Calvet, Jean-Henri, Emmanuelle Planus, Patricia Rouet, Sophie Pezet, Micheline Levame, Chantal Lafuma, Alain Harf, and Marie-Pia d’Ortho. Matrix metalloproteinase gelatinases in sulfur mustard-induced acute airway injury in guinea pigs. Am. J. Physiol. 276 (Lung Cell. Mol. Physiol. 20): L754–L762, 1999.—Respiratory tract lesions induced by sulfur mustard (SM), a chemical warfare agent, are characterized by epithelial damage associated with inflammatory cell infiltration. To test the potential role of matrix metalloproteinase gelatinases in these lesions, we evaluated gelatinase activity, albumin content, and total cell count in bronchoalveolar lavage fluid of guinea pigs 24 h after an intratracheal injection of 0.2 mg/kg of SM. The bronchial lavage and alveolar lavage fluids were analyzed separately. The increase in inflammatory cell content of the bronchial lavage fluid, mainly macrophages, observed in SM-intoxicated guinea pigs was accompanied by an increase in albumin and in 92-kDa gelatinase activity. There was a significant correlation between albumin content and 92-kDa gelatinase activity ($r = 0.67$) and between 92-kDa gelatinase and the number of macrophages. Immunohistochemistry performed on tracheal sections showed the presence of 92-kDa gelatinase at the site of intraepithelial cleavages. Zymography analysis of culture medium conditioned by guinea pig tracheal epithelial cells demonstrated that these cells produced in vitro 92-kDa gelatinase on stimulation. Culture of human bronchial epithelial cells obtained by the explant technique showed a marked increase in 92-kDa gelatinase after exposure to $5 \times 10^{-5}$ M SM that reinforced the relevance of our animal results to human exposure to SM. These results suggest that in SM respiratory intoxication, 92-kDa gelatinase of both inflammatory and epithelial cell origins could be involved in epithelial cell detachment.

SULFUR MUSTARD [SM; bis(2-chloroethyl)sulfide], an alkylating agent, was used as a vesicant chemical warfare agent during World War I. Its use in the past decade, as well as the growing capacity for the manufacture of chemical weapons, has increased the risk that soldiers or civilians may be exposed to SM. The injuries resulting from SM environmental exposure are mainly characterized by epithelial damage of the tissues through which it is absorbed, i.e., skin, eye, and respiratory tract (17, 24). These epithelial lesions are associated with subepithelial edema and inflammatory cell infiltration (3, 20). Although the respiratory tract lesions represent the major debility after SM exposure, only a few studies have investigated the pathophysiology of SM-induced airway lesions, in particular the mechanisms involved in inflammatory processes (23).

Among the mediators potentially involved, we have focused our attention on matrix metalloproteinases (MMPs), which degrade extracellular matrix components, and particularly on type IV collagenases/gelatinases. The MMP family members are divided into subclases characterized with respect to their structure: interstitial collagenases, stromelysins, metalloelastase, membrane-type MMPs, and type IV collagenases/gelatinases. The latter degrade components of the basement membrane (15) and anchoring collagen VII fibrils, as well as type XVII collagen of hemidesmosomes (22). Previous studies (7, 8, 14) have suggested involvement of MMPs in increased vascular permeability and inflammatory cell migration, both of which characterize acute lung injury. Besides, involvement of MMPs has already been shown in SM-induced dermal lesions (26). To test the potential role of MMPs in SM-induced lesions, we evaluated MMP activity present in the bronchi of guinea pigs 24 h after intratracheal injection of SM. To assess the cell origin of gelatinases found in bronchoalveolar lavage (BAL) fluid, we also performed cultures of both guinea pig and human bronchial epithelial cells (HBECs).

METHODS

Reagents

SM (purity > 98%) was obtained from the chemistry department of our institution. SM was stored in ethanol (100 mg/ml) and diluted 1:150 in 0.9% NaCl just before injection. Fluothane was purchased from Laboratoire Belamont (Paris, France). Phosphate-buffered saline (PBS) was obtained from Gibco BRL (Eargny, France). EDTA, N-ethylmaleimide (NEM), phenylmethylsulfonyl fluoride (PMSF), and phenolphthalein mono-b-β-glucosiduronic acid were supplied by Sigma (Saint Quentin Fallavier, France). Anti-human 92-kDa gelatinase antibodies were obtained from Vaibitech (Paris, France). The secondary antibodies used for Western blotting and immunocytochemistry were biotinylated goat anti-rabbit immunoglobulins from Dako (Trappes, France).

