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Quantification of Clara cell protein in rat and mouse biological fluids using a sensitive immunoassay

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ABSTRACT: Clara cell protein is a 16–17 kDa protein (CC16) secreted by Clara cells in the bronchiolar lining fluid of the lung. In order to investigate the potential of this protein as a pulmonary marker in animals, CC16 was isolated from rat bronchoalveolar lavage fluid (BALF) and a sensitive latex immunoassay applicable to both rat and mouse CC16 was developed.

The pattern of CC16 concentrations in rat biological fluids determined by the immunoassay was consistent with the hypothesis of a passive diffusion of the protein across the bronchoalveolar/blood barriers showing a difference of more than 5,000 fold between the concentration in the epithelial lining fluid (mean, 140 mg·L^{-1}) and that in serum (20 µg·L^{-1}) or urine (3 µg·L^{-1}). In BALF, the CC16 concentration averaged 5,500 µg·L^{-1} and was of the same magnitude as that determined on lung and trachea homogenates. CC16 was also detectable in amniotic fluid with a mean value of 800 µg·L^{-1} before delivery.

Damage of Clara cells produced by methylcyclopentadienyl manganese tricarbonyl resulted in a significant decrease of CC16 in BALF but did not affect the serum levels of the protein. The nephrotoxicant sodium chromate by contrast had no influence on the CC16 content of BALF but markedly increased CC16 levels in both serum and urine as a result of impaired glomerular filtration and tubular reabsorption, respectively.

In conclusion, mouse or rat Clara cell protein of 16–17 kDa can easily be quantified, not only in bronchoalveolar lavage fluid, but also in extrapulmonary fluids such as serum or urine. Thus, in rodents, Clara cell protein of 16–17 kDa follows the same metabolic pathway as in humans, diffusing from the respiratory tract into serum where it is eliminated by the kidneys. This serum Clara cell protein of 16–17 kDa may be useful as a peripheral marker of events taking place in the respiratory tract.
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Clara cell protein is a 16–17 kDa homodimeric protein (CC16) secreted by Clara cells in the lining fluid of bronchioles [1–4]. The protein has a molecular weight of 16,908 daltons in rats [5] and 15,840 daltons in humans [6]. It is frequently referred to as the 10 kDa Clara cell protein or CC10 but the abbreviation CC16, more in agreement with the size of the protein in humans and rodents, is used here. The exact physiological functions of CC16 are still unknown. There is growing evidence that CC16 plays an immunosuppresive and anti-inflammatory role in the respiratory tract. For instance, CC16 has been found to inhibit the activity of phospholipase (PL)A_2, a key enzyme in the production of prostaglandins and leukotrienes, as well as the production and the activity of interferon (IFN)-γ [3, 7, 8]. Studies on CC16-deficient mice suggest that it might also be involved in the tissue accumulation of lipophilic molecules (*e.g.* polychlorobiphenyls) or of endogenous macromolecules such as fibronectin [9, 10].

In humans, CC16 has been studied in biological fluids from healthy subjects and patients with lung disorders. The highest concentrations of CC16 are found in sputum and bronchoalveolar lavage fluid (BALF) due to the intense secretion of the protein into the airways [11]. High concentrations of CC16 are also found in amniotic fluid originating from the foetal lung [12, 13]. As a result of a passive transudation, the protein occurs in small concentrations in serum where it appears to mirror the amount secreted in the respiratory tract and/or the permeability of the bronchoalveolar/blood barrier [11]. For instance, CC16 has been found to be decreased in the serum of current smokers and of workers chronically exposed to various lung toxicants probably as a result of Clara cell damage. In other situations, such as sarcoidosis or following acute exposure to lung irritants, serum CC16 shows, in contrast, an elevation which can be attributed to an increased leakage from the respiratory tract [14, 15].

In animals, with the exception of amniotic fluid [12], no study has quantified CC16 in pulmonary and extrapulmonary biological fluids. Studies which have investigated CC16 synthesis and secretion in rat or mouse lung have

used only immunohistochemistry and molecular biology techniques [1, 4, 16–20]. In order to further explore in animals the potential of CC16 as a lung biomarker, we have developed a sensitive immunoassay applicable to both rat and mouse CC16 and measured CC16 concentrations in various biological fluids of these species.

Materials and methods

Animals

Sprague-Dawley rats aged 10 weeks and NMRI mice aged 8 weeks were used in this study. Pregnant rats were purchased from the ProefDieren Centrum (Catholic University of Louvain, Leuven, Belgium). All animals were allowed food (rodent chow type A03, Usine d'Alimentation Rationelle, Villemoisson-sur-Orge, France) and water *ad libitum*. They were housed on hardwood bedding in plastic cages in an air conditioned animal room (25°C, 50% relative humidity) with a regular 12 h light/dark cycle.

Treatments

Methylcyclopentadienyl manganese tricarbonyl (MMT; Aldrich Sigma, St Louis, Missouri) dissolved in arachid oil (5 mg·mL^{-1}) was given *i.p.* at a dose of 5 mg·kg^{-1}. Sodium chromate (Na_2CrO_4; Aldrich Sigma) was administered *s.c.* at the dose of 10 mg·kg^{-1}. Animals were sacrificed 24 h after MMT or Na_2CrO_4 administration. In each experiment, control rats given an equivalent volume of *i.p.* arachid oil were run in parallel with the treated animals. Each experimental group was composed of six animals.

