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Peptides of human bronchial mucus glycoproteins

Size determination by electron microscopy and by biosynthetic experiments

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Secreted human bronchial mucins, directly collected from macroscopically healthy bronchial mucosa, were prepared in the presence of six proteinase inhibitors, and analysed by electron microscopy. These mucins were similar in length distribution to molecules prepared from sputum [Slayter, Lamblin, Le Treut, Galabert, Houdret, Degand & Roussel (1984) Eur. J. Biochem. **142**, 209–218], although they were a little longer, their lengths ranging up to about 1650 nm. This length corresponds to an extended mucin peptide of about 450 kDa. In order to compare these peptide lengths with the molecular size of biosynthetic precursors, an antiserum raised against trifluoromethanesulphonic acid-treated highly glycosylated regions of human bronchial mucins was used to isolate mucin precursors synthesized in explants of human bronchial mucosa during pulse-labelling with [^{3}H]threonine or [^{3}H]glucosamine. A main precursor labelled with [^{3}H]-threonine and with an apparent molecular mass of about 400 kDa was detected by fluorography following SDS/polyacrylamide-gel electrophoresis. This band was observed as early as 20 min; it was more intense after a 40 min chase and had disappeared after a chase period of 280 min in unlabelled medium, presumably owing to glycosylation. Much fainter bands at about 200 kDa and between 200 and 400 kDa, also labelled with [^{3}H]threonine, were observed mainly after a 40 min chase and had disappeared after a 280 min chase. None of these bands was labelled with [^{3}H]glucosamine, nor did they disappear after multiple treatments with immobilized lectins. After a 280 min chase, [^{3}H]threonine-labelled material appeared in the stacking gel, which also contained [^{3}H]glucosamine label. The results indicate that the 200–400 kDa species are mucin precursors, whose size is comparable with that obtained by electron microscopy for respiratory mucins collected directly from the macroscopically healthy bronchial mucosa.

INTRODUCTION

Mucus glycoproteins, or mucins, are the most important components of the mucus layer that covers and protects the human respiratory mucosa. These macromolecules are very complex, and the determination of their molecular mass, like that of most mucins in general, is a matter of controversy (Carlstedt *et al.*, 1985; Laboisse, 1986). These high-molecular-mass glycoproteins are polydisperse and contain about 80% carbohydrate in the form of *O*-linked oligosaccharides. Mucins are believed to be made of highly glycosylated regions and of naked regions more or less devoid of carbohydrate chains (Roberts, 1976). The peptide part of the glycosylated regions is rich in hydroxylated amino acid. When examined by electron microscopy, mucins are observed as thread-like structures ranging in length from 200 nm to more than 1000 nm (Jenssen *et al.*, 1980; Slayter *et al.*, 1984; Rose *et al.*, 1984; Mikkelsen *et al.*, 1985; Sheehan *et al.*, 1986). The reasons for such polydispersity remain obscure, although some degradation of the mucin peptide is always possible.

In the present study, we have compared the peptide length of secreted bronchial mucins with the molecular size of biosynthetic precursors. Secreted mucins were directly collected in the bronchial tree. They were prepared under conditions that should limit peptide degradation as much as possible, and were analysed by electron microscopy.

In order to identify mucin precursors, an immune serum containing antibodies directed against uncovered peptide epitopes from the highly glycosylated regions of human bronchial mucins was prepared (Marianne *et al.*, 1986). This was used to identify non-glycosylated precursors of bronchial mucins in explants of human bronchial tissue.

EXPERIMENTAL

Materials

Leibovitz L15 medium, CMRL 1066 medium without glutamine, Hanks buffered salts solution and 1 M-Hepes buffer, pH 7.0, were obtained from GIBCO (Paisley, Renfrew, Scotland, U.K.). L-[^{3}H]Threonine (17 Ci/mmol) and Amplify were obtained from Amersham International (Amersham, Bucks., U.K.). D-[^{3}H]-Glucosamine hydrochloride (42.5 Ci/mmol) and ^{14}C-labelled standard proteins were obtained from New England Nuclear (Boston, MA, U.S.A.). Phenylmethanesulphonyl fluoride, lectin from *Ricinus communis* immobilized on beaded agarose (RCA_{120}–agarose), Non-

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idet P40, leupeptin, 1,10-phenanthroline monohydrate, L-tosylphenylalanylchloromethane ('TPCK') and pepstatin A were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Protein A–Sepharose CL-4B was from Pharmacia Fine Chemicals (Uppsala, Sweden). *Helix pomatia* agglutinin–Ultrogel (HPA–Ultrogel) and wheat-germ agglutinin–Ultrogel (WGA–Ultrogel) were from I.B.F. (Villeneuve la Garenne, France). X-ray films (X-Omat AR5) were from Kodak. Rabbit anti-(human IgA heavy chain) serum was from Cappel Laboratories (Cochranville, PA, U.S.A.). *N*-Ethylmaleimide was from Interchim (Montluçon, France). All other reagents were analytical grade.