SM Administration

Experiments were performed on male Hartley-strain guinea pigs (Charles River, Saint-Aubin les Elbeuf, France) weighing 250–300 g. The animals were housed in air-filtered temperature-controlled units (21°C) with food and water freely available. SM was administered intratracheally as follows. Guinea pigs were briefly anesthetized with fluothane via a face mask. A cervicotomy was performed, and a drop of 1% lidocaine was instilled subcutaneously. The trachea was gently exposed, and 0.1 ml of either SM (0.2 mg/kg) or solvent (0.5% ethanol in physiological saline) was injected into the trachea through a 24-gauge needle. The cervicotomy was closed by two agraftes.
Within 10 min of anesthesia, all guinea pigs had recovered. For security reasons, injections were performed in a controlled area under an adequate hood and with a face mask and butyl gloves in accordance with the security regulations of our institution.

Histology

Histological studies were performed in two groups of four guinea pigs each 24 h after an intratracheal injection of SM (0.2 mg/kg) or ethanol (0.5%; SM solvent). Guinea pigs were anesthetized intraperitoneally with 50 mg/kg of pentobarbital sodium, and a catheter connected to a fixative container was inserted into the trachea via a ventral incision of the neck. Simultaneously, the animals were exsanguinated by severing the abdominal aorta, the thorax was opened, and the lungs were immediately inflated in situ through the tracheal cannula with 4% formaldehyde in cacodylate buffer, pH 7.2, at a pressure of 25 cmH2O. The lungs were then removed and immersed in fixative for 8 days. A midsagittal slice was taken from each lung together with three samples from the lower third of the trachea and further processed for embedding in Paraplast Plus (Gassalem, Paris, France). Five-micrometer-thick sections were stained routinely with hematoxylin-phloxin-saffron. All observations were done by an observer who was not aware of the treatment given to the guinea pigs.

Obtaining BAL Fluid and Differential Cell Count

Twenty-four hours after an intratracheal injection of SM (0.2 mg/kg) or ethanol (0.5%; SM solvent), BAL was performed in two groups of 13 and 8 guinea pigs, respectively. Guinea pigs were anesthetized with pentobarbital sodium (50 mg/kg ip) and bled via section of the aorta. The heart and lung block was excised, and BAL was performed through the trachea with nine sterile syringes of 2.5-ml volumes of sterile pyrogen-free physiological saline. Fractional processing of the lavage fluid was performed to obtain samples enriched for bronchial and alveolar material (13, 19). The first 2.5-ml aliquot was defined as the bronchial portion of the lavage (BL) and was collected separately. The eight following aliquots were pooled and defined as the alveolar portion of the lavage (AL).

Small aliquots of the BL and AL samples were used for performing total cell counts and counting cytocentrifuge preparations with a Cytospin II (Shandon Southern Instrument, Cergy-Pontoise, France). Total cell count was performed manually with a hemocytometer. Cell smears were stained with standard May-Grünwald-Giemsa. Differential cell counts were performed by counting 200 cells. The total number of each cell type recovered in the BL and AL samples was estimated by multiplying the total number of cells per animal by the percentage of that cell type in the differential count.

Cell Isolation and Culture

The BL and AL samples were separated into their acellular (BL and AL fluids) and cellular components by centrifugation at 300 g for 7 min. Part of the BL and AL fluids were divided into aliquots and frozen at −80°C until used.

The cell pellet from the AL fluid was resuspended in 10 ml of PBS and homogenized by gentle suction into a Pasteur pipette. Cells suspended in PBS were separated with a discontinuous Hanks-Ficol-Histopaque gradient. After centrifugation at 550 g for 30 min, polymorphonuclear neutrophils (PMNs) were discarded, and the separated alveolar macrophages (AMs) were washed in PBS. The purity of this cell population was >90%. AMs were suspended in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 0.5% bovine serum albumin, 100 IU/ml of penicillin, 100 μg/ml of streptomycin, 0.25 μg/ml of amphotericin B, and 2 mM L-glutamine. The cells were cultured for 24 h at a density of 10⁶ cells/ml medium in a 5% CO2 atmosphere at 37°C. Cell-conditioned medium was collected, nonadherent cells were counted, and cell viability was determined with trypan blue exclusion (viability was >90%). Samples were frozen at −80°C until assayed.

