Mucins secreted by a transformed cell line derived from human tracheal gland cells¹

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High-molecular-mass glycoconjugates are secreted by the continuous cell line MM-39, which has been obtained from cultured human tracheal gland cells transformed by simian virus 40. They were purified on Sepharose® CL-4B and then by two steps of density-gradient centrifugation. High-molecular-mass glycoproteins resistant to digestion by hyaluronidase, chondroitin ABC lyase and heparitinase were obtained, in addition to hyaluronic acid and proteoglycans. They were susceptible to β -elimination. They contained polylactosaminoglycan chains as well as carbohydrate chains with a terminal sialic acid in the NeuAc $\alpha 2-3$ sequence. Most of them have a buoyant density of 1.45 g/ml in

CsCl-density-gradient centrifugation, except for MUC1. The MM-39 cells were also characterized by a high expression of *MUC1* and *MUC4* genes, but they did not express *MUC2*, *MUC3*, *MUC5B* and *MUC5AC*. Therefore the MM-39 cells synthesized mucin-like glycoproteins as well as lysozyme and mucous proteinase inhibitor [Merten, Kammouni, Renaud, Birg, Mattéi and Figarella (1996) *Am. J. Respir. Cell. Mol. Biol.* **15**, 520–528]; they should be considered as having a mixed, both serous and mucous, phenotype.

INTRODUCTION

The most important part of mucus is a broad family of polydisperse and highly glycosylated molecules called mucins. They have large molecular masses ranging from about 3×10^5 to $> 1 \times 10^6$ Da [1]. Recently, different stretches of human mucin cDNAs have been described and several mucin genes, *MUC1–MUC8*, have been identified (see review [2] and additional data [3–10]). Most of these apomucins contain extended domains of tandem repeats rich in hydroxylated amino acids, which are potential sites of O-glycosylation. The reactions of glycosylation are responsible for about 70% of the mass of the mucin molecules. Mucin carbohydrate chains are extremely diverse [11], and secreted mucins may be fucosylated, sialylated and/or sulphated.

Respiratory mucins are synthesized by goblet cells from the surface epithelium and by mucous cells from the submucosal glands. These glands are constituted by both serous cells and mucous cells [12]. Various proteins, such as lysozyme and mucous proteinase inhibitor (MPI) are predominantly synthesized by serous cells [13–17]. Mucus hypersecretion in the human airways occurs in bronchial diseases such as chronic bronchitis or cystic fibrosis, and there is a need for a better knowledge of the composition and the regulation of the glandular secretion in order to understand the pathophysiology of bronchial diseases.

Non-continuous epithelial cell cultures derived from human tracheal glands have been reported to express a mixed serous and mucous phenotype [18,19], but this approach is difficult due to the lack of actively proliferating cells, the small quantity of secreted material and the short lifespan of these cultures.

Chopra et al. [20,21] have already immortalized normal and cystic fibrosis cells from human tracheal glands by the explant/

outgrowth procedure; these cultures were found to secrete high-molecular-mass mucin-like glycoproteins and to express the *MUC2* mucin gene [21]. More recently, Merten et al. [22] succeeded in obtaining another immortalized cell line, MM-39, by infecting homogeneous cultured human tracheal gland cells with wild-type simian virus 40. They have demonstrated that these cells secreted lysozyme and MPI and that their stimulation was induced by adrenergic, cholinergic and purinergic agonists.

Alterations in glycosylation or sulphation of glycoconjugates have been observed in cystic fibrosis [23]. This disease is due to mutations in a gene encoding a membrane protein called CFTR (cystic fibrosis transmembrane conductance regulator). However, nothing is known about the mechanisms linking CFTR and mucin synthesis. MM-39 cells were also shown to express CFTR highly [22], like the *in vivo* original cells [24]. A better knowledge of the composition and the regulation of the glandular mucins secreted by these cells appears therefore relevant. As a basis for subsequent studies on regulation of mucin secretion linked to CFTR, the aim of the present work was to find out how far these MM-39 cells have conserved their ability to secrete glycoconjugates such as mucins.

