Isolation and Characterization of Two Glycoproteins from Patients with Alveolar Proteinosis

(pulmonary lavage/hydroxyproline/hydroxylysine/alveolar glycoproteins)

MICHAEL A. PASSERO*, ROSS W. TYE, KAYE H. KILBURN, AND WILLIAM S. LYNN

Departments of Biochemistry and Medicine, Duke University Medical Center, Durham, North Carolina 27710

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ABSTRACT The saline-insoluble particulate material obtained by pulmonary lavage from four patients with alveolar proteinosis was analyzed for total lipid (52%), protein (44%), and carbohydrate (4%). Sodium dodecyl sulfate-gel electrophoresis revealed only three major peptides in these patients. Molecular weights (from the gels) were 69,000, 62,000, and 36,000, and the latter two peptides were periodic acid-Schiff stain-positive. Amino-acid and sugar analyses were performed on the peptides cut from the gels and on peptides purified by Sephadex G-200 chromatography. The 69,000-molecular-weight peptide contained no carbohydrate. Antibody studies and amino-acid analysis indicated that it was albumin. The 62,000-molecular-weight peptide contained 1% hydroxyproline, 1% hydroxylysine, 10% glycine, and 9% carbohydrate. The 36,000-molecular-weight peptide contained 1.2% hydroxyproline, 1% hydroxylysine, 13% glycine, 1.4% sialic acid, 2.6% glucose, 2.4% galactose, 2% mannose, 0.8% fucose, 0.4% glycosamine, and 0.6% galactosamine. Proteins extracted from kidney glomeruli with similar amino-acid and carbohydrate composition have been observed previously.

Alveolar proteinosis, first described by Rosen, Castleman, and Liebow in 1958 (1), is a chronic pulmonary disease of unknown pathogenesis in which the alveoli and terminal bronchioles of the lung are filled with a periodic acid-Schiff-positive amorphous material. This material, which is insoluble in isotonic saline, can be removed in particulate form by pulmonary lavage (2, 3). The extractable lipid from the lavage material contains large amounts of phosphatidyl choline and cholesterol (4). In the present study, the major peptides from the saline-insoluble lavage material have been isolated and characterized.

METHODS

Four patients with alveolar proteinosis, diagnosed by a typical clinical course, radiographic and physiologic findings, and lung biopsy, underwent therapeutic pulmonary lavage with 15-20 liters of isotonic saline under the direction of either Dr. J. Ramirez-R. (1 patient) or Dr. J. Kylstra (3 patients) (2, 3). The lavage material was centrifuged at $5000 \times g$ for 20 min to obtain a thick, yellow precipitate and a clear supernatant. Both fractions were stored at -20° .

Solubilization and Sodium Dodecyl Sulfate (SDS) Gel Electrophoresis of the Lavage Precipitate. The lavage precipi-

tate was homogenized four times in cold isotonic saline and collected by centrifugation ($15,000 \times g$ for 15 min). Total protein was determined by the Lowry *et al.* method (5), and total carbohydrate was determined by the phenol-sulfuric-acid method (6), and the thiobarbituric-acid assay for sialic acid (7). Total lipid was estimated gravimetrically after extraction with chloroform-methanol (2:1 by volume).

The lavage precipitate was dissolved in 0.2% SDS (Fisher)-0.1% 2-mercaptoethanol (Eastman)-0.04 M Tris·HCl (Schwarz-Mann) (pH 7.5). Solubilization of this material could be achieved only in the presence of thiol reagents. A black substance, representing less than 5% of the material, was precipitated by centrifugation at $40,000 \times g$ for 1 hr. This was discarded. SDS-gel electrophoresis (all reagents were obtained from Eastman) was performed by the method of Weber and Osborn (8), and the gels were stained with Coomassie blue (Colab) or periodate-Schiff reagent (9) (all reagents were obtained from Fisher).

Solubilization was also achieved in 0.33 M lithium diiodosalicylate (10) (Eastman), 0.05 M Tris·HCl, and 0.1% 2-mercaptoethanol (pH 7.5). Most of the lipid was then removed by extraction with ethyl acetate (Allied Chemical). After extraction, the water phase was dialyzed against 0.04 M Tris·HCl (pH 7.5) at 4° for 24 hr, and then centrifuged at $30,000 \times g$ for 1 hr. No material could be removed by centrifugation, although the solution was slightly turbid. This solution was tested by immunodiffusion against antisera to human serum, human serum albumin, IgG, IgM, IgA, α -1-antitrypsin, α -2-macroglobulin, and the β -1-C fraction of complement (all antisera were obtained from Hyland).

SDS-Sephadex G-200 Gel Filtration of the Lavage Precipitate. An aliquot of the lavage precipitate from each of 2 patients was dissolved in 0.2% SDS and 0.1% 2-mercaptoethanol as described above and applied to a 2.5×63.5 cm (1×25 in.) column of Sephadex G-200 equilibrated with 0.2% SDS, 0.1% 2-mercaptoethanol, 0.02 M Tris·HCl (pH 7.5), and 0.02% sodium azide (Fisher). The two major peaks were checked for purity by SDS-gel electrophoresis.