Sacrifice and tissue sampling

The animals were anaesthetized with an *i.p.* injection of sodium pentobarbital (50 mg·kg^{-1}). The trachea was cannulated when the respiration had ceased and bronchoalveolar lavage was performed. The lungs were lavaged with normal saline using a total volume of 10 mL. The BALF was centrifuged (1,000×g, 10 min, 4°C) and the resultant cell free supernatant was analysed for the different biochemical parameters. The lungs were then quickly removed and tissue samples were immediately dissected and prepared for morphological analysis. Lungs and trachea were homogenized in cold saline with a Potter type homogenizer to obtain a suspension containing 1 g of lung tissue per 10 mL of saline. The homogenates were centrifuged at 25,000 ×g for 15 min and the supernatants were aliquoted and stored at -20°C until analysis. Following aortic cannulation, whole blood was collected and stored at 4°C for 3 h. Clotted blood was then centrifuged at 2,000×g for 10 min. Sera were stored at -20°C until further analysis. In pregnant rats, the pups were delivered by caesarean section. Amniotic fluid was collected by puncturing individual amniotic cavities from day 14 to day 21 of gestation. Fluid samples obtained from the different foetuses (between days 4 and 10) in each horn of the uterus were pooled.

Purification and characterization of rat CC16

CC16 was purified from 2 L of pooled BALF. Purification was carried out using the Pharmacia fast protein liquid chromatography (FPLC; Pharmacia-LKB Biotechnology, Uppsala, Sweden) system consisting of an automated injector ACT 100, a gradient programmer GP-250 Plus, two P-500 pumps and a Dual Path Monitor UV-2 (274 and 254 nm) with 1 and 20 mm flow cells (Pharmacia). All columns (Superdex 75 C26/100, Hiload 26/10 Q Sepharose and mono P HR 5/5) were from Pharmacia-LKB Biotechnology. Purification was achieved by the following procedure, in which CC16 was traced using the immunoassay for human CC16 (cross-reactivity of about 1/1,000). After a 100 fold concentration by lyophilization and dialysis overnight against 0.05 M Tris-hydrochloride (HCl) buffer (pH 7.4), BALF proteins were fractionated on a Superdex 75 column in the same buffer. The material showing an immunoreactivity with the human CC16 assay was eluted as a single peak with an apparent molecular weight of about 20 KDa. After dialysis against 0.01 M Tris-HCl buffer pH8, these fractions were chromatographed on a Hiload 26/10 Q Sepharose column with a 0.1–0.3 M sodium chloride (NaCl) gradient in 0.01 M Tris-HCl buffer (pH 8). Immunoreactive fractions were pooled, lyophilized and refractionated on Superdex 75 as described above. After dialysis against 0.02 MN-methylpiperazine pH 4.7, the CC6 peak was subjected to a final purification by chromatofocusing on a Mono P HR 5/5 column with 50 mL of Polybuffer 74 diluted 10 times and adjusted to pH 3.8. Purified CC16 was analysed by sodium dodecylculphate polyacrylamide gel (SDS-PAGE) electrophoresis (Pharmacia PhastSystem) on 20% polyacrylamide gel and stained with the PhastGel Silver Kit (Pharmacia).

Amino acid microsequence analysis

Amino acid microsequence analysis of the protein was performed by automated Edman degradation of a 1–10 pmol protein on a Beckman LF3400 proteinpeptide microsequencer equipped with an on-line model 126 Gold system microgradient high performance liquid chromatography (HPLC) and a model 168 diode array detector (Beckman Instruments). All samples were sequenced using standard Beckman sequencer procedure 40. The phenylthiohydantoin (PTH) derivatives were quantitatively identified by reverse phase HPLC on an spherogel micro PTH column (3 µm diameter particles, 2×150 mm, Beckman Instruments). All sequencing reagents were from Beckman. Sequences were compared to known protein sequences in the European Molecular Biology Laboratory (EMBL) databank using the T-FASTA search programme [21].

Antiserum production

Antiserum to rat CC16 was prepared by four injections of 100 µg of purified protein into two rabbits. The γ-globulin fraction was precipitated by ammonium sulphate (NH_4SO_4) and separated by chromatography (Mono Q column; Pharmacia) with 0.01 Tris HC1 buffer pH 8 in 0–0.3 M NaCl gradient.

Western blot analysis

Western blot analysis was performed on BALF and supernatant (25,000×g, 10 min) of lung homogenate samples. The BALF samples were concentrated 20 times by lyophilization and analysed in duplicate. They were subjected to electrophoresies on polyacrylamide gel (20%) in the presence of 0.1% SDS and electrophoretically blotted on nitrocellulose membranes (0.2 µm). The membranes were then incubated overnight with a blocking buffer (5% weight/volume (w/v) nonfat dry milk in Tris-buffered saline containing 0.02% tween-tris-buffered saline (TTBS)). They were then incubated at room temperature for 1 h with a rabbit polyclonal antibody against rat CC16 (dilution 1/1,000). The bands were revealed using the Amplified Alkaline Phosphatase Immuno-Blot assay kit of Bio-Rad (Bio-Rad Laboratories, Hercules, CA, USA). Briefly, after three washings with TTBS the membranes were incubated for 1 hr with a 1/3,000 dilution of a biotinylated goat anti-rabbit antibody. They were then washed twice with TTBS and incubated for 1 h with a streptavidin-biotinylated alkaline phosphatase complex. Colour development was performed by using a solution of nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP).

Morphological studies

Immediately after sacrifice, lung samples were fixed by immersion in 10% phosphate-buffered formalin or in Bouin's fluid for at least 48 h. Fixed tissues were rinsed in 70% ethanol, routinely dehydrated in graded ethanol and in butanol, and embedded in Paraplast Plus* paraffin according to standard procedures. Paraffin sections of 4–5 µm thickness were cut serially with a Reichert Autocut 2040 microtome and mounted on silane-coated glass slides. For each specimen, selected sections were stained with Masson's trichrome or with haematoxylin, erythrosin, orange G and anilin blue. To observe consecutive fields, the remaining adjacent slides were saved for immunocytochemical labellings.

