

Quantitative immunoelectrophoretic analysis of the plasma proteins in the sol phase of sputum from patients with chronic bronchitis

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SYNOPSIS An analysis of the plasma proteins in the sol phase of sputum was carried out using quantitative cross immunoelectrophoresis. The average concentrations of nine plasma proteins were estimated in the sol phase of sputum specimens from 30 patients with chronic bronchitis and the values were compared with the concentrations of these proteins in saliva and serum specimens from the same group of patients.

The results showed that α_1 antichymotrypsin and IgA concentrations were higher in the sol phase of sputum than would be expected if their presence were due entirely to passive transudation.

Chronic bronchitis, asthma, and cystic fibrosis are characterized by hypersecretion of mucus but the non-homogeneous nature of such secretions has placed considerable limitations on the study of these chronic chest diseases. The concentrations of soluble components of bronchial secretion, however, can be measured accurately in the sol phase of sputum (Ryley and Brogan, 1968) and it has been suggested that elevation of the relative concentration of plasma proteins in sputum may be indicative of inflammation or of damage to the lung parenchyma (Brogan, Ryley, Allen, and Hutt, 1971). Much of the work carried out on the characterization of the soluble sputum proteins has been restricted to qualitative study of plasma proteins, usually employing immunoelectrophoretic methods (Schultze and Heremans, 1966), and quantitative analysis of the serum proteins in sputum has mostly been confined to the immunoglobulins and to albumin (Falk, Okinaka, and Siskind, 1972). However, other serum proteins apart from immunoglobulins may have a biological role in the respiratory tract; for example, there appears to be a relationship between α_1 antitrypsin deficiency in serum and panlobular emphysema (Eriksson, 1965; Talamo, 1971).

The introduction of quantitative cross immunoelectrophoresis by Clarke and Freeman (1968) has now made possible the simultaneous estimation of many proteins in a single specimen. In the

present investigation, quantitative cross immunoelectrophoresis was used to characterize and measure the relative concentrations of the principal serum proteins found in the sol phase of sputum of a series of patients with chronic bronchitis. An attempt was also made to ascertain whether or not the plasma proteins were present in the ground substance of sputum purely as a result of passive transudation from the pulmonary vascular bed. It is not possible to obtain normal bronchial secretion in amounts sufficient for study so patients with chronic bronchitis were chosen for study as they constitute a group with less inflammatory transudate in their sputum than patients with other chest diseases (Brogan *et al*, 1971).

Patients and Methods

PATIENTS

A series of 30 patients who were suffering from chronic bronchitis on the criteria of the MRC Committee on the Aetiology of Chronic Bronchitis (1965) were studied; included in the group were patients suffering from simple, recurrent, and obstructive chronic bronchitis. The majority of those studied were inpatients of various of the hospitals of the University Hospital of Wales (Cardiff) Hospital Management Committee but three were under the care of their own general practitioners and were treated at home; all were receiving antibiotic therapy (tetracycline or

ampicillin) for exacerbations of their chest disease. The average age of the group was 59 ± 12 years and the number of women patients was nine.

Twenty-four-hour collections of sputum were made from each of the patients and the sol phase of the mucus was separated by ultracentrifugation at 120 000g ($r_{av} = 8.5$ cm) for four hours at 4°C (Brogan *et al*, 1971). The sol phases were stored at -70°C. It was only possible to obtain blood specimens at the same time as sputum from eight patients of the group; the serum was separated and stored at -70°C. Specimens of saliva were collected from 10 of the patients by the method described by Ryley (1972).

REAGENTS

Serum proteins

Albumin was obtained from Hoechst Pharmaceuticals Ltd, Portland House, London, SW1. Alpha₁ acid glycoprotein was prepared by the method of Bezkorovainy and Winzler (1961), IgA was prepared by the method of Zschocke, Griebler, Bach, and Anderson (1969) and IgG by the method of Joustra and Lundgren (1969). IgG heavy and light chains were prepared by the method of Fleischman, Pain, and Porter (1962) from the purified IgG. Alpha₁ antichymotrypsin was also prepared in this laboratory from human serum using CM Sephadex ion exchange, DEAE Sephadex ion exchange, ammonium sulphate precipitation, and a final separation in Sephadex G150; details of this method will be described elsewhere. A standard human serum was obtained from Hoechst Pharmaceuticals Ltd, and this reagent was used to prepare standard curves for the estimation of various of the serum proteins

Antiserum to plasma proteins

New Zealand white rabbits were used in the preparation of all antisera. In the preparation of antihuman serum protein, 1 part of a 1:2 dilution of human serum in water was emulsified with 1 part of Freund's complete adjuvant (Difco Laboratories, East Molesey, Surrey, England) and 1 ml of this mixture was injected into each of two sites along the back of the rabbit and intramuscularly into each hind leg. After four weeks, 1 ml amounts of the same adjuvant mixture were injected into each of the rabbits hind legs and, after a further week, the rabbits were bled from the marginal ear vein.