Assay of Protein and Albumin

The proteins in the BL and AL fluids were measured according to the method of Bradford, and their molecular masses were determined by comparison with a molecular-mass marker (Amersham, Les Ulis, France) in a sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) followed by Coomassie blue staining. Activities in the gel slabs were quantified with semiautomated image analysis. The corresponding density in grams per liter was calculated by measuring the density of known quantities of albumin on the range of obtained quantities.

Determination of β-Glucuronidase Activity

β-Glucuronidase activity was used as a marker of neutrophil degranulation. It was measured with phenolphthalein mono-β-D-glucosidic acid as the substrate as described by Fishman et al. (11). The incubation mixture was composed of 0.2 M sodium acetate buffer, pH 4.5, and 6 mM substrate, with a total volume of sample of 0.25 ml. The reaction was carried out at 37°C for 4 h and was stopped by the addition of 0.75 ml of H2O and 0.5 ml of glycine-NaOH buffer, pH 11.7, containing 0.2% SDS. Optical density was then determined by the absorbance at 540 nm (Uvikon 930 spectrophotometer, Kontron Instruments, Saint-Quentin-en-Yvelines, France).

Gelatinase Zymography

BL and AL fluids and conditioned medium from AMs were assayed for gelatinase activity by zymography. Aliquots of BL and AL fluids and AM-conditioned medium underwent electrophoresis in polyacrylamide gels containing 1 mg/ml of gelatin in the presence of SDS under nonreducing conditions. After electrophoresis, the gels were washed twice in 2.5% Triton X-100 for 1 h, rinsed briefly, and incubated at 37°C for 24 h in a buffer containing 100 mM Tris-HCl and 10 mM CaCl2, pH 7.40. After incubation, the gels were stained with Coomassie brilliant blue R250 and destained in a solution of 7.5% acetic acid and 5% methanol. Zones of enzymatic activities were indicated by negative staining: proteolytic areas appeared as clear bands against a blue background.

To determine the inhibition profile of the gelatinolytic activities found by zymography, we also used incubation in Tris buffer containing one of the following inhibitors: the metal chelator EDTA (10 mM), the cysteine protease inhibitor NEM (2 mM), or the serine protease inhibitor PMSF (2 mM).

Activities in the gel slabs were quantified with semiautomated image analysis (National Institutes of Health Image 1.52), which quantifies both the surface and the intensity of the lysis band after scanning the gels. Results are expressed as arbitrary units (AU): AU per hour per microliter for BL and AL fluids and AU per hour per 10⁶ cells for conditioned medium of AMs. To check that this method for measuring enzymatic activity on zymograms was linear over the range of activities in unknown samples, we evaluated the activities of increasing volumes of the same BAL fluid and found that the...
Isolation and culture of HBECs. HBECs were cultured as described above. Immune complexes were detected by immunoperoxidase with a Vectastain Elite Kit (Vector Laboratories). Sections were bleached with 1% hydrogen peroxide in PBS containing 0.025% Triton X-100 for 5 min. The cell pellet was resuspended in DMEM/F12 containing 10% FCS, 25 µg/ml of amphotericin B and conditioned for the next 24 h. Medium was collected and analyzed with zymography. In vitro stimulation by phorbol 12-myristate 13-acetate (PMA) was done for 24 h in serum-free medium at 10−7 M.

Cell cultures at confluence were stained with anti-cytokeratin antibodies to identify the cell type as previously described (27). Anti-macrophage (CD68 or KP1) and anti-fibroblast antibodies were also used as specific markers for macrophages and fibroblasts, respectively, to investigate culture purity. Immunostaining was revealed with the alkaline phosphatase and monoclonal anti-alkaline phosphatase method as described by Cordell et al. (5).