Amino-Acid and Carbohydrate Analyses. Amino-acid and amino-sugar analyses were performed by the method of Spackman (11) on a Beckman 120B analyzer on fractions eluted from the Sephadex columns, as well as stained bands cut from SDS-gels (12). All peptides were hydrolyzed in 6 N HCl at 110° for 24 hr. Hydroxylysine and hydroxyproline analyses were performed by the method of Piez and Morris (13). Sialic acids were determined by thiobarbituric-acid

Abbreviation: SDS, sodium dodecyl sulfate.

* Address all correspondence and reprint requests to Dr. Michael A. Passero, Box M3804, Duke University Medical Center, Durham, N.C. 27710.

FIG. 1. SDS-gel electrophoresis of alveolar lavage sediment from patients with alveolar proteinosis. The material was washed four times in cold isotonic saline, collected by centrifugation, and dissolved in 2% SDS-0.1% 2-mercaptoethanol (pH 7). 30–40 μ g of protein were applied to SDS-polyacrylamide gels. The gels show: (1) albumin, cytochrome *c*; (2–5) alveolar sediment from 4 different patients stained with Coomassie blue; (6) alveolar sediment stained with periodate-Schiff; (7) human serum. The sediment from all 4 patients contained the same three major peptides. Two of the peptides, molecular weights 62,000 and 36,000 are periodate-Schiff-positive.

assay (7). Glucose was determined by the glucose oxidase method after hydrolysis in 2 N H_2SO_4 at 100° for 4 hr (14). Neutral sugars were determined by the method of Lee *et al.* (15). Hydrolysis time was 4 hr.

Antibody Studies and SDS-gel Electrophoresis of Lavage Supernatant. The supernatant material was concentrated by lyophilization after dialysis and tested by immunodiffusion against antisera to human serum, human albumin, IgG, IgM, IgA, α -1-antitrypsin, and the β -1-C fraction of complement. SDS-gel electrophoresis was also performed on the supernatant material.

RESULTS

From 20 to 80 g of lavage precipitate were collected from each lung of each patient. This material contained up to 50% of the total protein in each lavage. About 50% of the lavage precipitate could be extracted into chloroform-methanol. Of the remainder, 91% was protein and 9% was carbohydrate. SDS-gel electrophoresis revealed that the SDS-mercaptoethanol-soluble material contained three major protein bands. Molecular weights (on gel) were 69,000, 62,000, and 36,000, and the latter two bands contained all of the carbohydrate. The electrophoretic pattern was similar in lavage material from each of the 4 patients (Fig. 1). As indicated (Fig. 1) by the Coomassie blue or Amido Black stains, the major glycopeptide is the one of molecular weight 36,000. The relative amounts of the peptides vary considerably from patient to patient. The lithium diiodosalicylate-solubilized material gave only one precipitin arc when tested against antiserum to human serum. When tests were performed with the specific antisera listed above, a reaction was seen only with the antiserum to human serum albumin.

Sephadex gel filtration

Two major peaks were obtained. The 36,000-molecular-weight peptide was obtained in relatively pure form, as assessed by gel electrophoresis. However, the 62,000-molecular-weight peptide could not be completely separated from the 69,000-molecular-weight peptide. SDS-gel electrophoresis indicated about a 10% contamination by serum albumin.

Amino-acid and carbohydrate analyses

Amino-acid analysis of the 69,000-molecular-weight peptide cut from SDS gels showed that it was similar to human albumin (16). Analyses of the 62,000- and 36,000-molecular-weight peptides cut from stained gels were similar to analyses of fractions obtained from Sephadex G-200 columns (Table 1). Sialic acid, mannose, galactose, fucose and glucose, galactosamine, and glucosamine are present in the 36,000-molecular-weight peptide. 4-Hydroxyproline was present; 3-hydroxyproline, if present at all, was there in very small amounts.

Antibody studies and SDS-gel electrophoresis of lavage supernatant

The lavage supernatants, which contained 40–50% of the total protein, gave precipitin bands with antisera to human albumin, IgG, IgA, IgM, α -1-antitrypsin, and the β -1-C fraction of complement. SDS-gel electrophoresis revealed one major band of molecular weight 69,000.