EXPERIMENTAL

Materials

Culture materials were obtained from Life Technologies (Paisley, U.K.). The anti-MUC1 monoclonal antibody (mAb) LICR-LON-M8 (culture supernatant; IgG1 isotype) was a generous gift from Dr. D. Swallow (Medical Research Council, London, U.K.). The preparation of the (19.2) polyclonal antiserum, raised against deglycosylated human bronchial glycopeptides of mucins,

Abbreviations used: MPI, mucous proteinase inhibitor; CFTR, cystic fibrosis transmembrane conductance regulator; mAb, monoclonal antibody; MAA, *Maackia amurensis* agglutinin; UEA I, *Ulex europeus* agglutinin; TFMS, trifluoromethanesulphonic acid; ECL, enhanced chemiluminescence; TBS, Tris-buffered saline; VNTR, variable number of tandem repeats; GAG, glycosaminoglycans.

¹ Dedicated to Roger W. Jeanloz on the occasion of his 80th birthday.

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and of the pre-immune serum (19.0) has been previously described [25]. The anti-(Lewis b) mAb 3D3 was a gift from Dr. N. Emery [26]. Heparitinase (heparinase III) from *Flavobacterium heparinum*, keratanase (*Pseudomonas* species), micrococcal nuclease from *Staphylococcus aureus* (Foggi strain), neuraminidase type VI from *Clostridium perfringens*, peroxidase-labelled lectin from *Ulex europaeus* (UEA I), peroxidase-labelled lectin from *Lotus tetragonolobus purpureas*, benzamidine hydrochloride, EDTA, *N*-ethylmaleimide, α -aminohexanoic acid, iodoacetamide, PMSF and trifluoromethanesulphonic acid (TFMS) were purchased from Sigma (St Louis, MO, U.S.A.). Peroxidase-labelled goat polyclonal anti-rabbit IgG (heavy + light) was purchased (1 ml) from Jackson ImmunoResearch (West Grove, PA, U.S.A.). RNase A from bovine pancreas, biotin-conjugated *Maackia amurensis* agglutinin (MAA) (1 mg/ml) and biotinylated *Sambucus nigra* agglutinin (1 mg/ml) were obtained from Boehringer Mannheim (Mannheim, Germany). Peroxidase-labelled streptavidin (1 mg/ml) was from Pierce (BA Oud-Beijerland, The Netherlands). The anti-(Lewis X) mAb and the purified anti-(sialyl Lewis X) mAbs (IgM isotype; 0.5 mg/ml) were purchased from Sera-Lab (Crawley Down, Sussex, U.K.). Chondroitinase ABC and hyaluronidase from *Streptomyces hyalurolyticus* were obtained from Miles Laboratories (Lisle, IL, U.S.A.). [6-³H]Glucosamine (40 Ci/mmol) and [6-³H]fucose (40 Ci/mmol), the enhanced chemiluminescence (ECL) Western-blotting detection system, the ECL glycoprotein-detection module, the nitrocellulose membrane Hybond-C extra, the Hybond-N sheets, Amplify[™] and Hyperfilm[™]-MP were purchased from Amersham (Amersham, Bucks., U.K.). CsBr, CsCl and guanidinium chloride were from Merck (Darmstadt, Germany). The Immobilon[™]-P membrane was purchased from Millipore (Bedford, MA, U.S.A.). cDNA probes have been already described: MUC1 was detected using PUM24P [27], MUC2 with SMUC41 [28], MUC3 with SIB139 [29], MUC4 with JER 64 [9], MUC5B with JER 57 [5] and MUC5AC with JER 58 [6]. β -Galactosidase-JER 64 fusion protein was a generous gift from Dr. J. P. Aubert (Unité INSERM n° 377, Lille, France).

Cell culture and metabolic radiolabelling

Cells were cultured as previously described [30], with some modifications. Briefly, they were grown on collagen-coated plastic culture flasks in Dulbecco's modified Eagle's medium/F12 medium supplemented with 10 % (v/v) adult bovine serum, 2 % (v/v) Ultrosor[®] G, glucose (10 g/l), sodium pyruvate (0.22 g/l), amino acids, adrenaline and antibiotics [30]. Cells were passaged at a split ratio of 1:4.