CC16-containing cells were detected in lung sections with the rabbit polyclonal antibody raised against purified rat CC16 according to a slightly modified streptavidin-biotin immunoperoxidase method [22]. Briefly, before immunostaining, rehydrated sections were immersed in 0.4% hydrogen peroxide (H_2O_2) for 5 min and pretreated by incubation in a wet autoclave at 120°C for 20 min. Prior to immunostaining, the pretreated sections were washed in phosphate-buffered saline (PBS) buffer (0.04 M sodium hydrogen phosphate (Na_2HPO_4), 0.01 M potassium hydrogen phosphate (KH_2PO_4) and 0.12 M NaCl, pH 7.4) and incubated in 5% normal goat serum (NGS) in PBS for 20 min. Slides were then rinsed and incubated sequentially at room temperature in the following solutions: 1) rabbit polyclonal anti-rat CC16 (diluted 1:400) for 1 h; 2) biotinylated goat anti-rabbit immunoglobulin (Ig)G (diluted 1:50) for 20 min and 3) avidin: biotinylated enzyme-complex (ABC) complexes for 20 min. Bound peroxidase activity was visualized by incubation with 0.02% 3,3'-diaminobenzidine - 0.01% H_2O_2 in PBS. The different solutions were prepared in 5% NGS-PBS buffer (pH 7.4) and after each step of the immunostaining procedure the sections were rinsed in the same buffer. The sections were finally counterstained with hemalun and luxol fast blue, dehydrated and mounted with a permanent mounting medium.

Control of the specificity of immunolabellings included the omission of the primary antibody or the substitution of the primary antibodies with nonimmune sera. The specificity of the CC16 immunostaining was also checked with an anti-rat CC16 antibody previously incubated with pure antigen (70 $\mu g \cdot mL^{-1}$ of pure rat CC16). In each case these assays confirmed the specificity of the observed immunolabellings. The anti-proliferating cell nuclear antigen (PCNA) antibody has been checked by the suppliers and claimed to be specific of its antigen. Monoclonal anti-PCNA antibody, biotinylated goat anti-rabbit IgG antiserum, biotinylated rabbit anti-mouse IgG antiserum and ABC complex were purchased from Dakopatts, Glostrup, Denmark.

Analytical methods

CC16 was determined by an automated latex immunoassay, similar to that described for the human protein, and using the anti-CC16 antibody and a standard based on the purified protein [23]. CC16 was determined in BALF, urine and lung homogenates samples without pretreatment. To eliminate possible interferences (complement, chylomicrons), the serum samples were pretreated by heating at 56°C for 30 min and by the addition of polyethylene glycol 600 (16%, volume/volume (v/v) 1/1) and trichloroacetic acid (10%, v/v 1/40). After overnight treatment at 4°C, the samples were centrifuged and CC16 was determined in the supernatant. The CC16 concentration in epithelial lining fluid was derived from that of BALF on the basis of the serum/BALF urea concentration ratio [24].

Albumin was determined in urine and serum by latex immunoassay using the antibody from USB (Cleveland, OH, USA) and a standard based on pooled sera from normal rats [25]. Total protein concentration in BALF and lung homogenates were determined using the Pyrogallol Red Kit of Technicon (Biodiffusion, Pont-Sainte-Maxence, France). Urea concentration in serum and BALF was measured by the enzymatic assay on Technicon. Creatinine was determined in serum and urine by the Jaffé's method.

Statistical analysis

Differences between groups were assessed by one-way analysis of variance (ANOVA) followed by the Dunnett's multiple comparison test. Associations between variables were evaluated by Pearson's correlation. Unless otherwise stated, values are given as the mean±SD. The level of significance was set at $p<0.05$.

Results

Protein purification and antibody specificity

At the last purification step by chromatofocusing, the material cross-reacting with the anti-human CC16 antibody was resolved in three peaks of about 16 kDa by SDS PAGE (fig. 1). These three peaks, corresponding presumably to the isotypes (A, B and C) previously described by

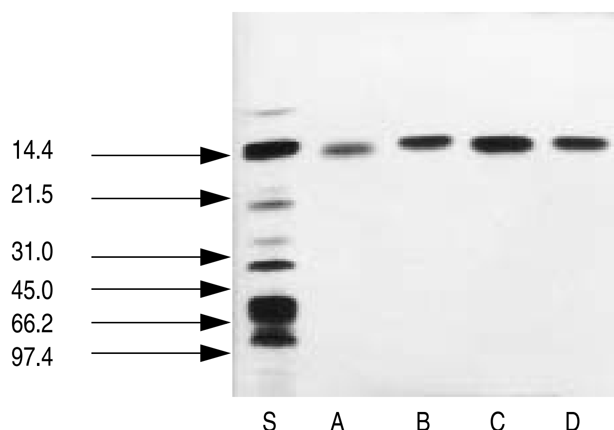

Fig. 1. – A sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) of the three isoforms of rat Clara cell protein of 16–17 kDa (CC16) isolated by chromatofocusing on a Mono P HR 5/5 column. The isoelectric point (pI) of the isoforms were 4.1 (lane A), 4.3 (lanes B and D), and 4.5 (lane C) respectively. Molecular weight standards (lane S) are indicated to the left.

UMLAND *et al.* [5], were pooled and subjected to amino acid microsequence analysis. Only one sequence was obtained: SSDICPGFLQVLEALLLGSESNYEAALKPF, which showed complete identity with the N-terminal sequence of rat CC16 after comparison with the sequence databases for similar or homologous entries in the EMBL databank using the T-FASTA search program [21]. This amino acid microsequencing analysis confirmed the high degree of purity of the purified rat CC16 sample.

Western blotting and immunocytochemistry

Western blot analysis of rabbit anti-CC16 antibody on BALF or lung homogenate from rats showed the presence of a single band corresponding to the purified protein (fig. 2). When tested on BALF from mice, the antiserum also recognized a protein at the same position corresponding to mouse CC16. Urine or serum samples were also tested but the protein was not detectable. We have also checked the specificity of our antiserum by immunostainings on control lung sections (rat or mouse). In each case the immunolabelling was confined to nonciliated cells of bronchioles corresponding to Clara cells and was mainly observed in terminal and respiratory bronchioles (fig. 3). No staining was observed in alveolar tissue nor in ciliated cells of the epithelial lining of the upper airways.