DISCUSSION

The above antibody studies confirm and extend previous demonstrations of serum proteins in lavage material from patients with alveolar proteinosis (17). It is not clear whether the presence of these proteins is a result of the illness or an

TABLE 1. Amino-acid analyses of lavage-sediment glycoproteins

	62,000 Peptide	36,000 Peptide		62,000 Peptide	36,000 Peptide
	<i>Residues per 1000 amino acids</i>				
Lys	62.1	42.4	1/2-Cys	12.5	19.8
His	24.9	23.3	Val	63.9	66.2
Arg	50.6	58.5	Met	14.2	20.5
Asp	96.1	80.7	Ile	29.5	44.5
Thr	51.5	47.5	Leu	86.0	96.9
Ser	48.2	48.8	Tyr	46.7	33.4
Glu	116.3	90.6	Phe	50.3	41.6
Pro	53.6	62.2	Hyp	9.8	12.0
Gly	98.7	131.5	Hyl	9.0	9.0
Ala	76.1	70.7	Total		
			carbohydrate	80.0	90.0
			Sialic acid		15
			Mannose		20
			Fucose		8
			Galactose		24
			Glucose		26
			Glu-NH ₂		4
			Gal-NH ₂		6

Amino-acid and carbohydrate analyses of the 62,000- and 36,000-molecular-weight glycoproteins obtained from alveolar lavage sediment. The peptides were purified by gel filtration on Sephadex G-200 equilibrated with 0.2% SDS and 0.1% 2-mercaptoethanol (pH 7.5). These results were similar with material from two different patients and with bands cut from the SDS gels.

artifact produced by pulmonary lavage. Serum proteins have been shown to be the major protein components in lavage material from normal animal lungs in which the pulmonary capillaries contain blood (18).

The saline-insoluble sediment from the lavage material, containing up to 50% of the total protein in the lavage, probably represents the periodate-Schiff-positive substance that fills the alveoli and terminal bronchi in this condition. The high lipid content (50%), disulfide content, and relative hydrophobic nature of two of the proteins (Table 1) may explain the insolubility of this material in isotonic saline.

Three major peptides have been shown to account for over 90% of the protein in the insoluble material obtained from 4 patients. The 69,000-molecular-weight peptide contains no carbohydrate, has an amino-acid analysis similar to that reported for human albumin, and forms a precipitin band with the antiserum to human serum albumin. The 62,000- and 36,000-molecular-weight glycoproteins are quite similar in amino-acid composition and are probably not serum glycoproteins. The glycine content is greater than would be expected in a serum protein, and the presence of hydroxyproline, hydroxylysine, and glucose also argue against a serum origin. These two proteins also fail to precipitate antibodies to human serum. However, their probable denatured state makes this argument against a serum origin less compelling than that based on amino-acid and carbohydrate composition.

Hudson and Spiro (19) have recently described several glycoproteins prepared by sonication of renal glomerular membranes, which, like the material in alveolar proteinosis, required SDS and reduction for solubilization. The amino-acid analyses of the various glomerular "basement membrane" peptides revealed that some fractions of low molecular weight contain similar percentages of cysteine, glycine, valine, serine, hydroxyproline, hydroxylysine, and carbohydrate (20) to those in the 62,000- and 36,000-molecular-weight glycoproteins from alveolar proteinosis material. An attractive hypothesis is that these two glycoproteins are lung basal laminar proteins, which are either overproduced or not appropriately utilized.

Recently, the synthesis of "basal lamina" by epithelial cells from human fetal glomeruli has been demonstrated *in vitro* (21). The basal laminar material was detected as granules in the glomerular epithelial cells and appeared to be secreted; 20% of the hydroxyproline in this material was 3-hydroxyproline. Theoretically, alveolar epithelial cells could also secrete basal laminar material, and overproduction or underutilization of the material would result in alveolar proteinosis. The electron microscopic observation that the "basement membrane" appears to be fragmented in some alveoli in the disease state (22) is consistent with this hypothesis. However, the observations of Vracko (23), indicating that normal lung, in contrast to glomerulus or nerve, is normally unable to deposit excessive layers of basal laminar material suggest that these two glycoproteins may not be derived from basal lamina.

Another possibility is that the two glycopeptides may have originated from alveolar cell membranes. At present nothing is known about the membrane proteins of the alveolar-lining cells. The alveolar macrophage membrane has been partially characterized by Nachman, Ferris, and Hirsch (24), but no glycoproteins of molecular weight below 100,000 were identified.

Evans and Gurd (25) have observed glycoproteins of about 70,000–90,000 molecular weight from mouse-liver plasma membrane. A glycoprotein of 100,000 molecular weight containing 5–8% carbohydrate and representing 25–30% of the total protein was obtained from human erythrocyte membranes (26). Unfortunately, hydroxyproline and hydroxylysine analyses have rarely been performed on isolated membranes, and it is not known whether these amino acids are present in some mammalian membrane proteins.

The quite similar composition of the two alveolar glycoproteins suggests that they may be derived from a larger protein.

Calcott *et al.* (27) have recently described a serum protein, Clq, that contains quite similar amounts of hydroxyproline, hydroxylysine, glycine, glucose, and sialic acid as those described here. This protein is composed of three subunits, the largest of which is only 29,000 in molecular weight (28). It appears unlikely, therefore, that the alveolar peptides derive from Clq. Whether other complement proteins are composed of similar amino acids and monosaccharides is not known.

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