At a time of 5 days after trypsinization, T75 flasks, containing cells at confluence, were incubated with 20 μ Ci/ml [³H]glucosamine or 20 μ Ci/ml [³H]fucose in the medium for 5 days with one medium change.

Preparation of total RNA and Northern-blot analysis

Total RNAs were extracted using the guanidinium thiocyanate/CsCl method according to Chirgwin et al. [31]. Northern-blot analysis was performed as previously described [6].

Separation of the radiolabelled products synthesized by the cell line

Gel-filtration chromatography

Gel-filtration chromatography of the dialysed and lyophilized culture supernatant was performed on Sepharose CL-4B (2.5 cm $\times$ 50 cm) [32]. When the culture supernatant was supple-

mented with proteinase inhibitors [33], it was directly applied on a Sepharose CL-4B column equilibrated at 4 °C and containing the same proteinase inhibitor cocktail [33].

Density-gradient centrifugations

The recovered material was submitted to a first density-gradient centrifugation according to Creeth et al. [34] and subjected to a second density-gradient centrifugation according to Carlstedt et al. [35] with a buffer containing 67 % (w/v) CsCl and 0.2 M guanidinium chloride.

Separation of the unradiolabelled products secreted by the cell line

Culture supernatant (2.4 l) was exposed to the same purification procedure. The material was loaded as 0.5 g per chromatography on Sepharose CL-4B. The first density-gradient centrifugation was performed with 18.5 mg of material contained in each centrifugation tube. For the second density-gradient centrifugation, the separation was performed in a single centrifugation tube containing the material recovered from the first density-gradient centrifugation and having a density higher than 1.35 g/ml.

SDS/PAGE analysis

Slab gel electrophoresis using a 5–15 % polyacrylamide gradient was performed as previously described [36]. The samples were treated with 2 % (w/v) SDS/5 % (v/v) β -mercaptoethanol overnight before being loaded on to the gel. Gels run with radiolabelled products were immersed in Amplify solution for 30 min, dried and then exposed to Hyperfilm-MP film at –80 °C.

Electroblotting and overlays with lectins or antisera

Blots were performed [37] on to nitrocellulose (Hybond-C extra) or PVDF membranes (Immobilon-P). PVDF membrane was exclusively used when a deglycosylation of the material immobilized on a transfer membrane was performed with TFMS [38].

Carbohydrate labelling (with sodium metaperiodate reagent) was performed with the ECL glycoprotein detection kit. It was carried out as indicated by the manufacturer in solid phase after SDS/PAGE and transfer to the nitrocellulose membrane.

In order to reveal MUC1 peptide epitopes with the anti-MUC1 mAb (LICR-LON-M8), the membranes were soaked in Tris-buffered saline (TBS) containing 4 % (w/v) defatted milk and 3 % (w/v) Tween[™]-20 before a 30 min incubation at room temperature with the mAb M8, which was diluted 1:100 in TBS containing 1 % (w/v) defatted milk. After washing, the membranes were incubated with the 1:25000-diluted secondary antiserum, conjugated to horseradish peroxidase, for 1 h at room temperature. The apomucin epitopes were revealed using the ECL Western-blotting detection system.

In order to reveal the epitopes recognized by the (19.2) antiserum, we used the procedure developed by Thornton et al. [38], allowing an efficient deglycosylation of mucin immobilized on PVDF membrane. The following steps were identical with those previously described for the LICR-LON-M8 mAb. The (19.2) antiserum was diluted 1:5000. The same dilution was used when the (19.2) polyclonal antiserum was preincubated with the fusion protein corresponding to the *MUC4* variable number of tandem repeats (VNTR) region. The control preimmune serum (19.0) was used 1:1000.