Methods

Collection of normal respiratory mucus. Samples of 'normal' respiratory mucus (about 1 ml of small mucus plug) were collected during bronchial endoscopy in macroscopically healthy areas of the bronchial trees of two individuals; they were immediately diluted in 10 ml of 16.7 mM-sodium phosphate buffer, pH 6.8, containing 33 mM-NaCl, 0.02% NaN_3 and various proteinase inhibitors: phenylmethanesulphonyl fluoride, *N*-ethylmaleimide, 1,10-phenanthroline and tosylphenylalanylchloromethane (each at 5 mM final concentration), and leupeptin (1 mg) and pepstatin A (1 mg). These mixtures were immediately cooled at 4 °C.

Isolation of normal respiratory mucins. The volume of the mixtures was adjusted to 26 ml with the buffer described above containing proteinase inhibitors. CsBr was added in order to obtain a final concentration of 42% (w/v), and the mixtures were very gently stirred on a horizontal shaker for 48 h at 4 °C. The mixtures were then subjected to density-gradient centrifugation (Woodward *et al.*, 1982). After centrifugation, 0.8 ml fractions were collected from the bottom of each tube and assayed as previously described (Houdret *et al.*, 1986).

Fraction 1 at a density of about 1.4 g/cm^3, corresponding to mucins, was dialysed at 4 °C against the phosphate buffer containing the same proteinase inhibitors in order to eliminate CsBr, and then studied by electron microscopy as follows.

Electron microscopy. Immediately before preparation for electron microscopy the mucin sample was passed through a Sepharose 4B exclusion column to remove residual CsBr and inhibitors and to change the buffer to 0.15 M-ammonium acetate.

Mucin specimens for electron microscopy were prepared from solutions containing 100 μg of mucin/ml in 0.15 M-ammonium acetate buffer, pH 7, and 25% (v/v) glycerol, as described previously (Slayter, 1976, 1978; Slayter *et al.*, 1984). Briefly, samples containing ammonium acetate and glycerol were applied as an aerosol to freshly cleaved mica. After 20 h of outgassing at 1.3 μPa (10^{-1} Torr), preparations were coated with a very thin layer of tungsten by electron-beam evaporation (0.093 μg/cm^2), and were subsequently coated with 2.5 nm of carbon. Micrographs were recorded very close to focus on a JEM 100 CX electron microscope at 100 kV, generally at a magnification of $\times$40000–53000, as high-resolution dark-field images obtained with matched annular condenser and objective apertures (Suzuki *et al.*, 1985).

Preparation of antiserum against trifluoromethanesulphonic acid-treated glycopeptides. The partially deglycosylated glycopeptides from human bronchial mucins were prepared by using trifluoromethanesulphonic acid (Marianne *et al.*, 1986). They contained high proportions of hydroxylated amino acid (31.2 residues of threonine and 14.1 residues of serine per 100 amino acid residues). The remaining sugars were also expressed as residues per 100 amino acid residues and values for *N*-acetylgalactosamine, *N*-acetylglucosamine, galactose and fucose were 34.6, 6.7, 4.0 and 1.3 respectively (before deglycosylation, they were 45.5, 94.2, 126.4 and 57.4 residues per 100 amino acid residues respectively). Immunization was carried out according to procedures previously described (Marianne *et al.*, 1986).

