Characterization of a High-Molecular-Weight Glycoprotein Isolated from the Pulmonary Secretions of Patients with Alveolar Proteinosis

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A high-molecular-weight glycoprotein was isolated, purified and partially characterized from the insoluble pulmonary secretions accumulating in lungs of patients suffering from pulmonary alveolar proteinosis. On electrophoresis in 5% polyacrylamide gel in the presence of sodium dodecyl sulphate and 2-mercaptoethanol, the purified protein gave one major band as detected by Coomassie Blue as well as with periodic acid/Schiff staining. An apparent mol.wt. of 250000 was estimated for this glycoprotein. Amino acid analysis showed that it contains hydroxyproline, and relatively high amounts of glycine, glutamic acid, aspartic acid and leucine. It contains approx. 6% hexose, 3% sialic acid and 2% glucosamine. The neutral sugars are galactose, mannose and fucose. An antiserum prepared in rabbits against this high-molecular-weight glycoprotein cross-reacted with two smaller glycoproteins (mol.wts. 62000 and 36000) isolated from the same pulmonary secretions of these patients. A complementary observation was also made when this large alveolar glycoprotein cross-reacted with an antiserum prepared in rabbits against the smaller glycoprotein (mol.wt. 36000). It appears that this high-molecular-weight glycoprotein may be the precursor of the two smaller glycoproteins present in the same diseased pulmonary secretions.

Normal alveolar secretions provide mechanical and immunological protection to the alveolar epithelium. Study of their properties are of interest because of their involvement in lung diseases such as respiratory-distress syndrome and pulmonary alveolar proteinosis (Rosen *et al.*, 1958). However, the normal secretions are scanty in amount and difficult to obtain. Less than 1 mg of material is obtained from the bronchoalveolar lavage of a healthy volunteer (Sahu *et al.*, 1976). Therefore the massive amounts of insoluble periodic acid/Schiff-positive amorphous material, which fills the alveoli and terminal bronchioles of patients suffering from pulmonary alveolar proteinosis, provides a unique source of alveolar secretions for investigation. This material is easily obtained by therapeutic bronchoalveolar lavage with sterile iso-osmotic saline (Ramirez-R., 1967; Kylstra *et al.*, 1971). As much as 20–80 g of insoluble lavage sediment is obtained from each patient, depending on the state of the disease (Passero *et al.*, 1973). This material is composed of approx. 55% lipid, 37% protein and 8% carbohydrate (Sahu *et al.*, 1976). Electrophoresis on 10% polyacrylamide gels showed that three proteins with mol.wts. of 69000 (albumin), 62000 and 36000 account for over 80% of the total protein present in

this material. The latter two are glycoproteins, of which the smaller is the major glycoprotein component (Passero *et al.*, 1973). The partial structural characterization of these two glycoproteins has been reported (Bhattacharyya *et al.*, 1976; Bhattacharyya & Lynn, 1977). Although lipids and the three proteins mentioned above are the major constituents, minor components such as phospholipase A₂ (Sahu & Lynn, 1977a,b) and hyaluronic acid (Sahu & Lynn, 1978) are also found in this alveolar secretion.

The studies reported in the present paper demonstrate the presence of a third glycoprotein with a very high molecular weight in this alveolar secretion.

Experimental

Isolation of alveolar secretions.

Patients suffering from alveolar proteinosis underwent therapeutic bronchoalveolar lavage with sterile iso-osmotic saline (0.9% NaCl) as previously described (Kylstra *et al.*, 1971) under the direction of Dr. J. A. Kylstra in the Duke University Medical Center. Immediately after the lavage, the material was centrifuged at 200g for 10 min at 4°C to remove alveolar macrophages and cellular debris. The supernatant was then centrifuged at 20000g for 30 min. This pellet was washed several times first with iso-osmotic saline and then with water, and

Abbreviation used: SDS, sodium dodecyl sulphate.

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finally collected by centrifugation at 20000g for 30 min as described previously (Sahu *et al.*, 1976). It was then freeze-dried and stored at -40°C .

Delipidation

The freeze-dried powder was delipidated as previously described (Sahu *et al.*, 1976) with chloroform/methanol (2:1, v/v).

Reduction and carboxymethylation

The delipidated proteins were reduced and carboxymethylated essentially by the method of Forstner *et al.* (1973). About 20 mg of protein was dissolved in 5 ml of 0.1 M-Tris/HCl, pH 8.5, containing 8 M-urea and 1% SDS. The solution was flushed with N_2 for 10 min and dithiothreitol was added to a concentration of 0.01 M. The reaction mixture was incubated at room temperature for 4 h. Then iodoacetamide was added to a concentration of 0.04 M and the reaction was allowed to proceed at room temperature for 30 min, in the dark. After the end of the reaction, it was diluted 4-fold with and dialysed against water at 4°C for 48 h with several changes of water. Finally the sample was freeze-dried. This procedure was repeated to ensure complete reduction and alkylation.