In order to reveal carbohydrate epitopes, the membranes were soaked in TBS containing 3% (w/v) Tween 20 exclusively. The biotin-conjugated MAA and *Sam. nigra* lectins were diluted 1:1000 and 1:500000 respectively. After washing, the nitrocellulose bands were incubated with peroxidase-labelled streptavidin (1:15000) for 1 h and revealed with the ECL Western-blotting detection system. The peroxidase-labelled lectins from UEA I and from *Lotus* were diluted 1:50000 and were directly revealed after washing. This dilution was also applied to the mAbs anti-(Lewis X), anti-(sialyl Lewis X and anti-(Lewis b).

The control of the MAA lectin reactivity was performed as indicated by Strub et al. [39].

Figure 1 Analysis of secreted ^3H glucosamine-radiolabelled glycoconjugates

(A) Gel-filtration chromatography of the dialysed and lyophilized culture supernatant on Sepharose CL-4B (2.5 cm $\times$ 50 cm) equilibrated with 0.1 mM Tris/HCl buffer, pH 8, containing 0.2 M NaCl and 0.02% (w/v) sodium azide. The flow rate was 15 ml/h. (B) Fluorogram pattern from SDS/PAGE (5–15% polyacrylamide) of radiolabelled materials contained in fraction I (lane 1) or fraction II (lane 4). Materials were treated with hyaluronidase [38] (lanes 2 and 5). A simultaneous treatment with hyaluronidase, chondroitinase ABC and heparitinase (GAG enzyme cocktail) [40] was also performed in lanes 3 and 6. The interface between the stacking gel and the separating gel is indicated by the dashed line. Positions of molecular-mass markers are shown on the right.

RESULTS

Analysis of radiolabelled glycoconjugates

Gel filtration

The ^3H glucosamine-radiolabelled material secreted by the MM-39 cells was separated into three fractions (I, II and III) by gel filtration on Sepharose CL-4B (Figure 1A); most of the radio-labelling was in peaks I and II. Peak III, which contained the proteins of the medium, was not studied. ^3H Fucose-radio-labelled macromolecules, excluded from Sepharose CL-4B, were not observed in the culture supernatant (results not shown).

SDS/PAGE of the high-molecular-mass fractions

The soluble material obtained from the secreted fraction I, after its digestion with the glycosaminoglycans (GAG) enzyme cocktail, migrated as a broad smear from the top of the stacking gel to the middle of the separating gel (at about 50 kDa) (Figure 1B, lane 1). The almost total degradation of this component by hyaluronidase [40] demonstrated the presence of hyaluronic acid (Figure 1B, lane 2). The band remaining at the top of the stacking gel was not degraded by the GAG enzyme cocktail (Figure 1B, lane 3). Treatment of the soluble material from the secreted fraction II with hyaluronidase [40] also indicated the presence of a small amount of polydisperse hyaluronic acid (Figure 1B, lane 4 as compared with lane 5). Additional treatment with chondroitinase ABC and heparitinase [38] allowed the degradation of some high-molecular-mass proteoglycans containing chondroitin sulphate and/or heparan sulphate (Figure 1B, lane 6 as compared with lane 5). However, these treatments left most radiolabelled products migrating as a large smear as well as discrete bands in the area of 20–46 kDa. The intensity of the smear was the highest in the stacking gel and decreased progressively in the separating gel (Figure 1B, lane 6). Identical electrophoretic patterns were observed when β -mercaptoethanol was omitted in the sample buffer.

The results above were not modified by the addition of proteinase inhibitors [33] in the culture supernatant, during the gel filtration at 4 °C and during the digestions with the same combination of GAG-degrading enzymes (added separately) [41] (results not shown). The remaining material from fraction I was investigated as to its elution position on Sepharose CL-2B and the effect of reduction by β -mercaptoethanol [33]. There was no shift of the material into the included volume (results not shown). This same material was not degraded by keratanase (results not shown). The material from fraction II disappeared after treatment by keratanase (0.1 unit/ml in Tris/acetate buffer, pH 7.3), except in the stacking gel (results not shown). The band at the top of the stacking gel (fraction I) and the smear from fraction II were susceptible to alkaline treatment [42] (results not shown).