Explant culture. After pneumonectomy or lobectomy performed in three patients with lung carcinoma (squamous carcinoma), specimens of macroscopically healthy bronchi were immediately removed from the surgical tissue, at a distance from the neoplastic tissue. Tissue portions were immediately placed into ice-cold sterile Leibovitz L15 medium and transported to the laboratory. The mucosa and submucosa were freed from cartilage and adhering lung parenchyma, cut into 0.3 cm^2 sections under sterile conditions and rinsed several times with sterile 0.9% NaCl. Then 24 explants were placed in a 35 mm plastic Petri dish that had been scratched with a scalpel to allow for better adherence of the tissue; 1.2–1.5 ml of Hanks buffered salts solution, supplemented with 20 mM-Hepes buffer, pH 7.5, was added to the Petri dishes. The explants were incubated at 37 °C in a humidified atmosphere of $CO_2/O_2/N_2$ (3:50:47) on a rocker platform at 3 cycles/min to allow the contact of explants with both atmosphere and medium (Trump *et al.*, 1980). A period of 1 h elapsed between removal at surgery and the beginning of incubation.

Incorporation of radioactive threonine and glucosamine into explant culture. After a 2 h incubation, the culture media were changed and replaced by Hanks buffered salt solution containing either L-[^{3}H]threonine or D-[^{3}H]-glucosamine (400 μCi/ml) for case no. 1 and exclusively L-[^{3}H]threonine for cases no. 2 and no. 3, and the explants were incubated as described above. After a 20 min pulse, labelled culture fluids were removed and the explants were washed repeatedly with CMRL 1066 medium supplemented by 2 mM-glutamine and 10 mM unlabelled threonine. Samples were processed immediately with no chase; others were re-incubated in CMRL 1066 medium containing unlabelled threonine for 40 min and then for a 280 min chase.

At each time interval, eight tissue explants were removed from the culture dishes and immersed in 1 ml of extraction buffer (10 mM-Tris/HCl buffer, pH 7.3, containing 150 mM-NaCl, 0.02% NaN_3, 1% Nonidet P40 and 1 mM-EDTA; 1 mM-phenylmethanesulphonyl fluoride, 0.2 mM-leupeptin, 0.14 mM-pepsatin and 1 mM-phenanthroline were included to prevent proteolysis). Cell extracts were obtained after homogenizing the labelled explants at 4 °C in a Potter–Elvehjem glass/Teflon homogenizer (for 15 s); cell debris was discarded after centrifugation at 4000 *g* for 5 min, and the supernatants were kept frozen.

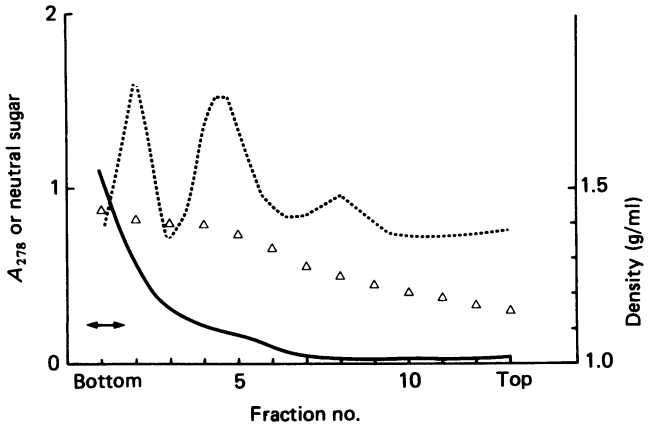

Fig. 1. CsBr-density gradient centrifugation of respiratory mucus sampled in the bronchial tree

The bronchial mucus was centrifuged at 125000 *g* for 48 h (Houdret *et al.*, 1986). Fractions (0.8 ml) were collected and analysed for absorbance at 278 nm (-----) and for neutral sugar (——). Density was measured by weighing (△). The mucin fraction collected in tube no. 1 was analysed by electron microscopy.

Immunoprecipitation. To remove the labelled IgA and IgG synthesized by the bronchial mucosa during the experimental period, the cell extract (1 ml) of case no. 1 was absorbed with 16 μl of anti-IgA serum for 1 h at room temperature, and then 32 mg of Protein A–Sepharose was added. For the other two cases, no precipitation of IgA was done, but a treatment with 16 mg of Protein A–Sepharose was carried out. After a 1 h incubation at room temperature, the insoluble material was removed by centrifugation at 4000 *g* for 5 min.

The extracts were then incubated with 16 μl of pre-immune rabbit serum for 1 h at 4 °C, again followed by a 1 h incubation with Protein A–Sepharose (16 mg) at room temperature. The Protein A–Sepharose was pelleted by centrifugation at 4000 *g* for 5 min and the supernatant was separated into two 0.5 ml portions.