Isolation and culture of HBECS were cultured as previously described (27). Briefly, human bronchial biopsies were obtained by fibroscope in three patients investigated for bronchopulmonary carcinoma. Biopsies were taken at a distance from the tumor as confirmed by pathological examination. All procedures were reviewed and approved by Henri Mondor Hospital (Creteil, France) Institutional Review Board, and written informed consent was obtained from the patients. One or two explants (~0.5 × 0.5 mm in size) were placed on sterile plastic dishes coated with collagen G (Polylabo, Strasbourg, France). The explants were incubated in a humidified incubator at 37°C under 5% CO2-95% air with DMEM/F12 (1:1) for 24 h and then with DMEM/F12 supplemented with 2% Ultroser G, 100 IU/ml of penicillin G, 100 µg/ml of streptomycin, 0.25 µg/ml of amphotericin B, and 2 mM L-glutamine. The culture medium was changed every 3–4 days. Explants were cultured for 2–3 wk until confluence was reached. The explants were transferred to new, sterile, collagen G-coated plastic dishes to initiate new primary HBECS cultures. Once the explant had taken off, confluent HBECS were incubated with Ultroser G-free culture medium in the presence of 0.2% lactalbumin for 24 h. These cultures were or were not subsequently exposed to 10−7 M PMA or 5 × 10−5 M SM. Conditioned culture medium was collected for zymography analysis.

Statistical Analysis

Data are presented as means ± SE. Results in the control and SM-intoxicated groups were compared with the nonparametric Mann-Whitney U-test. Linear regression was used in the SM-intoxicated group to evaluate relationships between the cell count or albumin content and gelatinase activity in BL and AL fluids. Data were analyzed with a Macintosh computer and standard statistical software Statview II. P values < 0.05 were considered significant.

RESULTS

Bronchial Portion of the BAL Fluid

Cytology and protein content. The volume recovered from the first 2.5 ml injected was 1.3 ± 0.1 ml in SM-injected guinea pigs and 1.2 ± 0.1 ml in control guinea pigs (not significant). The total number of cells recovered was clearly higher in SM-intoxicated guinea pigs than in control guinea pigs (0.96 ± 0.20 × 106 and 0.29 ± 0.06 × 106 cells, respectively; P < 0.05). Macrophages accounted for most of the increase in the different cell types when expressed in absolute number (0.69 ± 0.14 × 106 and 0.22 ± 0.06 × 106, respectively; P < 0.05; Fig. 1).

In the BL samples, we observed clumps of epithelial cells in all guinea pigs. In control guinea pigs, only a few epithelial cells were observed, whereas in BL

AU obtained with the image-analysis system increased linearly with the volume of the samples (r = 1.00) (8, 9, 27).

Western Blot

Aliquots of BAL fluid containing high levels of gelatinase activity were separated by SDS-PAGE and transferred to an Immobilon-P filter. Nonspecific sites were blocked by incubating the membranes for 90 min in 10 mM Tris-HCl buffer (Tris-buffered saline (TBS), pH 7.40, containing 150 mM NaCl, 5% nonfat dried milk, and 0.05% Tween 20. The transfers were then incubated overnight with polyclonal rabbit anti-human gelatinase B diluted 1:500 in TBS. The blots were washed three times in TBS-0.05% Tween 20 and incubated for 90 min with biotinylated goat anti-rabbit immunoglobulins diluted 1:1,000 as the secondary antibody. The blots were visualized with alkaline phosphate and Fast Red TR/Naphthol AS-MX. Purified human gelatinase B was used as a positive control, and the absence of reactivity without primary antibody was also checked.

Immunohistochemistry

Embedded paraffin tracheal sections 5 µm thick were deparaffinized and rehydrated. Sections were bleached with 1% hydrogen peroxide in PBS containing 0.025% Triton X-100 (PBS-T) for 15 min to inactivate endogenous peroxidase, then rinsed three times in PBS for 5 min, and incubated with “blocking serum” (10% newborn calf serum in PBS-T) for 1 h to saturate cellular Fc receptors. Sections were incubated with the primary antibody (rabbit anti-human 92-kDa gelatinase) at a 1:50 dilution in a buffer (1% bovine serum albumin and 0.25% Triton X-100 in PBS) overnight at room temperature. They were rinsed three times for 5 min in PBS-T. After the third rinse, the sections were incubated with the secondary antibody, biotinylated goat anti-rabbit immunoglobulin, at a 1:200 dilution for 1 h. Then three washings were done as described above. Immune complexes were detected by immunoperoxidase with a Vectastain Elite Kit (Vector Laboratories). Rinsing with water was followed by mounting the tissue sections with Eukitt and coverslips. Negative controls were done in parallel with nonimmune rabbit immunoglobulins and nonimmune goat serum.