Purification by density-gradient centrifugation of the secreted high-molecular-mass glycoproteins

Fractions I and II radiolabelled with ^3H glucosamine were pooled and subjected to a density-gradient centrifugation. In order to limit the contamination of fraction II by fraction III, only the tubes between $K_{av} 0$ and $K_{av} 0.2$ were collected. A major peak of radioactivity (Fa*, * indicating the presence of radio-labelled material in this peak), with a buoyant density higher than 1.4 g/ml, was found in the bottom of the tube (Figure 2A); it may correspond to the presence of proteoglycans and mucins. Two other fractions (Fb* and Fc*) with lower densities and a high absorbance at 280 nm were also observed (Figure 2A).

Figure 2 Purification of high-molecular-mass radiolabelled glycoconjugates secreted by the MM-39 cell line

(A) The [^3H]glucosamine-labelled fractions I and II, which were obtained after Sepharose CL-4B gel filtration of culture supernatant, were submitted to a density-gradient centrifugation [34]. Absorbance at 260 nm is not indicated because its variations followed the variations at 280 nm. (B) The [^3H]glucosamine-labelled fraction Fa*, which was obtained after the first density-gradient centrifugation, was treated with hyaluronidase, chondroitinase ABC and heparitinase [40] before being submitted to a second density-gradient centrifugation [35]. The hatched areas corresponded to tubes that were not collected.

Fraction Fa* was treated by enzymes degrading hyaluronic acid and proteoglycans and exposed to a second centrifugation (Figure 2B). The three fractions Fa1*, Fa2* and Fa3* were obtained (Figure 2B). Fraction Fa1*, with the highest density, had a high absorbance at 260 and 280 nm. Two other fractions were obtained: a major fraction, Fa2*, at a 1.45 g/ml density, which did not absorb at 260 or 280 nm, and a minor fraction, Fa3*, at a 1.40 g/ml density. The material from fraction Fa2* migrated as a smear in SDS/PAGE, which disappeared after β -elimination. The smear was degraded by keratanase, except at the top of the stacking gel (results not shown). Consequently, polylactosamine chains were carried by polydisperse glycoconjugates having the characteristics of mucins.

The amounts of recovered radiolabelled material were too low to enable a detailed study. Therefore, high-molecular-mass material contained in a pool of unlabelled culture supernatant was isolated by collecting the tubes from the void volume to the K_{av} 0.2 volume of a Sepharose CL-4B column. The material (445 mg) underwent density-gradient centrifugation using CsBr (Figure 3A). The hexose content was measured [43], as were the absorbances at 260 and 280 nm. Two fractions, Fa and Fb (18.5 mg and 6.6 mg respectively) were collected. They corresponded to radiolabelled fractions Fa* and Fb* respectively (compare Figure 2A with Figure 3A). A third fraction (Fc;

Figure 3 Purification of non-radioactive high-molecular-mass glycoconjugates secreted by the MM-39 cell line

(A) First density-gradient centrifugation performed according to Creeth et al. [34] and applied to the unlabelled fraction obtained after Sepharose CL-4B gel filtration of culture supernatant from cells cultivated at late confluency. Absorbance at 260 nm is not indicated because its variations followed the variations at 280 nm. The neutral sugars were detected by an automated orcinol assay [43]. (B) Second density-gradient centrifugation performed according to Carlstedt et al. [35] and applied to the unlabelled fraction Fa, which was obtained after the first density-gradient centrifugation. (C) Carbohydrate labelling of glycoconjugates by the ECL glycoprotein detection system. This reaction was performed in solution as indicated by the manufacturer, with 10 μl from the different fractions aspirated from the tube bottom of the second density-gradient centrifugation and taken before SDS/PAGE and Western blotting.

4.8 mg), with a high absorbance at 280 nm, was also identified. To ensure that these fractions were not contaminated by any high-molecular-mass components from bovine serum or Ultrosor G, a sample of adult bovine serum or of Ultrosor G was fractionated in the same way by density-gradient centrifugation. No high-density fraction corresponding to fraction Fa was detected, even using the ECL glycoprotein detection module. Since all the components of bovine serum appeared in fractions having densities identical to those of fractions Fb and Fc (results not shown), these two fractions were not further analysed. Consequently, the high-molecular-mass material found in fraction Fa2 is not contaminated by bovine serum and is secreted by the MM-39 cells.