Assay

A typical standard curve of CC16 is shown in fig. 4. The limit of detection defined as the concentration giving a 10% agglutination is around 0.1 μg·L^{-1}. As the lowest dilution allowing an accurate determination of the protein is 20 times in serum and five times in other fluids, this corresponds to practical limits of detection of 2 and 0.5 μg·L^{-1}, respectively. The analytical recovery of the assay, tested by supplementing 10 sera samples with 50 μg·L^{-1} of purified CC16, averaged 90±7%. When lung homogenates or BALF samples were fractionated by gel filtration on a Superdex 75 column, the immunoassay detected only a single peak of CC16 at a molecular weight around 16 kDa.

Occurrence in biological fluids

The concentrations of CC16 in BALF, serum and urine from normal rats are shown in table 1. No significant influence of sex was found. The concentrations of CC16 were particularly high in BALF with average values corresponding to about 7% of that of albumin (albumin in BALF: 64±19 mg·L^{-1} in males and 76±19 mg·L^{-1} in females). The values of CC16 in serum and urine were on average more than 200 and 1,000 lower, respectively. These differences are illustrated in figure 5 which also compares the levels of CC16 in lung and trachea homogenates and that estimated in the epithelial lining fluid (ELF). As expected, the highest concentrations of CC16 were found in ELF. The CC16 concentration in ELF averaged 140 mg·L^{-1}, a value 75 times higher than that in the corresponding BALF. It is interesting to note that CC16 measured in BALF, lung and trachea homogenates fall in about

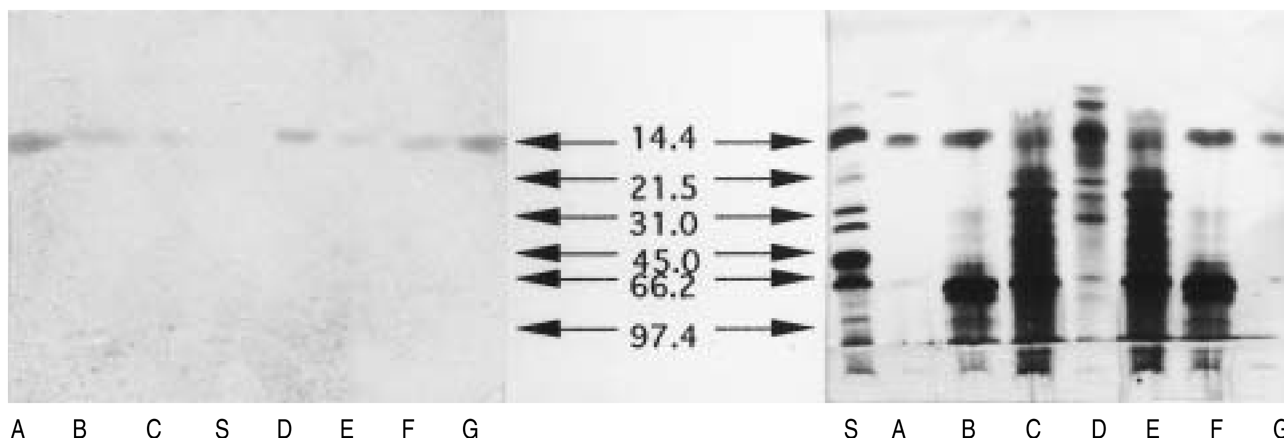

Fig. 2. – Western blot analysis of rabbit anti-Clara cell protein of 16–17 kDa (CC16) antibody tested against: the 10–20 kDa Superdex 75 fraction of rat bronchoalveolar lavage fluid (BALF) (lane A); pooled specimens of mouse (lanes B and F); pooled specimens of rat BALF (lanes C and E); the 10–20 kDa Superdex fraction of a rat lung homogenate (lane D); and purified rat CC16 (lane G). The immunoreactive bands (left panel) were detected with a biotinylated goat anti-rabbit antibody and the formation of a streptavidin-biotinated alkaline phosphatase complex. The right panel shows the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) patterns of protein in the tested samples. Molecular weight standards are indicated to the left (lane S).