A 0.5 ml portion of supernatant was then incubated once (cases no. 2 and no. 3) or twice (case no. 1) with a mixture of immobilized lectins (75 μl of HPA–Ultrogel, 75 μl of WGA–Ultrogel and 75 μl of RCA_{120}–agarose) for 1 h at room temperature in order to remove glycosylated precursors or labelled mucins; the mixture was then centrifuged and only the supernatant was kept. The other 0.5 ml was not treated with lectins.

The two portions were then incubated overnight with 7 μl of immune serum at 4 °C; in some experiments the immune serum was absorbed with desialylated bovine submaxillary mucin (Marianne *et al.*, 1986). Protein A–Sepharose (7 mg) was added to each tube, and the adsorbed immunoprecipitate was collected by centrifugation at 4000 *g* for 5 min after incubation for 1 h at room temperature. The pellet was washed with 0.5 ml of 10 mM-Tris/HCl buffer, pH 7.2, containing 150 mM-NaCl and 0.25% Nonidet P40 and centrifuged (three times).

The adsorbed products were then eluted by boiling the pellets for 3 min in 100 μl of Laemmli sample buffer (125 mM-Tris/HCl buffer, pH 6.8) containing 1% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 10% (v/v) glycerol (Laemmli, 1970).

SDS/polyacrylamide-slab-gel electrophoresis and fluorography. Electrophoresis was performed in 5–18%-gradient polyacrylamide gels with a Tris/glycine buffer at pH 8.6 (Slayter *et al.*, 1984). Samples containing 1% SDS, together with a small amount of Bromophenol Blue as tracking dye, were loaded on to the gel. They were electrophoresed at 10 V/cm for 17 h, and the slabs were stained for proteins with Coomassie Brilliant Blue R-250. The apparent molecular mass of the proteins was determined by comparison with thrombospondin (420 kDa) and with ^{14}C-labelled markers: myosin (220 kDa), phosphorylase *b* (97.4 kDa), albumin (69 kDa), ovalbumin (46 kDa) and lactoglobulin A (18.4 kDa).

For detection of radioactive bands on polyacrylamide gels, the slab gels were bathed for 30 min in Amplify, placed on wetted Whatman 3 MM filter paper and dried *in vacuo* at 80 °C. The dried gels were exposed to Kodak X-Omat AR5 film at −80 °C in the dark for 10 days and developed.

RESULTS AND DISCUSSION

Electron microscopy of human bronchial mucus glycoproteins

Bronchial mucus was collected during bronchial endoscopy. It was immediately mixed with various proteinase inhibitors (phenylmethanesulphonyl fluoride, *N*-ethylmaleimide, 1,10-phenanthroline, tosylphenylalanylchloromethane, leupeptin and pepstatin A) to minimize proteolytic degradation by serine, thiol and metallo-endopeptidases (Barrett, 1977). It was then gently stirred for 48 h at 4 °C in order to solubilize mucins, but avoiding excessive mechanical shearing. Density-gradient centrifugation is one of the methods that can be used to purify bronchial mucins (Creeth *et al.*, 1977; Woodward *et al.*, 1982).

Human bronchial mucins were prepared by CsBr-density-gradient centrifugation (Fig. 1), which resulted in a carbohydrate-rich band at a density of 1.4 g/cm^3 near the bottom of the gradient. The possibility of contamination of this fraction by nucleic acid could be discounted, since (i) no nucleic acid could be observed on agarose electrophoresis (Roussel *et al.*, 1972) and (ii), on the basis of the u.v. spectra of this fraction, the maximum amount of nucleic acid that might be present would be much less than 1%. Moreover, the bronchial secretion sample was not infected. The presence of the six proteinase inhibitors was maintained during the whole purification procedure. Under these circumstances, the degradation of secreted mucins should have been eliminated as much as possible.