Gel filtration

The reduced and alkylated protein powder was dissolved in a small volume of 10 mM-Tris/HCl, pH 7.2, containing 0.1% SDS and 0.02% NaN_3 . It was then applied to a column (80 cm $\times$ 2.5 cm) of Bio-Gel A-5m, equilibrated and eluted by the same buffer.

Protein determination

Total protein was measured by the method of Lowry *et al.* (1951), by using bovine serum albumin as the standard. Alternatively, protein concentration was estimated by measuring the A_{280} (Kalckar, 1947).

Carbohydrate determination

Total hexoses were determined by the phenol/ H_2SO_4 method (Dubois *et al.*, 1956). For determination of individual hexose, the glycoprotein was hydrolysed in 1 M-HCl for 6 h at 100°C under vacuum (Spiro, 1972). The hydrolysate was dried under vacuum to remove HCl. It was then dissolved in small amounts of water and applied to a column (20 cm $\times$ 2 cm) of Dowex 50-X4 (H^+ form). The sugars were eluted with 10 column volumes of water and freeze-dried. The individual sugars were determined by g.l.c. of their trimethylsilyl derivatives as previously described (Sahu *et al.*, 1976).

Hexosamines were determined as previously described (Bhattacharyya *et al.*, 1976) on a Beckman 120B amino acid analyser after hydrolysis of the

glycoprotein in 4 M-HCl for 6 h at 100°C under vacuum (Spiro, 1972).

Sialic acid was determined by the thiobarbituric acid method (Warren, 1959) after the protein was hydrolysed in 0.05 M- H_2SO_4 at 80°C for 1 h as described previously (Bhattacharyya *et al.*, 1976).

Polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was carried out on 5% acrylamide gels by the method of Fairbanks *et al.* (1971). Electrophoresis was carried out at constant current of 4 mA/gel until the Bromophenol Blue tracking dye was near the end of the gel. Proteins were stained with Coomassie Blue and the carbohydrates with periodic acid/Schiff reagent (Glossman & Neville, 1971).

Molecular-weight measurement

Apparent molecular weights of the proteins were measured by gel electrophoresis (Weber & Osborn, 1969). Approximate molecular weights were also estimated by gel filtration on the Bio-Gel A-5m column. Cytochrome *c*, concanavalin A, ovalbumin, cross-linked bovine serum albumin (mol.wts. 66000, 132000, 198000 and 264000 respectively; obtained from Sigma) and serum fibronectin (see the Discussion section) were used as protein standards.

Amino acid analysis

Amino acid analysis was carried out as previously described (Bhattacharyya *et al.*, 1976; Sahu & Lynn, 1977b) on a Beckman 120B amino acid analyser by the method of Spackman (1967). The protein was hydrolysed in 6 M-HCl at 110°C for 24 h in evacuated sealed tubes. Amino acid analyses were carried out on the protein isolated by gel filtration as well as on the protein eluted from SDS/polyacrylamide gels by the method of Weiner *et al.* (1972). Both methods gave identical results. Hydroxyproline and hydroxylysine analyses were carried out as before (Bhattacharyya *et al.*, 1976) by using the temperature gradient. Cystine was determined as cysteic acid (Moore, 1963).

Preparation of antisera

Antisera were prepared in rabbits against two purified glycoproteins (mol.wts. 250000 and 36000) isolated as described above from the insoluble pulmonary secretions of patients with alveolar proteinosis. New Zealand White adult rabbits were injected intramuscularly in the hindquarter with 1 mg of the purified glycoprotein with 1 ml of complete Freund's adjuvant. After 3 weeks, they were reinjected intramuscularly with 0.3 mg of the purified glycoprotein without adjuvant. Three weeks after the second injection, the rabbits were boosted with 0.3 mg of the glycoprotein intraperitoneally. One week after

the booster injection, the antisera were collected from the ear vein.

Materials

All chemicals were of reagent grade. Polyacrylamide and all other reagents for gel electrophoresis were obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A., as was Bio-Gel A-5m. Cross-linked bovine serum albumins for standards were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Serum fibronectin was a gift from Dr. E. Engvall, Department of Immunology, City of Hope National Medical Center, Duarte, CA, U.S.A.