Alpha₁ acid glycoprotein, IgA, and IgG were prepared in the same manner using aqueous solutions containing 1 mg per ml of the purified protein. Antiserum to IgA was made specific to the alpha chain by absorption with IgG immunoabsorbent. Antiserum to IgG was made specific to the gamma

chain by absorption with IgG light chains. Antisera to alpha₁ antitrypsin, alpha₁ antichymotrypsin, alpha₁-B-glycoprotein, alpha₁ lipoprotein, alpha₂ macroglobulin, ceruloplasmin, haptoglobin, transferrin, beta_{1c} globulin, beta₂ globulin, and haemopexin were obtained from Hoechst Pharmaceuticals Ltd.

QUANTITATIVE IMMUNOELECTROPHORESIS AND IDENTIFICATION OF PRECIPITIN ARCS

Cross immunoelectrophoresis was carried out by a method closely similar to that of Clarke and Freeman (1968) on 10 × 10 cm glass slides; the details of the technique have been described previously (Ryley, 1972). Each analysis was carried out with 4 μl volumes of sputum sol phase or with 4 μl volumes of a 1:40 dilution in 0.9% NaCl of the serum specimens. Of antihuman serum protein antiserum 0.2 ml was incorporated into the antibody agarose phase. The immunoprecipitation arcs were identified with specific antisera and the purified proteins by methods described by Kröll (1969a). Areas under the arcs were measured by projection and the relative concentrations of the proteins were estimated from standard curves. These were constructed by plotting the areas occupied by the arcs in the immunoelectrophoretograms against the corresponding concentrations of the proteins when a series of dilutions of the standard serum were subjected to cross electrophoresis. The concentration of alpha₁ antichymotrypsin in the standard serum was not determined by the manufacturer so pure alpha₁ antichymotrypsin was used to prepare the standard curve. IgG was estimated by the electroimmunodiffusion method (electroimmunoassay) described by Lopez, Tsu, and Hyslop (1969).

Results

DISTRIBUTION OF PLASMA PROTEINS IN THE SOL PHASE OF SPUTUM

Quantitative cross immunoelectrophoresis of the sputum sol phase against antihuman serum gave patterns similar to that shown in the figure. Albumin, alpha₁ antitrypsin, transferrin, and IgA were detected in all 30 specimens and alpha₁ antichymotrypsin, alpha₁ acid glycoprotein, haptoglobin, and beta_{1c} globulin were detected in over 90% of specimens; those specimens in which they were absent were characterized by an overall low protein content. Although IgG was not seen in the cross immunoelectrophoretic patterns, all 30 specimens contained amounts of IgG that could be estimated by the electroimmunoassay method.

Alpha₁-B-glycoprotein and haemopexin were detected in about 40% of the specimens; haemopexin may have been present in more of the specimens

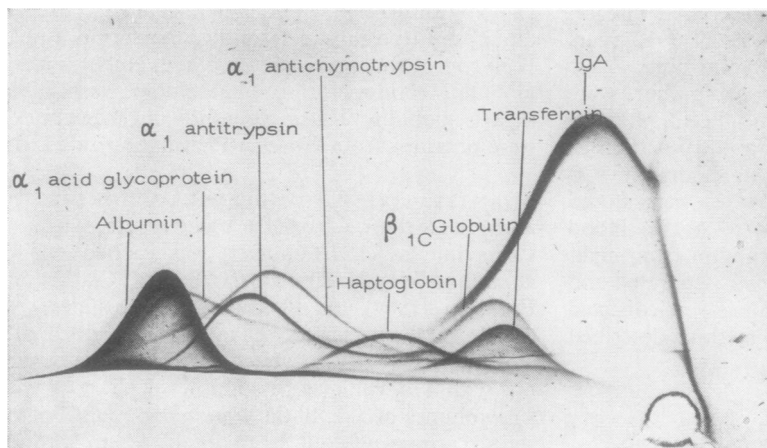


Fig Photograph of a cross immunoelectrophoretic pattern of the sol phase of sputum from a patient with chronic bronchitis showing eight of the nine plasma proteins commonly found in the continuous phase of the mucous ground substance. The antihuman serum protein antiserum was incorporated into the antibody agarose phase.

but the arc was faint and often obscured by the transferrin and β_{1C} globulin arcs. Ceruloplasmin was found in about 25% of the specimens, α_2 macroglobulin was found in only one specimen and α_1 lipoprotein and β_2 globulin were never detected. The α_1 antitrypsin arc gave two peaks in just over 50% of the specimens; the more cathodal peak was probably due to an antitrypsin-proteolytic enzyme complex (Ohlsson, 1971). The α_1 antichymotrypsin arc gave a single peak in all specimens though it was seen to be broader in the sputum than in the serum specimens.