Tracheobronchial Epithelial Cell Culture

Guinea pig tracheal epithelial cell culture. Guinea pig tracheal epithelial cells were isolated and cultured following the method of Nahori et al. (16) with slight modifications. The trachea was aseptically removed from 250- to 300-g male guinea pigs and immersed immediately in DMEM-Ham's F-12 medium (DMEM/F12) supplemented with 2 mM glutamine, 100 IU/ml of penicillin, 100 µg/ml of streptomycin, and 25 µg/ml of amphotericin B. Both ends of the trachea were tied, and it was filled with 0.1% Pronase in supplemented DMEM/F12, incubated at 4°C overnight, and then gently rinsed with 10 ml of supplemented DMEM/F12 containing 10% FCS, 25 µg/ml of amphotericin B and conditioned for the next 24 h. Medium was changed to serum-free DMEM/F12 containing 2 mM glutamine and 0.29 ± 0.06 × 106 cells/ml seeded on Primaria culture plates (Falcon, Le Pont de Claix, France). Cultures were maintained at 37°C in a humidified incubator at 5% CO2-95% air with DMEM/F12 supplemented with 2% Ultroser G, 100 IU/ml of penicillin G, 100 µg/ml of streptomycin, 0.25 µg/ml of amphotericin B, and 2 mM L-glutamine. The culture medium was changed every 3–4 days. Explants were cultured for 2–3 wk until confluence was reached. The explants were transferred to new, sterile, collagen G-coated plastic dishes to initiate new primary HBECS cultures. Once the explant had taken off, confluent HBECS were incubated with Ultroser G-free culture medium in the presence of 0.2% lactalbumin for 24 h. These cultures were or were not subsequently exposed to 10−7 M PMA or 5 × 10−5 M SM. Conditioned culture medium was collected for zymography analysis.

Statistical Analysis

Data are presented as means ± SE. Results in the control and SM-intoxicated groups were compared with the nonparametric Mann-Whitney U-test. Linear regression was used in the SM-intoxicated group to evaluate relationships between the cell count or albumin content and gelatinase activity in BL and AL fluids. Data were analyzed with a Macintosh computer and standard statistical software Statview II. P values < 0.05 were considered significant.

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In the BL samples, we observed clumps of epithelial cells in all guinea pigs. In control guinea pigs, only a few epithelial cells were observed, whereas in BL
samples of SM-intoxicated guinea pigs, there was a high number of epithelial clumps. SM significantly raised the protein content in the BL samples (1.61 ± 0.71 g/l in SM-intoxicated guinea pigs and 0.20 ± 0.02 g/l in control guinea pigs; \( P < 0.05 \)). SDS-PAGE demonstrated that albumin accounted for a small part of this increase (0.23 ± 0.05 g/l in SM-intoxicated and control guinea pigs, respectively). *\( P < 0.05 \) compared with respective control value.

Gelatinase activity. Gelatin zymograms of control guinea pig BL fluids (Fig. 2) demonstrated that small amounts of gelatinase were present, mainly in the active 68-kDa form associated with low levels of 92-kDa gelatinase activity. After SM intoxication, total gelatinolytic activities rose. This was due not only to the presence of the 92-kDa form of gelatinase but also to the 68-kDa form and to that of a species with a molecular mass > 92 kDa (Fig. 2). All gelatinase activities were inhibited by 10 mM EDTA but not by NEM or PMSF. Shifts in relative molecular mass after treatment with 4-aminophenylmercuric acetate demonstrated that the 92-kDa band corresponds to the pro form of this gelatinase and that 72 and 68 kDa correspond to the pro and active forms of 72-kDa gelatinase, respectively. Quantification of gelatinase activities with automated image analysis found 6.0 ± 1.3 AU·h⁻¹·µl fluid⁻¹ in SM-intoxicated guinea pigs vs. 1.6 ± 0.2 AU·h⁻¹·µl fluid⁻¹ in control guinea pigs for the 92-kDa species and 6.4 ± 1.0 AU·h⁻¹·µl fluid⁻¹ in SM-intoxicated guinea pigs vs. 3.1 ± 0.4 AU·h⁻¹·µl fluid⁻¹ in control guinea pigs for the 68-kDa gelatinase (\( P < 0.05 \) for both; Fig. 3).

Analysis of the correlation between 92-kDa gelatinase activity and the number of macrophages demonstrated that the greatest increases in the number of macrophages were associated with higher levels of total 92-kDa gelatinase activity (\( r = 0.85; P < 0.0005 \); Fig. 4). In contrast, no correlation was found between the number of PMNs and 92-kDa gelatinase activity. A
significant correlation between albumin content and 92-kDa gelatinase activity was found ($r = 0.67; P < 0.05$; Fig. 5).