Results

Bronchoalveolar lavage of each patient suffering from pulmonary alveolar proteinosis yielded 10–50g

Fig. 1. SDS/polyacrylamide-gel electrophoresis of the crude delipidated alveolar proteins stained with Coomassie Blue

For details see the Experimental section.

of dry insoluble alveolar secretions, depending on the state of the disease. When lipids were removed, the proteins constituted approx. 45% of the dry material. The crude delipidated proteins on SDS/polyacrylamide-gel electrophoresis (Fig. 1) showed two major bands, one corresponding to a glycoprotein of mol.wt. 36000 and the other containing another glycoprotein of mol.wt. 62000 together with albumin, and at least two minor bands corresponding to higher molecular weight proteins, the highest one being a glycoprotein of mol.wt. 250000, whose purification and characterization are the subject of the present paper. The delipidated crude proteins, after reduction and alkylation, when subjected to gel filtration on a Bio-Gel A-5m column produced three major peaks as determined by the A_{280} (Fig. 2).

Gel electrophoresis of the protein obtained from the shaded area of peak I (Fig. 2) on SDS/polyacrylamide gels gave a single band, which stained with Coomassie Blue as well as with periodic acid/Schiff reagent, indicating it as a glycoprotein (Fig. 3). An apparent mol.wt. of 250000 was estimated for this glycoprotein. This value was essentially identical with the approximate molecular weight of the protein determined by the gel filtration. It was estimated that this high-molecular-weight glycoprotein represents

Fig. 2. Separation of the alveolar glycoproteins, reduced and alkylated, by gel filtration on a Bio-Gel A-5m column. Volume per fraction was 1.3ml. Void volume, as determined by Blue Dextran, was 71.5ml. Protein obtained from the shaded area of peak I was used for analysis. For further details see the Experimental section.

Fig. 3. *SDS/polyacrylamide-gel electrophoresis of the purified alveolar glycoprotein stained with periodic acid/Schiff reagent (a) and Coomassie Blue (b) and the purified serum fibronectin (c) stained with Coomassie Blue*
For details see the Experimental section.

Table 1. *Amino acid composition of the high-molecular-weight glycoprotein isolated from the alveolar secretions*
The results are averages of triplicate analyses. Cystine was determined as cysteic acid. Details are given in the Experimental section.

Amino acids	Content (residues/1000 residues)
4-Hydroxyproline	17.4
Aspartic acid	97.5
Threonine	52.7
Serine	56.2
Glutamic acid	117.6
Proline	71.3
Glycine	148.7
Alanine	63.4
Half-cystine	15.1
Valine	68.4
Methionine	15.2
Isoleucine	41.3
Leucine	92.4
Tyrosine	32.6
Phenylalanine	32.1
Lysine	37.8
Histidine	21.5
Arginine	46.3

Table 2. *Carbohydrate composition of the high-molecular-weight glycoprotein isolated from the alveolar secretions*
The results are averages of triplicate analyses. Details of measurements are given in the Experimental section.

Carbohydrate	Content (mg/100mg of protein)
Sialic acid	3.02
Glucosamine	2.21
Hexoses	6.51
Mannose	2.87
Galactose	2.49
Fucose	1.12

approx. 5–10% of the total protein present in this insoluble alveolar secretion.

SDS/polyacrylamide-gel electrophoresis of the material from peak II (Fig. 2) showed the presence of a mixture of proteins with apparent mol.wts. 130000, 95000, 68000 (albumin) and 62000 (results not shown). Similar treatment of material from peak III (Fig. 2) showed the presence of a mixture of proteins with mol.wts. 36000 and 29000 (results not shown). The proteins with mol.wts. 62000 and 36000 are glycoproteins that have been partially characterized (Bhattacharyya *et al.*, 1976; Bhattacharyya & Lynn, 1977). The high-molecular-weight glycoprotein (250000) was not detected in previous studies because of the use of 10% polyacrylamide gels, into which this glycoprotein does not penetrate. In the present study, when we used 5% acrylamide gels, we were able to detect it. Prior reduction and alkylation were found to be required for the separation of the glycoprotein of mol.wt. 250000 from the smaller two glycoproteins present in this alveolar secretion, indicating an association by disulphide linkage into higher molecular-weight forms.

Amino acid analysis (Table 1) of the purified glycoprotein of mol.wt. 250000 showed the presence of hydroxyproline, relatively high amounts of aspartic acid, glutamic acid, glycine, alanine and leucine, and relatively low amounts of methionine.

Carbohydrate analysis (Table 2) of the high-molecular-weight glycoprotein showed the presence of sialic acid, glucosamine, galactose, mannose and fucose.