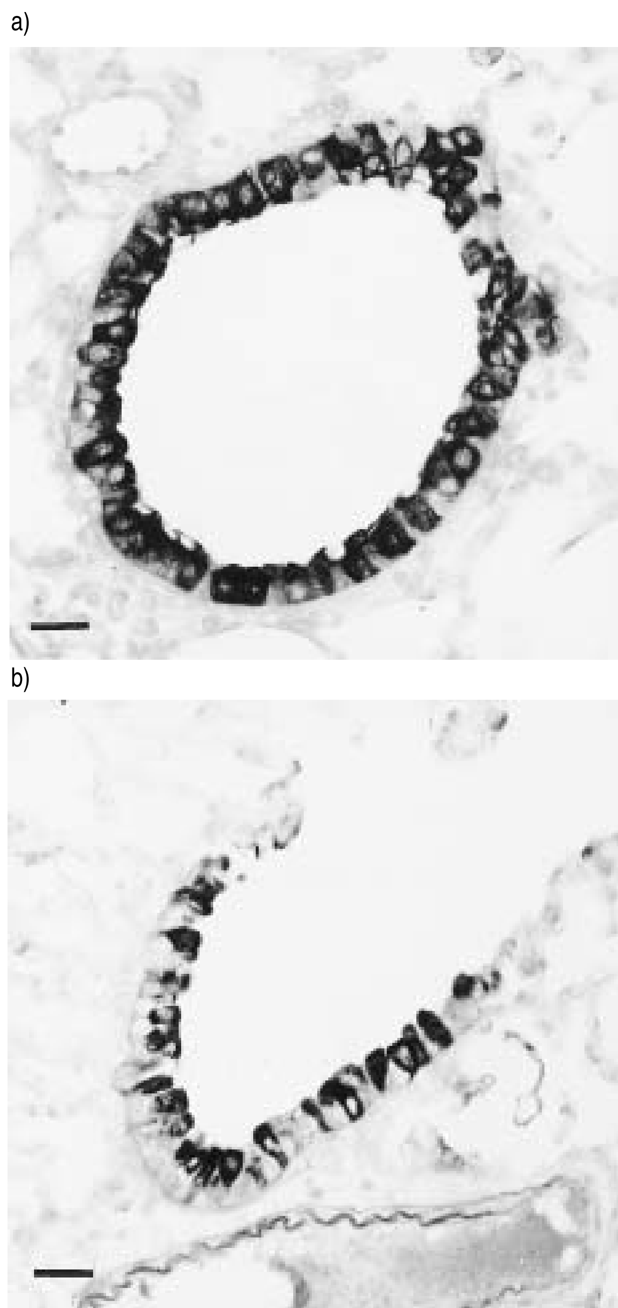

Fig. 3. – Immunocytochemical demonstration of Clara cell protein of 16–17 kDa (CC16) immunoreactivity in lung tissue of a control rat. The immunostaining was restricted to Clara cells as observed in a terminal bronchiole in a) a transverse section or b) in a respiratory bronchiole. ABC method. (Internal scale bar = 25 µm).

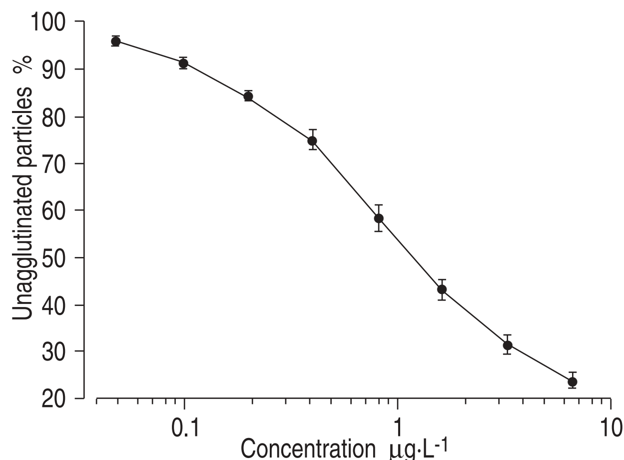

Fig. 4. – Standard curve for the determination of rat or mouse Clara cell protein of 16–17 kDa (CC16) by latex immunoassay.

Table 1. – The concentrations of Clara cell protein of 16–17 kDa (CC16) in bronchoalveolar lavage fluid (BALF), serum and urine of normal rats (n=10)

	BALF $\mu g \cdot L^{-1}$	Serum $\mu g \cdot L^{-1}$	Urine $\mu g \cdot L^{-1}$
Males	5640±990	17.0±8	2.8±1.3
Females	5550±810	23.8±12.3	3.2±1.3

Values are presented as mean±SD.

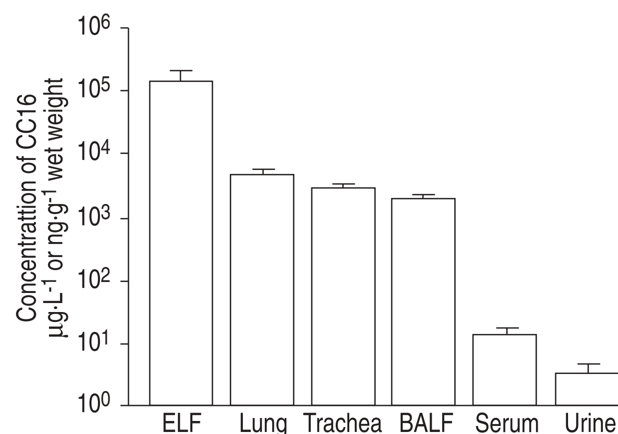

Fig. 5. – Concentrations of Clara cell protein of 16–17 kDa (CC16) in different biological media from normal rats (n=10). Values are presented as means and are given in nanograms per gram of wet weight for lung or trachea homogenates and in micrograms per litre for other media. Bars represent the SD. ELF: epithelial lining fluid; BALF: bronchoalveolar lavage fluid.

the same range of values. In a separate experiment, we examined the relationship between CC16 in BALF, lung homogenate and serum. The concentration in BALF from 20 rats (10 males and 10 females) was significantly correlated with that in lung homogenate (fig. 6). By contrast no correlation was found between CC16 measured in serum and values determined in lung homogenate or BALF. In amniotic fluid, CC16 was detectable from day 17 and increased continuously to reach a mean value of 800 $\mu g \cdot L^{-1}$ at the end of gestation (fig. 7).

The immunoassay was also tested in NMRI mice. An average concentration of 9.2±1.6 $mg \cdot L^{-1}$ was observed in the BALF of a group of six females. The corresponding serum concentration was 62.0±9.8 $\mu g \cdot L^{-1}$. These values were significantly higher than those measured in rats.

Effects of MMT and Na_2CrO_4

Administration of MMT resulted in a significant decrease of CC16 in BALF (table 2) whereas albumin concentration was significantly increased (3,270±98 *versus* 43.4±9.8 $mg \cdot L^{-1}$). MMT did not produce any significant effect on the serum concentrations of CC16 nor on the

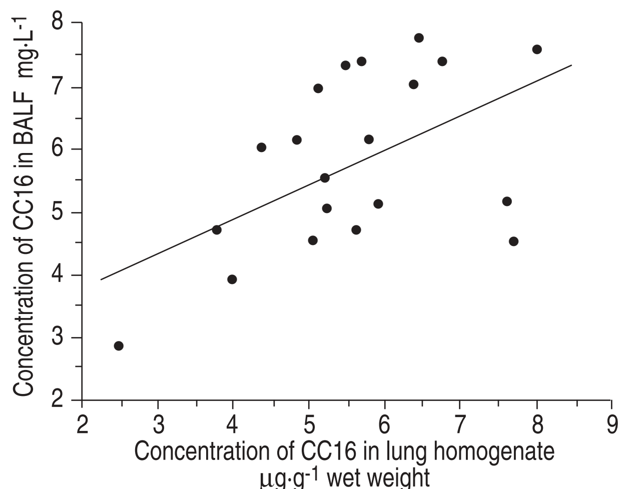

Fig. 6. – Correlation between the concentrations of Clara cell protein of 16–17 kDa (CC16) in bronchoalveolar lavage fluid (BALF) and in lung homogenate of normal rats (n=20).

