant enteral modification of α_1 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 concentrations α_1 antitrypsin becomes more efficient in inhibiting its own proteolysis. In most of the patient samples (26/34) faecal α_1 antitrypsin concentrations were higher (>0.49 mg/g) than in normal samples. Consequently, these samples showed higher molecular weight fragments of α_1 antitrypsin compared with normal samples. Molecular weight patterns of faecal α_1 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_1 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*,^{11 12} we could not find an association between certain molecular forms of faecal α_1 antitrypsin and Crohn's disease. We therefore think that morphological differences in faecal α_1 antitrypsin can hardly be used as a diagnostic tool.

In the light of these data and the findings of others^{9 11 12} it remains uncertain whether faecal α_1 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_1 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_1 antitrypsin, although it yields semiquantitative data, which reflect rather than represent the faecal concentration of α_1 antitrypsin. Various systems of measurement, such as nephelometry and radial diffusion, and sample preparation methods¹⁷ are probably affected to some extent by molecular antigen alterations.^{9 11 12} This can, in part, explain the controversial results in published reports on the use of faecal α_1 antitrypsin in estimating the inflammatory activity of inflammatory bowel diseases.

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