Electron micrographs, prepared with attention to the inhibition of proteolysis, show that mucins are filamentous (Fig. 2) with a length distribution extending to higher values than was found previously (Slayter *et al.*, 1984). In studies with epiglycanin Wold *et al.* (1985) have found that the most probable extended length for this mucin-type molecule is that obtained by following the distribution up to the point where it essentially cuts off. In the present study on human bronchial mucins, the maximum length observed with significant frequency is in the range 1500–1650 nm; only a few per cent are longer (Fig. 3). Assuming that the mucin peptide is fully extended (0.364 nm per fully extended amino acid

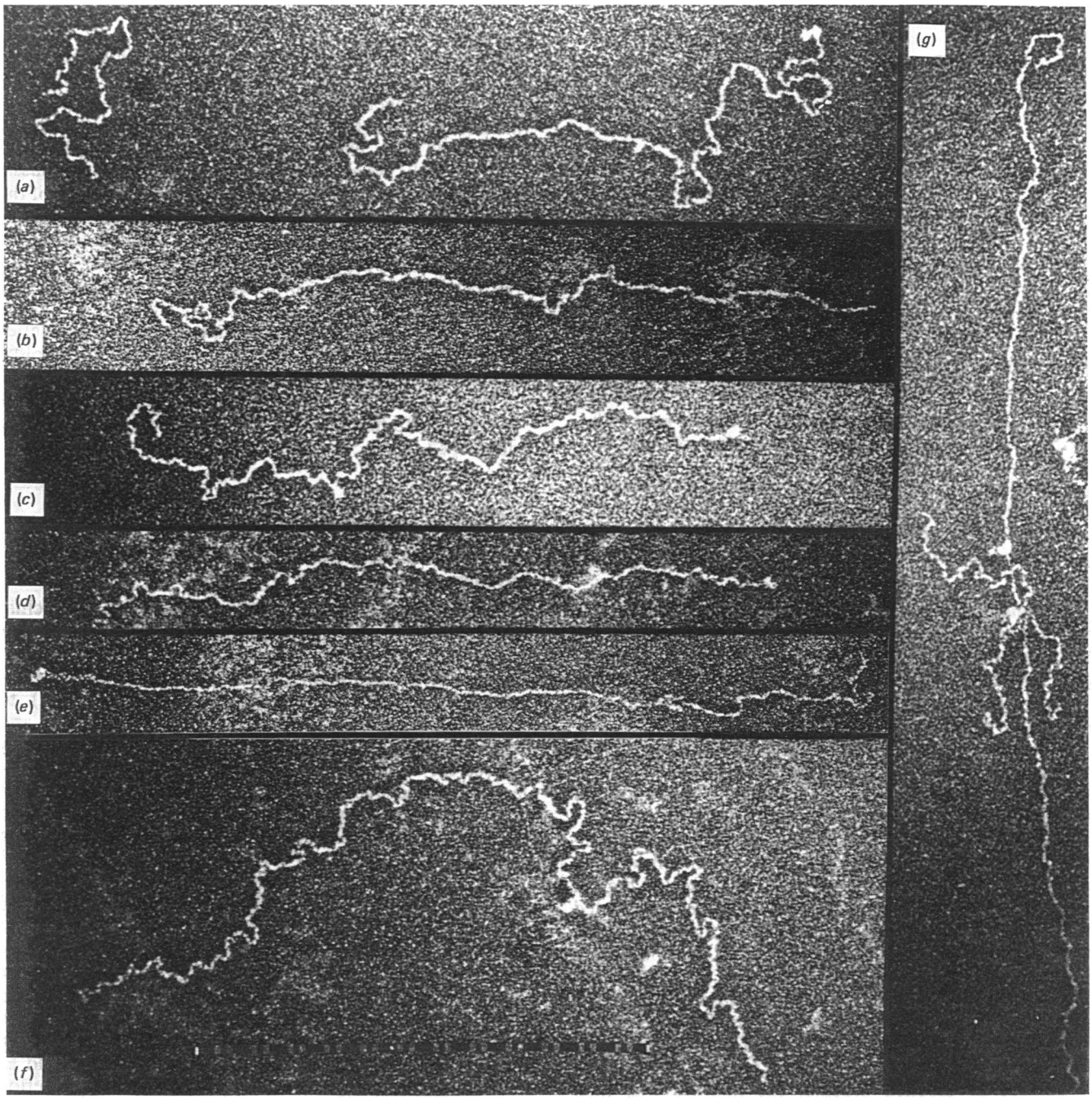

Fig. 2. Dark-field electron micrographs (*a–f*) of high-molecular-mass human bronchial mucins obtained from lightly tungsten-shadowed specimens

The cut on the right (*g*) displays an aggregate. Magnification ×115000.

residue), mucin species in the range 1500–1650 nm would correspond to a peptide with molecular mass in the range 400–450 kDa.