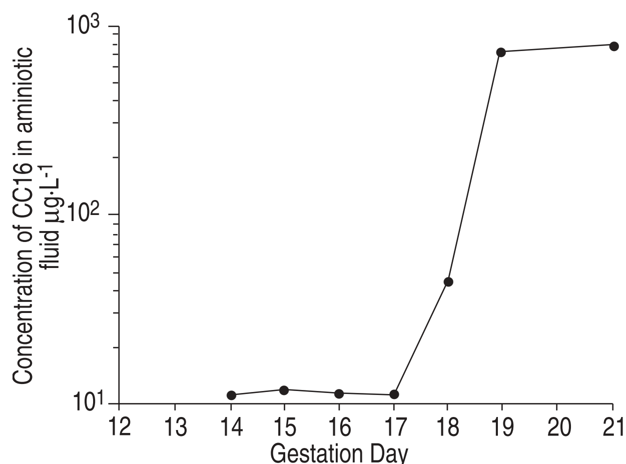

Fig. 7. – Time course of Clara cell protein of 16–17 kDa (CC16) in amniotic fluid during gestation in rats. Each point represents the value in pooled amniotic fluid samples from two mothers.

Table 2. – Effect of sodium chromate (Na_2CrO_4) and methylcyclopentadienyl manganese tricarbonyl (MMT) on the concentrations of Clara cell protein of 16–17 kDa (CC16) in bronchoalveolar lavage fluid (BALF), serum and urine of rats (n=6 in each group)

	BALF µg·L-1	Serum µg·L-1	Urine µg·L-1
Controls	5160±790	14.9±2.7	3.1±1.9
Na_2CrO_4	4960±600	178±147*	7050±6170*
MMT	2284±420*	30.9±35.0	21.5±44.0*

Values are presented as mean±SD. *: values significantly different compared to control values

renal function parameters (serum creatinine and albuminuria were unchanged). The urinary excretion of CC16 was, however, significantly increased.

Na_2CrO_4 administration, by contrast, did not affect the CC16 concentrations in BALF but markedly increased the concentrations of the protein in both serum and urine. These animals presented with severe renal dysfunction as shown by the marked elevation of creatinine in serum (58.4±18.4 *versus* 4.3±1.1 mg·L-1) and albumin in urine (2,640±2,170 *versus* 52.5±37.5 mg·L-1). The concentration of albumin in BALF was significantly decreased compared to that of controls (25.1±8.2 *versus* 43.4±9.8 mg·L-1).

Histopathological analysis after MMT or Na_2CrO_4 treatment

One day after MMT administration, the lung parenchyma was characterized by a slight interstitial thickening and a mild oedema both in the alveolar walls and in the perivascular connective tissue. By comparison with controls, the alveolar lining showed an increased number of enlarged type II pneumocytes while the alveolar spaces were occupied by an increased population of alveolar macrophages. Marginated neutrophils were observed in blood vessels but neutrophil infiltration in lung parenchyma remained discrete and frequently associated with peribronchial tissue. Small aggregates of lymphocytes were also seen in peribronchial or perivascular tissues both in control and treated animals but were generally regarded as normal lymphoid tissue involved in the mucosal immune defence system [26]. In the bronchioles, ciliated cells of the epithelial lining seemed unaffected but focal Clara cell necrosis was evident, in particular in the distal segments of airways. Morphologically, these degenerating cells were characterized by apical swelling, cytoplasmic vacuolization or picnotic nuclei. No abnormality was seen in the lungs of rats treated with Na_2CrO_4.

Discussion

The present study describes the development of an immunoassay for rat CC16 using a polyclonal antiserum against the protein from rat BALF. CC16 purified from rat BALF shown by chromatofocusing three forms of similar size corresponding most likely to the three isoforms described by UMLAND [5]. The amino acid sequence of the pooled isoforms was identical to that determined on CC16 purified from rat [3, 5]. The rabbit antiserum produced against this material stained a single band at a molecular size around 16 kDa on Western blot analysis of BALF or lung homogenates from rats or mice. This is at variance with the results obtained by SINGH *et al.* [2] who also used a rabbit antiserum to rat CC16 and described three reactive bands, at 12, 55 and 200 kDa, respectively. We have also checked that the antiserum used in the CC16 immunoassay gave an immunostaining pattern specific to Clara cells. In each experimental group, the immunolabelling was observed only at the level of nonciliated cells in the bronchiolar epithelium and was particularly prominent in terminal and respiratory bronchioles. This immunostaining pattern was in accordance with the observations reported in the literature for the histological distribution of Clara cells in airways [1, 4, 18].

With the exception of the amniotic fluid, this study is the first to report the normal concentrations of CC16 in various biological fluids of the rat including serum and urine. High levels of CC16 were measured in BALF where, as in humans [9], CC16 emerges as one of the most abundant lung secretory protein with a concentration averaging 7% of that of albumin. High levels were also found

in lung and trachea homogenates, which clearly indicates that the protein is synthesized along the tracheobronchial tree. An estimation of the CC16 concentration in the ELF yielded an average value of 140 mg·L^{-1}, confirming that very large amounts of CC16 are secreted in the airways lining fluid. Across the bronchoalveolar/blood barriers there is thus a CC16 concentration gradient of more than three orders of magnitude which undoubtedly provides a strong driving force to the passive diffusion of the protein from the respiratory tract into serum. The CC16 concentrations in rat serum (on an average between 10 and 20 µg·L^{-1}) are very similar to those found in humans [6]. There is thus little doubt that as in humans most serum CC16 is derived from the respiratory tract since, so far, CC16 expression in rat has been confined to the Clara cells [16]. In urine, CC16 concentrations are even lower, averaging only a few micrograms per litre. Interestingly, no difference was found in either serum or urinary concentrations of CC16 relating to sex. This is in contrast with observations in humans, where higher concentrations of CC16 in the urine of male subjects from puberty onwards have been shown as a result of a postrenal secretion of the protein by the urogenital tract [27, 28].