β-Glucuronidase activity, used as a marker of neutrophil degranulation, was not increased in the BL fluid from SM-intoxicated guinea pigs compared with that in control guinea pigs (2.8 ± 0.6 and 2.6 ± 0.4 µg phenolphthalein·h$^{-1}$·ml fluid$^{-1}$, respectively).

**Alveolar Portion of the BAL Fluid**

Cytology and protein content. The volume recovered from the eight 2.5-ml aliquots was 19.4 ± 0.2 ml in SM-intoxicated guinea pigs and 19.0 ± 0.2 ml in control animals (not significant). The total number of cells in the AL fluid was not significantly increased in SM-intoxicated guinea pigs (13.6 ± 1.7 $\times$ 10$^6$ vs. 9.6 ± 1.3 $\times$ 10$^6$ cells in the control animals). In contrast to the bronchial portion, no clumps of epithelial cells were observed in this part of the lavage.

The protein content in the AL fluid was significantly increased in SM-intoxicated guinea pigs (1.3 ± 0.5 vs. 0.2 ± 0.0 g/l in the control animals; $P < 0.05$). Albumin accounted for a small part of this increase (0.23 ± 0.04 vs. 0.12 ± 0.01 g/l; $P < 0.05$).

Gelatinase activity. Gelatin zymograms of the AL fluid from control guinea pigs demonstrated that small amounts of gelatinase were present, mainly as the 68-kDa form. We observed an increase in 92-kDa gelatinase in only 4 of 13 SM-intoxicated guinea pigs. For the other animals, no change in gelatinase activity was seen. Accordingly, there was no significant increase after SM intoxication for the entire group of guinea pigs.

AMs secreted a 92-kDa gelatinase in the basal state. This production was significantly increased in AMs from SM-exposed guinea pigs (25.3 ± 3.8 AU·h$^{-1}$·10$^6$ cells$^{-1}$ in the SM-exposed group vs. 9.7 ± 2.2 AU·h$^{-1}$·10$^6$ cells$^{-1}$ in the control group; $P < 0.05$; data not shown).

**Western Blot**

Western blot of AL and BL fluids identified 92-kDa gelatinase as immunologically identical with human gelatinase B (Fig. 6) and demonstrated the specificity of the antibody because only one band was shown.

**Histology and Immunohistochemistry**

The main lesions were observed in the trachea and large bronchi. At this level, vacuolar degeneration and detachment of ciliated cells were observed, leading, in some places, to bullous spaces between degenerating cells and basal cells (Fig. 7). Subepithelial edema with inflammatory cell infiltration was observed in the most severe epithelial lesions. In some places, there was complete detachment of ciliated cells. Immunostaining with specific antibodies demonstrated the presence of gelatinase B at the site of intraepithelial cleavages (Fig. 8B). Sections of trachea obtained from control
guinea pigs were negative for gelatinase B (Fig. 8A). Sections of trachea obtained from SM-intoxicated guinea pigs stained with nonimmune rabbit immunoglobulins used as the primary antibody were also negative (Fig. 8C).

In the lower respiratory tract, unevenly distributed foci of alveolar hemorrhage (<20% of the total surface of the slide) were observed, with inflammatory cell infiltrates in the interstitium. The epithelium and endothelium appeared intact at this magnification (Fig. 7B).

Tracheobronchial Epithelial Cell Cultures

Guinea pig tracheal epithelial cell culture. Tracheal epithelial cell culture was pure at 99% as demonstrated by anti-cytokeratin staining (data not shown). Analysis of conditioned medium by zymography showed that tracheal epithelial cells produced mainly a gelatinase activity found as a doublet around 70 kDa, with a minor activity at 92 kDa (Fig. 9A). Stimulation by PMA induced a marked increase in 92-kDa gelatinase (Fig. 9A).

HBEC culture. In the basal state, major 92-kDa gelatinase and minor 72-kDa gelatinase activities were found in conditioned medium of HBECs, in keeping with previous results (27, 28). Stimulation by PMA induced a marked increase in 92-kDa gelatinase as did exposure to $5 \times 10^{-5}$ M SM (Fig. 9B).