The relatively broad range of the length distribution below 1650 nm is analysed as follows.

The variable extension of the peptide bond, the variable content of sialic acid, frequency of carbohydrate side chains, and secondary and tertiary structure are all factors that may contribute to the observation of variable lengths for a given molecular mass. Equally important may be the method of specimen preparation, which allows a mucin to spread on a surface in the presence of glycerol. Previous work with epiglycanin with the use of this method indicates that even relatively homogeneous preparations of mucin molecules (determined in the ultracentrifuge) may be observed by electron microscopy to be heterogeneous in length (Slayter & Codington, 1973). Pauling *et al.* (1951) have set limits on the variable extension of the peptide bond from a lower limit of 0.15 nm, equivalent to the α-helical configuration, to approx. 0.364 nm per residue, when fully extended. Thus there is a range of more than 2-fold over which the peptide bond can extend. Examination of the contour-length distribution of bronchial mucin molecules ob-

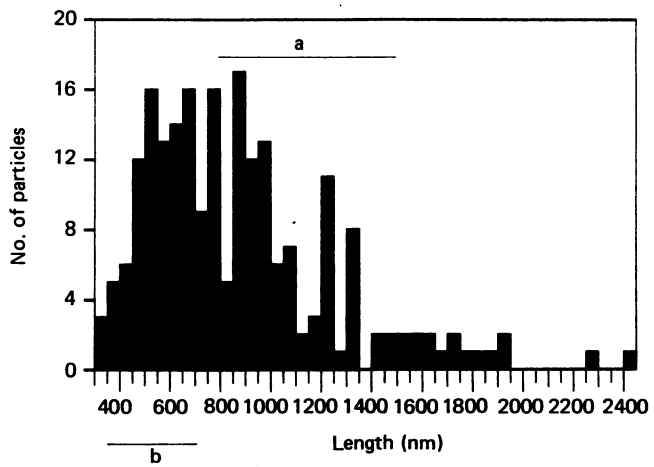

Fig. 3. Length distribution of purified respiratory mucins obtained from micrographs, showing also ranges of lengths calculated for extensions of 0.15 nm (b) and 0.364 nm (a) per peptide bond to be equivalent to ranges 200–400 kDa

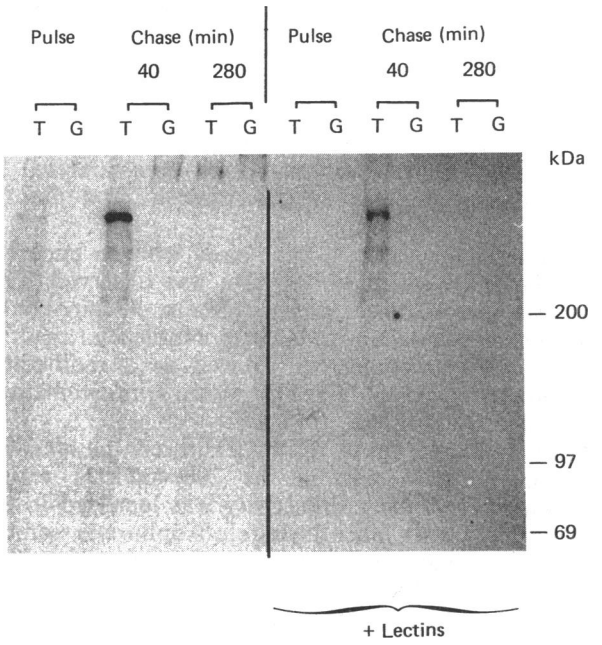

Fig. 4. Fluorography pattern of a polyacrylamide-slab-gel electrophoretogram of precipitatable [^{3}H]threonine-labelled (T) or [^{3}H]glucosamine-labelled (G) mucin peptide precursors from patient no. 1

The cell extracts were treated twice with lectins (six lanes on the right) or not at all (six lanes on the left). Electrophoresis was carried out after a 20 min pulse, after a 40 min chase and after a 280 min chase.

tained here in the presence of inhibitors, isolated from a CsBr gradient, shows lengths well in excess of 1500 nm. However, a very large proportion of the distribution is seen to be of the order of a half of this length. The shorter lengths might be due either to an 'accordion' effect on the peptide backbone or to proteolysis, which might have occurred before collection.