Figure 4 Electroblotting and overlays with lectins or antisera of SDS/PAGE patterns from the fractions Fa1, Fa2 and Fa3 obtained by the second density-gradient centrifugation

Carbohydrate labelling (with sodium metaperiodate reagent; IO_4^-) was performed with the ECL glycoprotein detection kit. It was carried out as indicated by the manufacturer in solid phase after SDS/PAGE and transfer to the membrane. Carbohydrate determinants were detected with MAA lectin. MUC1-type peptide epitopes were revealed with the anti-MUC1 mAb LICR-LON-M8 (M8). MUC4-type peptide epitopes were detected with the (19.2) polyclonal antiserum; the preimmune (19.0) serum was used as control. These two lanes corresponded to material treated with TFMS. Each lane was loaded with 50 μg of material, except for the lanes incubated with the polyclonal antiserum (19.2) and the corresponding preimmune serum (19.0); in these cases, 100 μg of material was loaded on the polyacrylamide gel. Positions of molecular-mass markers are shown to the right of each fraction pattern.

The purification of non-radioactive fraction Fa was improved by performing a second density-gradient centrifugation using CsCl after treatment by the GAG enzyme cocktail (Figure 3B). After SDS/PAGE, the ECL glycoprotein detection kit revealed the presence of high-molecular-mass glycoconjugates, localized in the stacking gel in tubes 1–11 and migrating as a smear in the separating gel for tubes 4–6. Bands with molecular masses lower than 200 kDa were only visible in tubes 7–13 (Figure 3C). The material contained in tubes 1–3 (fraction Fa1; 3 mg), 4–6 (fraction Fa2; 2 mg) and 8–11 (fraction Fa3; 1.7 mg) presented the same densities as those of the [^3H]glucosamine-radiolabelled fractions Fa1*, Fa2* and Fa3* respectively (compare Figure 3B with Figure 2B). These results demonstrate the presence of purified high-molecular-mass mucin-like glycoconjugates in fraction Fa2.

Characterization of the high-density fractions

Fraction Fa1

A major band of fraction Fa1 was stained with Toluidine Blue and had the same anodic mobility as nucleic acids in agarose-gel electrophoresis [38] (results not shown). This fraction Fa1 was incubated with micrococcal nuclease and RNase A [40]. Despite this treatment, it contained a material stained with the ECL glycoprotein detection kit and located in the stacking gel (Figure 4). These glycoconjugates did not react with MAA lectin nor with the mAb LICR-LON-M8 nor with the (19.2) antiserum (results not shown).

Consequently, this fraction contains nucleic acid and unidentified glycoconjugates having a high buoyant density.

Figure 5 Northern-blot analysis of total RNA extracted from MM-39 cell line

Total RNA (25 μg) was loaded on the agarose gel in order to perform the blots. The staining was positive with MUC4 and MUC1 probes after the blots were exposed for 6 h to the film. Exposures of 24 h were tried with MUC2, MUC3, MUC5B, MUC5AC and MUC6 probes without obtaining a signal. The positions of 28 S and 18 S rRNAs are indicated by arrows.

Fraction Fa2

Fraction Fa2 was submitted to SDS/PAGE followed by transfer on to a membrane. The ECL glycoprotein detection module stained intensively the blot in the region corresponding to the stacking gel and to the interface between the stacking gel and the separating gel; a faint smear was also revealed in the 100–400 kDa range (Figure 4). No reactivity was found with lectins specific for fucose residues (UEA I and *Lotus*) or with mAbs directed against fucosylated epitopes [anti-(Lewis X), anti-(sialyl Lewis X) and anti-(Lewis b)] (results not shown). An intense staining appeared with MAA lectin (reacting with sialic acid in the $\alpha 2-3$ sequence [44]), migrating as a smear in the stacking gel and in the separating gel from 400 to 150 kDa (Figure 4); it disappeared when the blot was treated with neuraminidase before being incubated with MAA lectin (results not shown). No signal was detected with *Sambucus nigra* lectin, which is specific for sialic acid in a NeuAc $\alpha 2-6$ sequence [45] (results not shown), indicating that NeuAc was linked $\alpha 2-3$ and not $\alpha 2-6$.