In amniotic fluid, the time course of CC16 was very similar to that previously reported following either CC16 quantification [13] or CC16 gene expression analysis [14]. The protein concentration increased rapidly in amniotic fluid from day 18 to reach values around 800 µg·L^{-1} at day 21. SINGH *et al.* [13] also detected CC16 from day 18 but reported only a 3.8 fold increase till day 20 which was much lower than that reported here (18 fold increase between day 18 and 21 and 80 fold increase between day 17 and 21). The pattern of increase of CC16 in rat amniotic fluid was very similar to that described in humans [12] and it is likely that it also reflects the foetal lung growth.

Results obtained in rats with lung or kidney damage provide interesting insight into the metabolism of CC16 and its response to lung injury. MMT was selected as a model of pneumotoxicant because it produces by systemic administration extensive lung lesions characterized by a severe alveolar inflammation and bronchiolar injury [29, 30]. As for other systemic toxicants damaging Clara cells [31–33], the lung toxicity of MMT appears to be mediated by cytochrome P450-dependent mono-oxygenases [34]. The lesions of Clara cells induced by MMT and confirmed histologically were associated with a decrease of the CC16 in BALF, most likely as a result of reduced synthesis and/or secretion of the protein. This reduction of CC16 in BALF was, however, not reflected by the levels of the protein in serum which on the contrary tended to increase. We interpret this difference by the fact that lung-damage induced by MMT extends also to the bronchoalveolar/blood barrier and gives rise to an increased transepithelial leakage of proteins such as albumin and thus probably also CC16.

Renal failure was induced by Na_2CrO_4, a well known nephrotoxic agent causing severe tubulopathy [35]. Rats treated with this compound developed a severe renal dysfunction resulting in elevated serum creatinine and albuminuria. These changes were accompanied by a marked elevation of the protein in serum and urine which is in agreement with human studies showing that CC16 is cleared from plasma by glomerular filtration and then reabsorbed and catabolized in the proximal tubules [19, 36]. These findings indicate that rat CC16 follows the same metabolic pattern as in humans, diffusing from its main synthesis site in the respiratory tract into serum from where it is rapidly eliminated by glomerular filtration.

Although we have not systematically studied CC16 in all mouse biological materials, the results indicate that the immunoassay we have developed against the rat protein can also be used to quantify the mouse protein with an apparently equal sensitivity. This excellent crossreactivity is easily explicable in view of the close homogeneity between the two proteins [37].

In conclusion, mouse and rat Clara cell protein of 16–17 kDa can easily be quantified not only in bronchoalveolar lavage fluid but also in extrapulmonary fluids such as serum or urine. This lung secretory protein indeed follows the same metabolic pathway in rodents as that proposed in humans, diffusing from the respiratory tract into serum from where it is rapidly cleared by the kidneys. The results presented here strongly support the view that serum Clara cell protein of 16–17 kDa can be used as a peripheral reporter of events taking place in the respiratory tract.

References

1. Singh G, Katyal SL. An immunologic study of the secretory products of the rat Clara cells. *J Histochem Cytochem* 1984; 32: 49–54.
2. Singh G, Katyal SL, Gottron SA. Antigenic, molecular and functional heterogeneity of Clara cell secretory proteins in the rat. *Biochim Biophys Acta* 1985; 829: 156–163.
3. Singh G, Katyal SL, Brown WE, Kennedy AL, Singh U, Wong-Chong ML. Clara cell 10 KDa protein (CC10): comparison of structure and function to uteroglobin. *Biochim Biophys Acta* 1990; 1039: 348–355.
4. Bedetti CD, Singh J, Singh G, Katyal SL, Wong-Chong ML. Ultrastructural localisation of rat Clara cell 10 kDa secretory protein by the immunologold technique using polyclonal and monoclonal antibodies. *J Histochem Cytochem* 1987; 35: 789–794.
5. Umland TC, Swaminathan S, Furey W, Singh G, Pletcher J, Sax M. Refined structure of rat Clara cell 17 kDa protein at 3 A resolution. *J Mol Biol* 1992; 224: 441–448.
6. Bernard A, Dumont X, Roels H, *et al.* The molecular mass and concentration of protein 1 or Clara cell protein in biological fluids: a reappraisal. *Clin Chim Acta* 1993; 223: 189–191.
7. Dierynck I, Bernard A, Roels H, De Ley M. Potent inhibition of both human interferon gamma production and biologic activity by the clara cell protein CC16. *Am J Respir Cell Mol Biol* 1995; 12: 205–210.
8. Lesur O, Bernard A, Arsalane K, *et al.* Clara cell protein (CC16) induces a Phospholipase A2-mediated inhibition of fibroblasts migration *in vitro*. *Am J Respir Crit Care Med* 1995; 152: 290–297.
9. Stripp BR, Lund J, Mango GW, *et al.* Clara cell secretory protein: a determinant of PCB bioaccumulation in mammals. *Am J Physiol* 1996; 271: L656–L664.
10. Zhang Z, Kundu G, Yuan C-J, *et al.* Severe fibronectin-deposit renal glomerular disease in mice lacking uteroglobulin. *Science* 1997; 276: 1408–1411.
11. Bernard A, Marchandise FX, Depelchin S, Lauwerys R, Sibille Y. Clara cell protein in serum and bronchoalveolar lavage. *Eur Respir J* 1992; 5: 1231–1238.
12. Singh G, Katyal SL, Wong-Chong ML. A quantitative assay for a Clara cell-specific protein and its application

in the study of development of pulmonary airways in the rat. *Pediatric Res* 1986; 20: 802–805.