It is noticed in the micrographs that some regions of molecules tend to present rather straight (Fig. 2*e*), whereas others tend to be kinked, sometimes throughout their length (Figs. 2*a*, 2*c* and 2*f*). Moreover, although measurements of the contour length are very carefully made, there may be unresolved micro-kinks related to the 'accordioning' of the peptide bond. Thus the breadth of the histogram may be generally related to the molecular mass of the polypeptide backbone by the following analysis: first, a peak on the low side, from roughly 300–700 nm, corresponding to a range of 200–400 kDa peptide assuming the extension per peptide bond to be 0.15 nm; secondly, the upper range for the extended molecules from roughly 700–1650 nm may also represent a 200–450 kDa polypeptide backbone but with 0.364 nm extension per peptide bond; the small number of particles above 1650 nm may be considered to represent aggregates.

Sheehan *et al.* (1986) have reported lengths of bronchial mucins in the same general range as those reported here, except for a somewhat larger proportion above 1500 nm. There was significant evidence of aggregation in their micrographs, which, coupled with the fact that glycerol was not used as a spreading agent, suggests that aggregation may account for their reported skewing to larger lengths. Also, aggregation in solution would account for the much higher molecular mass reported from their light-scattering measurements. Mikkelsen *et al.* (1985) have reported lengths for bronchial mucins cutting off at 1500–1650 nm, but qualify their results by suggesting that the measured contour length may be significantly shorter than the 'actual' contour length, although this possibility has not been supported by evidence.

Thus, even by limiting the proteolytic degradation, bronchial mucins present a broad distribution of length. Therefore it was necessary to find out whether the properties of the mucin peptide precursors could explain this polydispersity.

Identification of human bronchial mucin precursors

The preparation of an immune serum specifically directed against mucin peptide epitopes is probably a prerequisite for the isolation of mucin precursors, but raises several problems.

Human respiratory mucin preparations obtained from sputum are frequently contaminated by small quantities of peptide, and may not be suitable to raise an immune serum because it might contain antibodies against peptide contaminants as well as antibodies directed against mucin peptide epitopes.

Peptide contaminants as well as naked regions can be eliminated by proteolysis during the preparation of glycopeptides corresponding to the highly glycosylated regions of human bronchial mucins. But, then, these glycopeptides have to be deglycosylated in order to uncover peptide epitopes. Deglycosylation with endo-β-*N*-acetylgalactosaminidase has proven to be rather limited (Feldhoff *et al.*, 1979), and chemical deglycosylation, although more extensive, does not completely remove all the sugars, especially the *N*-acetylgalactosamine residues of the carbohydrate–peptide linkages (Mian *et al.*, 1986; Marianne *et al.*, 1986).

In the present study, an antiserum was raised against the mixture of peptides and glycopeptides obtained by treatment of human bronchial glycopeptides with trifluoromethanesulphonic acid (Marianne *et al.*, 1986). This antiserum was shown specifically to reveal human respiratory goblet cells and mucous glands (M. Mazzuca, personal communication).

Therefore, explants from macroscopically healthy human bronchi were pulse-labelled with [^{3}H]threonine for 20 min, followed by a chase incubation in the presence of unlabelled threonine for either 40 min or 280 min. The radioactive products were immunoprecipitated with this antiserum and then analysed by SDS/polyacrylamide-gel electrophoresis and fluorography.

After a 20 min pulse, a faint band with an apparent molecular mass of about 400 kDa was observed. This band was much more intense after a 40 min chase. However, radioactive material with a molecular mass of approx. 200 kDa appeared, as well as a radioactive smear in the region 200–400 kDa with a reinforcement at about 300 kDa (Fig. 4).

After a 280 min chase, all the [^{3}H]threonine labelling at about 400 kDa and in the 200–400 kDa region disappeared, and the radioactivity was localized in the stacking gel (Fig. 4). Since during electrophoresis secreted human bronchial mucins are usually retained in the stacking gel (Slayter *et al.*, 1984), these labelled products probably correspond to heavily glycosylated mucin peptides. These results suggest that the labelled products at about 400 kDa and in the 200–400 kDa region are mucin precursors.

Radioactive bands with a lower molecular mass were also visible in experiments corresponding to case no. 2 and case no. 3 (results not shown) when the cell extracts were not treated with anti-IgA serum. These smaller-molecular-mass species are not observed in the experiment with case no. 1 (Fig. 4), in which the cell extracts were treated with anti-IgA serum. Therefore they probably do not correspond to mucin precursors.