An antiserum prepared in rabbits against the high-molecular-weight glycoprotein (250000) cross-reacted with the two smaller glycoproteins (mol.wts. 62000 and 36000) isolated from insoluble alveolar secretions of these patients. However, serum fibronectin failed to cross-react with this antiserum (Fig. 4). The alveolar glycoprotein of mol.wt. 250000 also cross-reacted with the antiserum prepared against the purified glycoprotein of mol.wt. 36000 (results not shown). Thus, the reactions were complementary.

Fig. 4. Comparison by immunodiffusion of alveolar glycoproteins of nominal mol.wts. 250000, 62000 and 36000 and purified serum fibronectin

The centre well contained an antiserum against the mol.wt. 250000 alveolar glycoprotein prepared as described in the Experimental section. The peripheral wells contained purified serum fibronectin (1-3) (Engvall & Ruoslahti, 1977), mol.wt. 250000 alveolar glycoprotein (4), mol.wt. 62000 alveolar glycoprotein (5), and mol.wt. 36000 alveolar glycoprotein (6).

The immunodiffusion experiment (Fig. 4) indicates that the mol.wt. 250000 glycoprotein used for immunization was not a single component. Contamination in a concentration less than the detection limits of SDS/polyacrylamide-gel electrophoresis is a strong possibility.

Discussion

Materials accumulating in the alveoli of patients suffering from pulmonary alveolar proteinosis are an excellent source of alveolar secretions for investigation. Since glycoproteins are known to be responsible for the rheological properties of respiratory secretions either directly (Gibbons & Glover, 1959; Sturgess *et al.*, 1971) or by virtue of their association with proteins (Gibbons, 1969), it is of interest to characterize the glycoproteins present in alveolar secretions. Two alveolar glycoproteins of mol.wts. 62000 and 36000 have partially been characterized (Bhattacharyya *et al.*, 1976; Bhattacharyya & Lynn, 1977). The present paper reports the partial characterization of a third glycoprotein with apparent mol.wt. 250000, representing approx. 5-10% of the total proteins present in the insoluble materials accumulating in the diseased alveoli of these patients.

Chemical analysis of the three alveolar glycoproteins with apparent mol.wts. 250000, 62000 and 36000 shows similar, but not identical, composition. The high-molecular-weight (250000) glycoprotein (a) contains hydroxyproline, sialic acid and glucos-

amine, (b) shows relatively high contents of aspartic acid, glutamic acid, glycine, alanine and leucine, (c) shows relatively low content of methionine, and (d) contains the same hexoses as do the two smaller (mol.wts. 62000 and 36000) glycoproteins (Passero *et al.*, 1973; Bhattacharyya *et al.*, 1976). The two smaller glycoproteins cross-react with an antiserum prepared in rabbits against the mol.wt. 250000 glycoprotein. Thiol reduction experiments show that these three alveolar glycoproteins are linked to higher-molecular-weight forms by disulphide bonds. It therefore appears that the high-molecular-weight glycoprotein may be the precursor of the two smaller glycoproteins.

The source of this high-molecular-weight glycoprotein is not known. Non-collagenous glycoproteins of apparent mol.wts. over 200000 have been reported in basement membrane (Kefalides, 1972). These glycoproteins show somewhat similar amino acid and carbohydrate composition to the mol.wt. 250000 alveolar glycoprotein. However, hydroxylysine and glucose are important constituents of the basement-membrane glycoproteins, but are absent in the purified alveolar glycoprotein. Similarly, the molar ratio of glucosamine to galactosamine in the basement-membrane glycoprotein is 7:1, whereas the alveolar glycoprotein contains only glucosamine. Fibronectin, found in human plasma and on the surface of cultured normal fibroblasts, is a glycoprotein with an apparent mol.wt. of 250000 (Vuento *et al.*, 1977; Engvall & Ruoslahti, 1977). This glycoprotein is synonymous (Engvall & Ruoslahti, 1977) with the cold-insoluble globulin (Morrison *et al.*, 1948) and large-external-transformation-sensitive protein (Hynes & Bye, 1974). It has also been shown that fibronectin in tissues is particularly enriched in various basement membranes (Linder *et al.*, 1975). The cold-insoluble globulin, purified from human plasma, consists of two polypeptide chains each of mol.wt. approx. 200000, held together by disulphide bonds (Mosher, 1975). The amino acid composition of this protein shows relatively high contents of aspartic acid, threonine, serine, glutamic acid, proline, glycine and valine (Mosher, 1975), unlike the alveolar glycoprotein. Furthermore, serum fibronectin does not cross-react with an antiserum prepared in rabbits against the high-molecular-weight alveolar glycoprotein. It is therefore unlikely that the high-molecular-weight alveolar glycoprotein is similar to fibronectin. The cellular source, biological function and the role that the mol.wt. 250000 alveolar glycoprotein plays in the aetiology of pulmonary alveolar proteinosis require study.

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