Using the anti-MUC1 mAb M8, a staining of fraction Fa2 was localized at the top of the stacking gel (Figure 4). Such a discrepancy between the signal obtained with the ECL glycoprotein detection module (or the MAA lectin) and that obtained with the anti-MUC1 mAb indicated the presence of other mucin-like glycoproteins, associated to MUC1 glycoproteins in fraction Fa2.

In order to exhibit the reactivity of fraction Fa2 with the (19.2) polyclonal antiserum raised against deglycosylated glycopeptides of bronchial mucins [25], the blots first had to be deglycosylated with TFMS for 30 min. Under these conditions, a smear ranging from the stacking gel to 150 kDa was revealed with the (19.2) antiserum (Figure 4). The staining did not disappear when the (19.2) polyclonal antiserum was pre-incubated with a fusion protein corresponding to the MUC4 VNTR region (results not shown). It was not observed with the preimmune serum (19.0) (Figure 4).

This fraction contains polydisperse sialomucins.

Fraction Fa3

SDS/PAGE of fraction Fa3 showed high-molecular-mass glycoproteins, stained by the ECL glycoprotein detection kit in the stacking gel (Figure 4). The pattern obtained after incubation with MAA lectin was similar to that performed with the anti-MUC1 mAb M8 (Figure 4). The reactivity of this mAb with the fraction Fa3 material was markedly higher than with the fraction Fa2, even though the two fractions were blotted and revealed simultaneously. When the Fa3 blot was exposed to TFMS before incubation with the (19.2) antiserum, no reactivity was observed in the stacking gel or in the separating gel (results not shown) (Figure 4).

MUC1 glycoprotein is abundant in this fraction and seems less polydisperse than the other sialomucins found in the fraction Fa2.

Expression of mucin mRNA by the MM-39 cell line

MUC1 and MUC4 mRNAs were the most abundant messages expressed by these cells. Transcripts of 5 and 4 kb, respectively, were detected with the MUC1 probe, whereas a smear with reinforcement at 4 kb was observed with the MUC4 probe (Figure 5). The hybridization signals corresponding to MUC1 and MUC4 were as intense as the signal corresponding to MUC1 in a control experiment performed with the total RNA from the HT29 MTX cell line, which did not express MUC4 [46] (results not shown).

DISCUSSION

The MM-39 cells derived from human tracheal submucosal glands were described as secreting lysozyme and MPI, indicative of their serous nature [22]. However, in the present work, we give evidence in favour of mucin secretion. This is demonstrated through the following. (i) A secretion of high-molecular-mass glycoconjugates is sensitive to β -elimination and resistant to the GAG-degrading enzymes. (ii) Fa2, the major fraction of these high-molecular-mass glycoconjugates, has a buoyant density of 1.45 g/ml [35]. (iii) This fraction is revealed by the MAA lectin, which is specific for sialic acid in the $\alpha 2-3$ sequence, like mucins collected from human airways [11]; it also contains apomucin epitopes. (iv) MM-39 cells express *MUC1* and *MUC4* genes.

However, these mucins have unusual features also. The mucins secreted by the MM-39 cell line correspond to a population of molecules with different molecular size. A part of this population is excluded from a Sepharose CL-4B column and remains in the stacking gel of SDS/PAGE, but a larger part of the secreted mucins is formed by molecules that are partially included and appear in SDS/PAGE as a smear migrating from the stacking gel to the upper part of the separating gel. Since the mucins secreted by the MM-39 cells were purified in the presence of proteinase inhibitors, the smaller size of these mucins is not the result of proteolysis occurring during the dialysis step, the gel filtration or the treatment with the GAG enzymes. Recently, a novel goblet-cell line, SPOC1, derived from rat trachea has been prepared [47]: the mucins secreted by these cells are included in Sepharose CL-4B after thiol reduction and alkylation [48]. However, in the case of the MM-39 cells, there is currently no evidence for disulphide linkages between mucin monomers, since the electrophoretic pattern and the chromatographic profile of the secreted mucins are not influenced by reduction.