A number of α_1 globulins have been under preparation in this laboratory and partially purified fractions, when used as reagents, suggested the presence in the sputum sol phase of α_1 -B-glycoprotein and α_1 antichymotrypsin before their identity was confirmed with commercially prepared specific antisera. However, no evidence has been obtained, when the partially purified fractions were used as reagents, of the presence in the sputum sol phase of either inter- α -trypsin inhibitor or Gc globulin.

CONCENTRATION OF PLASMA PROTEINS IN THE SOL PHASE OF SPUTUM, IN CORRESPONDING SERUM SPECIMENS, AND IN SPECIMENS OF SERUM FROM NORMAL SUBJECTS

The sensitivity of the method of estimating plasma proteins by cross electrophoresis lay between 0.1 and 0.5/100 ml for most plasma proteins. The concentrations of the plasma proteins most commonly found in sputum were also estimated in corresponding serum specimens from eight of

the patients and in a group of eight normal subjects (see table). Although mean concentrations of plasma proteins in sera from the bronchitic patients differed from those of the normal subjects, the differences were only statistically significant in the case of albumin, α_1 acid glycoprotein, and haptoglobin. The values obtained mostly lay within expected limits with the exception of β_{1C} globulin; the concentration of this plasma protein was almost one third of that reported as the normal plasma concentration (Becker, Rapp, Schwick, and Storiko, 1968) but it is likely that this protein was consumed during the clotting of the sera and was converted to β_{1A} globulin (Krøll, 1969b).

A comparison of the average plasma protein concentrations of matched specimens of sputum in the sol phase and serum showed that the sol phase IgA concentration was approximately 1:6 of that in serum. Sputum sol phase IgA, however, is a mixture of 7S and secretory IgA and estimation of this immunoglobulin by cross electrophoresis as 7S globulin was thus only semiquantitative and may have been an underestimate of the true concentration. Corresponding concentration ratios in the sol phase of sputum and serum showed that the sol phase α_1 antichymotrypsin was approximately 1:35 of that in serum, and the sputum sol phase concentrations of albumin, α_1 acid glycoprotein, α_1 antitrypsin, transferrin, and IgG were all approximately 1:120 of that in serum since the individual ratios of the proteins did not differ significantly from one another. The average ratio of the concentrations of albumin : IgA in serum was 21.8 ± 11.5 and this was significantly higher than the corresponding average ratio in

Plasma Protein	Average Concentration (mg/100 ml) in Serum of Eight Normal Subjects	Average Concentration (mg/100 ml) in Serum of Eight Bronchitic Patients	Average Concentration (mg/100 ml) in Sputum of 30 Bronchitic Patients	Protein Concentration Ratios : Serum/Sputum Matched Specimens
	Mean \pm SD	Mean \pm SD	Mean \pm SD	Mean \pm SD
Albumin	4390 \pm 667	3230 \pm 616	29.7 \pm 8.8	139 \pm 65
Alpha ₁ acid glycoprotein	61.4 \pm 18.4	111 \pm 59.5	1.2 \pm 1.0	147 \pm 133
Alpha ₁ antitrypsin	177 \pm 59.8	208 \pm 81.1	2.7 \pm 1.6	139 \pm 155
Alpha ₁ antichymotrypsin	55 \pm 17.1	48.5 \pm 16.1	1.5 \pm 1.1	33 \pm 27
Haptoglobin	157 \pm 64.6	298 \pm 98.1	2.2 \pm 1.6	230 \pm 152
Transferrin	269 \pm 54	218 \pm 55.5	2.9 \pm 1.0	114 \pm 53
Beta _{1c} globulin	44 \pm 8.7	48 \pm 21.4	1.4 \pm 1.3	70 \pm 27
IgA ^a	198 \pm 82.6	216 \pm 173	80.0 \pm 41.8	5.9 \pm 3.7
IgG	1174 \pm 468	957 \pm 267	13.3 \pm 8.2	105 \pm 6.4

Table I Average concentrations of principal plasma proteins in the sol phase of sputum from a group of patients with chronic bronchitis compared with their concentrations in corresponding serum specimens and in specimens of serum from a group of normal subjects¹

¹The plasma protein concentrations were estimated by cross immunoelectrophoresis with the exception of IgG, the concentrations of which were determined by electroimmunoassay.