DISCUSSION

In a recent study, Calvet et al. (3) observed severe lesions in the trachea and main bronchi early after SM intoxication in guinea pigs. Lesions were characterized by columnar cell shedding of the epithelium, with edema, vasodilatation, and inflammatory cell infiltration of the submucosa. To get further insight into this inflammatory process, we decided to analyze BAL samples. To avoid the severe bronchoconstriction observed after a 0.3 mg/kg intratracheal injection that would have precluded BAL processing, we used a lower dose of SM, i.e., 0.2 mg/kg, as suggested by a previous study (18) demonstrating that the SM-induced inflammatory reaction was dose dependent. As expected, we found a pattern of histological lesions similar to the previous study by Calvet et al. (3), which were, however, less extensive.

Because the airways sustained the main lesions, we used fractionated analysis of recovered BAL fluid, divided into proximal (BL) and peripheral (AL) portions. The separate processing of the first aliquots from the subsequent aliquots of the BAL fluids was proposed by Helleday et al. (13) and Rennard et al. (19) to obtain
samples enriched for bronchial and alveolar material. This method, especially when small volumes were used to obtain relatively pure bronchial material, was shown to improve the analysis of airway inflammation. The presence of clumps of epithelial cells in the first aliquot only, in accordance with the observation by Rennard et al., further supports the assumption that this first aliquot is composed mainly of bronchial material.

SM was responsible for an increase in total protein content in the bronchial portion of the BAL fluid. Because this increase can reflect leakage of plasma as well as epithelial cell destruction, the albumin content was determined. We found a twofold increase in albumin content, indicating plasma leakage resulting from some alteration of the endothelial and epithelial barriers. This result is in accordance with the increase in Evans blue dye extravasation in the trachea that Calvet et al. (3) previously documented 5 h after an intratracheal injection of 0.3 mg/kg of SM. Evans blue dye extravasation was also found by Harada et al. (12) in the dermis of rabbits bearing SM skin lesions.

The pathological mechanisms underlying SM-induced epithelial lesions remain undefined, and it has been suggested that enhanced proteolytic activity could be involved (18). In this regard, Cowan et al. (6), using synthetic substrates, have shown that cytotoxic doses of SM caused an increase in serine-proteinase activities of human peripheral blood lymphocytes after in vitro SM exposure. They also found a similar increase in the skin of hairless guinea pigs after SM-induced lesions. Furthermore, Woessner et al. (26) have shown that collagenase and proteoglycanase release was increased in the supernatant fluid of culture medium from rabbit skin explants obtained during the acute and healing
phases of SM-induced lesions. No data have been published for the respiratory epithelium.

Our study demonstrates an increase in total gelatinase activity in the fluid originating from the airways where the main lesions were observed. The molecular mass of gelatinase (68/72 and 92 kDa) and inhibition of their activities by EDTA suggested that they were MMP gelatinases A and B, respectively. Western blot confirmed that the 92-kDa species was identical to human gelatinase B. Both the 92-kDa pro form of gelatinase B and the 68-kDa active form of 72-kDa gelatinase were clearly enhanced after SM exposure.

The 72-kDa gelatinase present in BL and AL fluids could originate from epithelial cells as suggested by the results of primary culture. Indeed, zymography of medium conditioned by guinea pig tracheal epithelial cells showed a major gelatinolytic activity as a doublet at 70 kDa in the basal state. This result contrasts with previous data (27) obtained with human epithelial cells cultured from bronchial explants that showed that these cells produce both gelatinases A and B, the latter representing the major activity in the basal state and after in vitro stimulation by lipopolysaccharide. The discrepancy is probably explained by the difference in species. Production of 72-kDa gelatinase by tracheal epithelial cells in vitro suggests an epithelial origin for the 72-kDa gelatinase found in control BL and AL fluids. In SM-intoxicated guinea pigs, plasma leakage could also contribute, at least in part, to the presence of a 72-kDa gelatinase in both BL and AL fluids.