Since the antiserum used had an affinity for peptide epitopes as well as for incomplete carbohydrate structures (Marianne *et al.*, 1986), it was necessary to determine whether the precursors in the 200–400 kDa region, especially the band of about 400 kDa, were glycosylated or not.

Consequently, a similar experiment was simultaneously carried out with[^{3}H]glucosamine. A radioactive product was barely visible after a 20 min pulse in the stacking gel (Fig. 4). It increased after a 40 min pulse and was still visible after a 280 min pulse (Fig. 4). No labelling was observed at 400 kDa or in the 200–400 kDa area. This experiment suggests that the 200–400 kDa [^{3}H]threonine-labelled precursors are not glycosylated after a 40 min chase and that their disappearance after a 280 min chase occurs at the same time as their glycosylation.

Ricinus communis and wheat-germ agglutinins have been shown to bind to material contained in bronchial glandular cells (Mazzuca *et al.*, 1982). These lectins have also an affinity for most secreted bronchial mucins (Lhermitte *et al.*, 1981). *Helix pomatia* agglutinin has an affinity for the content of mucous cells (Mazzuca *et al.*, 1982).

In order to determine whether any of the [^{3}H]-threonine-labelled components of the 200–400 kDa region are glycosylated, a comparative study was performed with and without treatment of the cell extracts with *Helix pomatia*, *Ricinus communis* and wheat-germ agglutinins bound to beads (Fig. 4). These treatments were designed to remove all the radioactive precursors with complete or growing carbohydrate structures having an affinity for those lectins, and especially those containing the *N*-acetylgalactosamine–peptide linkage (Marianne *et al.*, 1986). Experiments were also carried out after adsorption of the antiserum with desialylated bovine submaxillary mucin, which contains *N*-acetylgalactosamine–peptide linkages.

Prior adsorption of the antiserum with desialylated bovine submaxillary mucins did not change the relative intensities of the radioactive bands (results not shown).

After treatment with the lectins, the [^{3}H]threonine-labelled 400 kDa fraction was still clearly visible, although slightly attentuated (Fig. 4); the bands in the 200–300 kDa region after a 40 min pulse were not diminished by the lectin treatment, indicating that these are not glycosylated (Fig. 4). No [^{3}H]glucosamine- or [^{3}H]threonine-labelled material could be detected in the stacking-gel area (Fig. 4). Similar results were obtained with cases no. 2 and no. 3 (results not shown).

Altogether these data indicate that the [^{3}H]threonine-labelled 200–400 kDa bands contain non-glycosylated mucin peptide precursors, which with time disappear, presumably because they are progressively glycosylated to form the [^{3}H]glucosamine- and [^{3}H]threonine-labelled products that remain in the stacking gel, just as do secreted bronchial mucins (Slayter *et al.*, 1984). The glycosylated products can be removed with lectins.

The band at about 400 kDa does not appear to be labelled with [^{3}H]glucosamine, although some radioactivity is already detected in the stacking gel after a 20 min pulse, and is even more apparent after a 40 min chase. However, this band is attenuated by lectin treatment; this attenuation does not seem to differ after one or two series of lectin treatments. The band at about 400 kDa does not disappear after prior adsorption of the immune serum with desialylated bovine submaxillary mucin. Therefore this band may contain a mixture of mucin peptide precursors, including some slightly glycosylated peptides containing sufficient carbohydrate to be bound by the lectins but not enough to induce a marked change of molecular mass and mobility in polyacrylamide-gel electrophoresis or to be labelled with [^{3}H]-glucosamine under present conditions. An alternative possibility is that non-glycosylated precursors are non-specifically adsorbed on the lectins.

The correlation of the mucin lengths observed by electron microscopy with the molecular size of the mucin precursors is relatively good. The distribution of lengths observed by electron microscopy may be due to the synthesis of different mucin peptides in the range 200–450 kDa, to an 'accordion' effect related to the extension of the peptide backbone of the secreted mucins, or to the possibility of limited proteolytic phenomena occurring either at the glandular cell level or immediately after secretion into the bronchial lumen. Nevertheless, the maximum significant lengths correspond to the maximum predicted by the biosynthetic experiments. Thus, for the first time, several respiratory mucin peptide precursors in the range 200–400 kDa have been defined by explant-culture experiments, and these are shown to be completely consistent with actual lengths of mucins.

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