None of the mucin genes that are mainly expressed in the airways (*MUC2*, *MUC5B* and *MUC5AC*) [49] has been detected in the MM-39 cells, which, in our experimental conditions, express *MUC1* and *MUC4*. Those two genes are highly expressed

in normal lung tissue [50]. One should also point out that the *MUC5B* gene is highly expressed in gallbladder epithelial cells only when the cells are cultured on Matrigel, a reconstituted basement membrane that promotes cell differentiation [51]. Using standard culture conditions to propagate the cells, the pattern of *MUC* genes expression from the MM-39 cell line is obviously very different from that of glandular cells in the human respiratory mucosa. Nevertheless, this cellular model may give the opportunity to study MUC4-type glycoproteins and their secretion by respiratory cells. Like *MUC1*, the *MUC4* gene is not clustered on 11p15.5 [52], and its regulation may be completely independent from that of the *MUC2*, *MUC5AC* and *MUC5B* genes. In the literature, the *MUC4* expression pattern corresponds to a weak polydisperse smear and a band with a size varying from 5 kb to 12–16 kb [53–57]. In the present study, the size of the main band was in the range of 4 kb and is analogous to that of the band observed in normal and cancer cells by Ogata et al. [53].

The mucins with a buoyant density of 1.35 g/ml contain MUC1 epitopes revealed by an mAb specific for MUC1 apomucin. The mucins with a buoyant density of 1.45 g/ml contain epitopes revealed by the polyclonal antiserum (19.2) [25] prepared against deglycosylated human respiratory mucins. This immune serum has been used already to localize the peptide precursors of human respiratory mucins within bronchial cells: in the respiratory mucous glands, the labelling was localized around or below the nucleus. In the present study, the apomucin epitopes recognized by the (19.2) antiserum cannot be related to the *MUC4* VNTR region, but it is possible that this antiserum contains antibodies directed against a non-repetitive domain of MUC4.

Another interesting feature of the carbohydrate part of the polydisperse mucins secreted by MM-39 cells is their susceptibility to keratanase, indicating the presence of polyactosaminoglycans. A sensitivity to keratanase has been previously reported by Wu et al. [58] for mucin-like molecules secreted by cultured hamster tracheal epithelial cells, by Davies et al. [59] for cat mucin-like glycoproteins and by Varsano et al. [60], who have described the release of keratanase-sensitive mucin-like glycoproteins by dog respiratory epithelial cells deprived from secretory granules. Polyactosaminoglycans have also been observed in episialin-related molecules [61,62].

Interestingly, the secreted mucins are not fucosylated, although low amounts of [3 H]fucose may be incorporated in intracellular mucins (J. M. Périni, unpublished work). In contrast, Merten et al. [19] have observed a secretion of fucosylated mucin-like glycoproteins in non-immortalized cultures of human tracheal submucosal glands. Important differences in glycosylation have been already observed between colonic mucins secreted *in vivo* [63,64] and mucins secreted by a clone from the HT-29 colonic cell line, which does not secrete fucosylated glycoproteins [65].

Owing to their special patterns in glycosylation and mucin genes expression, these MM-39 cells may represent an interesting model to study the correlation between a given profile of mucin genes expression and the glycosylation of these apomucins.

In conclusion, MM-39 cells derived from submucosal glands have a mixed phenotype. Like serous cells, they secrete lysozyme and MPI [13–17], but they have also the capacity to secrete mucins in addition to other glycoconjugates, such as hyaluronic acid and small amounts of proteoglycans with chondroitin and/or heparan sulphate GAG. Therefore, this model could be helpful in the study of the biosynthesis of mucins different from MUC1 and, since these cells express CFTR [24], in looking for abnormalities in mucin secretion by altering the expression of CFTR.

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