We focused the study on gelatinase B because SM intoxication was followed by a greater increase in this MMP than in the 72-kDa gelatinase. The 92-kDa gelatinase is secreted by inflammatory cells, i.e., macrophages and PMNs, as previously reported (21). Several lines of evidence suggest that the 92-kDa gelatinase present in BL fluid comes mainly from macrophages and not from PMNs. Indeed, even though a 10-fold increase in PMN number was observed, this recruitment (<10% of total cells) remained moderate compared with that of macrophages (75% of total cells). In addition, the absence of an increase in β-glucuronidase activity indicated that there was no PMN degranulation, and no correlation was found between PMN number and 92-kDa gelatinase activity. In contrast, there was a significant correlation between the presence of the 92-kDa gelatinase present in BL fluid and the number of macrophages in BL fluid, and AMs from SM-intoxicated guinea pigs secreted more 92-kDa gelatinase than those from control animals, suggesting that locally recruited macrophages were in an activated state. We analyzed AMs, although lesions were predominantly observed in the airways. Indeed, SM-induced lesions of parenchymal lung, as shown by the increase in albumin concentration in the AL fluid, was associated with the presence of focal alveolar hemorrhage and inflammatory cell infiltration found on histology.

Another possible source for gelatinase B could be tracheal and bronchial epithelial cells, as well as inflammatory cells. Indeed, in our model, immunohistochemistry showed the presence of gelatinase B within the bronchial epithelium at the site of intraepithelial cleavages. This result is in accordance with previous studies that have demonstrated production of MMPs, particularly gelatinases, by bronchial epithelial cells in different in vitro models. Buisson et al. (2) have shown gelatinase B expression by epithelial cells originating from human nasal polyps after an in vitro injury. Moreover, Yao et al. (27) have found expression of MMP gelatinases A and B by cultured bronchial epithelial cells from explants obtained by bronchial fibroscopy. Exposure to lipopolysaccharide induced an increase in both gelatinases and in their mRNAs, suggesting that bronchial epithelial cells may be actively involved in the physiological and pathophysiological remodeling of airway extracellular matrix (27). In the present study, we performed cultures of HBECS and found that exposure to PMA induced an increase in the 92-kDa gelatinase, in keeping with the previous results by Yao et al. (27). We also demonstrated that exposure to SM induced a marked increase in 92-kDa gelatinase. Altogether, our data obtained with both guinea pig and human bronchial epithelial cells strongly support the hypothesis that the 92-kDa gelatinase found in BAL fluid after exposure to SM originated, at least in part, from bronchial epithelial cells. Our observation that in vitro exposure to SM induced an increase in 92-kDa gelatinase secreted by HBECS underlines the clinical relevance of our data obtained in guinea pigs.

Our results showed a highly significant correlation of albumin content with gelatinase B activity. This correlation suggests a possible role for gelatinases in inflammatory involvement of MMPs in plasma extravasation has already been suggested in acute lung edema (4) and multiple organ failure (10, 25) and is supported by experimental models of acute lung injury (7, 8). Proteinases could also be involved in epithelial lesions, as suggested by the presence of gelatinase B at the site of intraepithelial cleavages. This hypothesis is supported by the demonstration that the 92-kDa gelatinase contributes to blister formation in bullous pemphigoid via the degradation of type XVII collagen of hemidesmosomes (22). Moreover, our result is in accordance with a previous study (1) that has shown, in a model of SM-induced skin lesions, that proteinases selectively attack the attachment of the basal cells to the basal lamina, producing lesions reminiscent of the classic subepidermal blistering observed after SM exposure in humans. On the other hand, gelatinases could be involved in the repair process of the respiratory epithelium, as suggested by results obtained by Buisson et al. (2) that showed that gelatinase B was upregulated during the wound repair process, with a maximum peak observed at wound closure.

In summary, analysis of BL fluid by zymography and Western blotting demonstrated an increase in both gelatinases A and B after SM intoxication. Zymography of AM-conditioned medium and correlation between gelatinase B activity and macrophage numbers in BL fluid together with the absence of β-glucuronidase activity in BL fluid suggest that gelatinase B could originate from macrophages. The presence of gelatin-
ase B at the site of intraepithelial cleavages, as evidenced by immunohistochemistry, suggests that gelatinase B could be involved in epithelial cell detachment. Zymography analysis of culture medium conditioned by guinea pig tracheal epithelial cells demonstrated that these cells produced in vitro a 92-kDa gelatinase on stimulation. Culture of HBECs obtained by the explant technique showed a marked increase in 92-kDa gelatinase after exposure to 5 × 10^{-5} M SM that reinforced the relevance of our animal results to human exposure to SM. These results suggest that in SM respiratory intoxication 92-kDa gelatinase could be of both inflammatory and epithelial cell origin.

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