^aEstimated as 7S globulin

the matched specimens of sputum sol phase which was 0.8 ± 0.5 ($t = 4.8$, $P < 0.001$). In contrast, the average of the ratios of the concentrations of albumin : IgG in serum was 3.5 ± 1.0 and this was not significantly different from the corresponding average in the matched specimens of sputum sol phase which was 3.7 ± 2.7 . However, the average of the ratios of alpha₁ antitrypsin : alpha₁ antichymotrypsin in serum was 4.4 ± 1.3 and this was significantly higher than the corresponding average in the matched specimens of sputum which was 1.2 ± 0.5 ($t = 6.45$, $P < 0.001$).

PLASMA PROTEINS IN SALIVA SPECIMENS

The plasma protein content of the saliva specimens that were collected from 10 of the patients was estimated to assess the possible effect of salivary contamination of the plasma proteins in the sputum sol phase. The average concentration of albumin was 7.0 ± 2.8 mg/100 ml and the average concentration of IgA was 4.5 ± 3.8 mg/100 ml. Haptoglobin was detected in only two of the saliva specimens and the mean concentration was 0.4 mg/100 ml. No other plasma proteins were detected in amounts that could be estimated.

Discussion

Sputum is a mixture of normal and pathological secretions variably contaminated with saliva and we have described precautions that can be taken to obviate the inclusion of gross amounts of saliva in sputum specimens before their analysis (Ryley and Brogan, 1968). Nonetheless, the presence of saliva in mucus could vitiate the results of an analysis of the soluble proteins of sputum but the present investigation has shown that concentrations

of plasma proteins in saliva are such that they would constitute a correction of the second order; the net effect of salivary contamination, therefore, would be to contribute to variance in the analytical results only by causing dilution. The high coefficients of variance in the analytical results were not unexpected since electrolyte, protein, and carbohydrate estimations have shown high variance from specimen to specimen in individual patients (Ryley and Brogan, 1968) and between specimens of sputum from groups of patients with similar chest diseases (Brogan *et al*, 1971).

Apart from immunoglobulins and possibly beta_{1c} globulin, no other plasma proteins have been demonstrated as being synthesized in lung tissue (Asofsky and Thorbecke, 1961). Immunoglobulins are synthesized in the lung by plasma cells which are located under the bronchial epithelium and immunofluorescence has revealed that most bronchial plasma cells contain IgA and only a few synthesize the other classes (Martinez-Tello, Braun, and Blanc, 1968); thus it would appear that the major part of sputum IgG is derived from plasma. Falk *et al* (1972) found that the ratios of albumin and IgG in sputum and serum differed and suggested that the IgG content of sputum is higher than would be expected if the presence of this immunoglobulin was due entirely to passive transudation. We found, however, that the corresponding ratios did not differ significantly in matched specimens of sputum and serum. One could have expected, however, the albumin : IgG ratio to have been significantly higher in sputum than in serum if the sole source of IgG was passive transudation since IgG has the higher molecular weight of the two proteins.

The ratio of alpha₁ antitrypsin : alpha₁ antichymotrypsin was found to be significantly higher

in serum than in sputum and this suggested selective concentration of α_1 antichymotrypsin in sputum. This finding is of particular interest in view of the recent work describing the relationship between α_1 antitrypsin deficiency and emphysema that was recently reviewed by Talamo (1971). The value of α_1 antichymotrypsin as a potential proteolytic inhibitor in the lung is not clear. Although α_1 antitrypsin inhibits a broad spectrum of proteolytic enzymes (Lieberman and Kaneshiro, 1972), α_1 antichymotrypsin is more specific than this and does not inhibit the action of trypsin, plasmin, or thrombin (Heimburger and Haupt, 1965); Ohlsson (1971) has even suggested that its affinity for chymotrypsin is not great. Lieberman, Trimmer, and Kurnick (1965), however, have described the presence of two neutral proteases that are released from degenerating leucocytes, one of which has 'chymotrypsin-like' properties; and it is therefore possible that this protease is inhibited by α_1 antichymotrypsin. The detection of α_1 antichymotrypsin in the sol phase of sputum thus raises the question of the presence in sputum of a protein that is capable of neutralizing enzymes originating from leucocyte disintegration.

Cross electrophoresis permits the direct and simultaneous determination of the concentrations of plasma proteins in the sol phase of sputum; this is a great advantage in that the method does not require prior concentration of the 'sputum sol phase since this dilute protein solution is metastable (Ryley, 1970). Clarke and Freeman (1968) suggested that internal controls should be incorporated in cross immunoelectrophoretic determinations but this was not possible as many patients produced insufficient quantities of sputum; the absence of such controls may have lowered the accuracy of individual determinations but it was nonetheless possible to show a general trend in the distribution of sol phase plasma proteins in sputum in a group of patients with bronchitis. Our findings have suggested that an enzyme inhibitor, α_1 antichymotrypsin, is selectively concentrated in the bronchial lumen in patients with chronic bronchitis.

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