13. Bernard A, Thielemans N, Lauwerys R, Langhendries J-P, Van Lierde M, Freund MM. Clara Cell Protein in human amniotic fluid: a potential marker of fetal lung growth. *Pediatric Res* 1994; 36: 771–775.
14. Hermans C, Bernard A. Clara cell protein (CC16): characteristics and potential applications as biomarker of lung toxicity. *Biomarkers* 1996; 1: 3–8.
15. Bernard A, Hermans C, Van Houte G. Transient increase of serum Clara cell protein (CC16) after exposure to smoke. *Occup Environ Med* 1997; 54: 63–65.
16. Hackett BP, Shimizu N, Gitlin JD. Clara cell secretory protein gene expression in bronchiolar epithelium. *Am J Physiol* 1992; 262: L399–L404.
17. Ten Have-Opbroek M, De Vries EC. Clara cell differentiation in the mouse: ultrastructural morphology and cytochemistry for surfactant protein A and Clara cell 10 kDa protein. *Microscopy Res Techn* 1993; 26: 400–411.
18. Ray MK, Wang G, Barrish J, Finegold MJ, DeMayo FJ. Immunohistochemical localization of mouse Clara cell 10-KD protein using antibodies raised against the recombinant protein. *J Histochem Cytochem* 1996; 44: 919–927.
19. Stripp BR, Maxson K, Mera R, Singh G. Plasticity of airway cell proliferation and gene expression after acute naphtalene injury. *Am J Physiol* 1995; 269: L791–L799.
20. Van Winkle LS, Buckpitt AR, Nishio SJ, Isaac JM, Plopper CG. Cellular response in naphtalene-induced Clara cell injury and bronchiolar epithelial repair in mice. *Am J Physiol* 1995; 269: L800–L818.
21. Pearson WR, Lipman DJ. Improved tools for biological sequence comparison. *Proc Natl Acad Sci USA* 1988; 85: 2444–2549.
22. Nilaver G, Kozlowski GP. Comparison of the PAP and ABC immunocytochemical techniques. *In:* Bullock, GR. and Petrusz P, eds. Techniques in Immunocytochemistry. London, Academic Press, 1989 pp. 199–215.
23. Bernard A, Lauwerys R, Noël A, Vandeleene B, Lambert A. Determination by latex immunoassay of protein 1 in normal and pathological urine. *Clin Chim Acta* 1991; 201: 213–246.
24. Rennard SI, Basset G, Lecossier D, O'Donnell KM, Pinkston P, Maran PG, Crystal RG. Estimation of volume of epithelial lining fluid recovered by lavage using urea as marker of dilution. *Am J Physiol* 1985.
25. Bernard A, Lauwerys R. Continuous flow system for the automation of latex immunoassay. *Clin Chem* 1983; 29: 1007–1011.
26. Greaves P, Faccini JM. Rat histopathology. A glossary for use in toxicity and carcinogenicity studies. Elsevier, Amsterdam, 1992.
27. Bernard A, Lauwerys R, Noël A, Vandeleene B, Lambert A. Urine protein 1: a sex-dependent marker of tubular or glomerular dysfunction. *Clin Chem* 1989; 35: 2141–2142.
28. Ishii S, Itoh Y, Okutani R, *et al.* Sex-associated differences in protein 1 values in urine: immunochemical detection of protein 1 in genital tissues. *Eur J Clin Chem Clin Biochem* 1994; 32: 31–36.
29. Hakkinen RP, Haschek WM. Pulmonary toxicity of methylcyclopentadienyl manganese tricarbonyl: nonciliated bronchiolar epithelial (Clara) cell necrosis and alveolar damage in the mouse, rat, and hamster. *Toxicol Appl Pharmacol* 1982; 65: 11–22.
30. Haschek WM, Hakkinen PJ, Witschi HP, Hanzlik RP, Traiger GJ. Nonciliated bronchiolar epithelial (Clara) cell necrosis induced by organometallic carbonyl compounds. *Toxicology Letters* 1982; 14: 85–92.
31. Boyd MR. Evidence for the Clara cell as a site of cytochrome P-450 dependent mixed-function oxidase activity in lung. *Nature* 1977; 269: 713–715.
32. Boyd MR. Metabolic activation and lung toxicity: a basis for cell-selective pulmonary damage by foreign chemicals. *Environ Health Perspect* 19B4; 55: 47–51.
33. Richards RJ, Oreffo VIC, Lewis RW. Clara cell cultures from the mouse and their reaction to bronchiolar toxins. *Environ Health Perspect* 1990; 85: 119–127.
34. Verschoyle RD, Wolf, CR, Dinsdale D. CYP2B1 is responsible for the pulmonary bioactivation and toxicity of butylated hydroxytoluene, O,O,S-trimethylphosphothiotate and methylcyclopentadienyl manganese tricarbonyl. *J Pharmacol Exp Ther* 1993; 226: 958–963.
35. Bernard A, Lauwerys R. The effects of sodium chromate on the urinary excretion and tissue distribution of cadmium in cadmium-pretreated rats. *Toxicol Appl Pharmacol* 1981; 57: 30–38.
36. Bernard A, Thielemans N, Lauwerys R, Vandeleene B, Lambert AE. The renal handling of protein 1 (Clara cell protein): effect of age, sex and renal dysfunction. *Contrib Nephrol* 1993; 101: 66–70.
37. Hashimoto S, Nakagawa K, Sueishi K. Monkey Clara Cell 10 kDa (CC10): a characterization of the amino acid sequence with an evolutional comparison with humans, rabbits, rats, and mice. *Am J Respir Cell Mol Biol* 1996; 15: 361–366.