TOXIC EFFECTS OF SULFUR MUSTARD ON RESPIRATORY EPITHELIAL CELLS IN CULTURE

MARTINE CHEVILLARD*, PIERRE LAINEE†, PASCALE ROBINEAU†, AND EDITH PUCHELLE*

*** INSERM Unité 314**
CHR Maison-Blanche
Université de Reims, France

† Laboratoire de Pharmacodynamie
Centre d'Etudes du Bouchet
Vert le Petit, France

Sulfur mustard (SM) is known to induce cutaneous injury and to cause acute damage to the respiratory tract. Although skin vesication has been demonstrated on human epidermal keratinocytes in culture, no study has been carried out to analyze the effects of SM on the ultrastructural and functional activity of surface respiratory epithelial cells. To evaluate this SM toxicity, we developed an in vitro *model of respiratory epithelial cells in primary culture. The study was performed on surface epithelial cells from rabbit trachea cultured according to the explant-outgrowth technique. The functional activity of the cultures was evaluated by measuring the ciliary beating frequency (CBF) of the ciliated cells with a videomicroscopic method. The morphological aspects of the cells were analyzed by light and electron microscopy. Addition of 0.1 mM SM directly into the culture medium produced a sudden and irreversible CBF inhibition, first observed after 2 hr on the ciliated cells of the outgrowth periphery. The arrest of the ciliary beating progressively reached the whole surface of the outgrowth and was simultaneously observed with a detachment of the outgrowth cells. It began at the outgrowth border, leading to the exfoliation of cell sheets, and then to the whole culture after 48 hr. Morphological damage was expressed by intense vacuolisation and disorganization of cytoplasmic and nuclear structures. These findings suggest that the detachment of the respiratory epithelial cells from the matrix represents a major toxic effect of 0.1 mM SM. SM dramatically affects the viability of respiratory epithelial cells in culture. Moreover, the sudden CBF inhibition is more likely due to the death of the ciliated cells than to a specific ciliotoxic effect of SM.*

1. Address all correspondence to: Martine Chevillard, INSERM U314, 45 rue Cognacq-Jay, F-51092 Reims Cedex, France.

2. Abbreviations: CBF, ciliary beating frequency; HEPES, N2-hydroxyethylpiperazine-N'2-ethanesulfonic acid; PBS, phosphate buffer saline; SM, sulfur mustard; TEM, transmission electron microscopy.

Cell Biology and Toxicology, Vol. 8, No. 2, pp. 171-181

ISSN: 0742-2091

INTRODUCTION

Sulfur mustard (SM), 2,2'-dichlorodiethyl sulfide $(ClCH_2CH_2)_2S$, is an alkylating agent of the vesicant family. This chemical compound was used as a weapon during the First World War and more recently in the Iran-Iraq conflict. Usually referred to as mustard gas, SM is in fact an oily liquid which is extremely toxic. The main targets of this vesicant compound are the skin, the eyes, and the respiratory tract (Balali, 1984; Sohrabpour, 1984; Colardyn, 1986). SM induces inflammatory lesions and injuries at skin level. Many studies report an inflammatory response and blister formation on the epidermis following exposure of different animal species (McAdams, 1956; Vogt et al., 1984; Dannenberg et al., 1985) or of human skin graft on nude mice (Papermeister et al., 1984) to SM. It is well known that SM possesses mutagenic properties (Fox and Scott, 1980), but the biochemical effects of this compound in the cell are not fully understood and no effective antidote is available. Papermeister et al. (1985) hypothesized that the generation of SM-induced pathology includes different steps: DNA alkylation followed by the depletion of the cellular NAD^+. More recently, Smith et al. (1990) verified on human epidermal keratinocytes in culture that the biochemical mechanisms of SM toxicity induced a decrease of cellular NAD^+ levels. Therefore, the toxic events following exposure to SM were essentially demonstrated for skin epithelial cells. Up to now, the only data related to SM-induced respiratory injuries were restricted to the clinical observations of surviving subjects exposed to the gases (Wada et al., 1968; Balali, 1984; Colardyn et al., 1986; Sohrabpour, 1984; Leipner et al., 1987). The first targets of the toxin were the larynx and the tracheal and bronchial epithelia, whereas the bronchiolar and alveolar epithelia were more rarely affected. But no experimentation concerning this toxicity on the respiratory tract has been recently published.

In order to evaluate the pathogenicity of SM on the respiratory tract, we have developed an *in vitro* model of respiratory epithelial cells from rabbit trachea. Primary cultures produced according to the explant-outgrowth technique, already developed in the laboratory (Chevillard et al., 1991), seem to be a suitable model for studying the cytotoxic and ciliotoxic effects of SM, because ciliated and nonciliated cells retain their morphological and functional characteristics *in vitro*. In this report, observations using videomicroscopy and light and electron microscopy demonstrated the sensitivity of respiratory epithelial cells to SM. Because of technical requirements in the culture method, and in order to maintain the highest degree of safety during the experiments, SM was applied directly onto the culture cells by dissolution of the toxic into the culture medium. After different concentrations of SM had been tested, the 0.1 mM dose was chosen, allowing us to follow the different parameters investigated over an acceptable period of time (up to 48 hr), in order to avoid the more drastic phenomena that take place at 1 and 10 mM SM.

METHODS

Cell Culture Methods

Respiratory epithelial cells were obtained from rabbit tracheae and cultured according to the explant-outgrowth technique (Kennedy and Ranyard, 1983). Tracheae were removed from six

adult male New Zealand white rabbits and washed in RPMI 1640 culture medium supplemented with 100 U/ml penicillin and 100 μg/ml streptocmycin. Mucosae were dissected free from the underlying connective tissue and cut into small pieces (about 2 mm^2). After microscopic examination, pieces exhibiting an active ciliary beating were selected and used for explant culture. These explants were seeded on the substratum of culture dishes (Nunc, Denmark), coated with collagen I and IV, and cultured in a RPMI serum-free medium supplemented with hormones and growth factors (1 μg/ml insulin, 1 μg/ml transferrin, 10 ng/ml EGF, 0.5 μg/ml hydrocortisone, 10 ng/ml retinoic acid). These culture conditions have been shown to improve cell adhesion, migration, and differentiation (Chevillard et al., 1991). The dishes were placed in an incubator at 37° and 5% CO_2 and the medium was renewed every two days. All the experiments were performed after 5 days of culture.

Functional Studies

For all experiments, when culture dishes were removed from the incubator, 20 mM HEPES was added to the defined medium to ensure pH stability. Ciliary beating frequency (CBF) was analyzed by a videomicroscopic method developed by Zahm et al. (1990a). Briefly, cell culture dishes were placed on a heating stage (37°) of an inverted phase contrast microscope. One to three cellular fields were then randomly selected in the outgrowth and were recorded using a CCD camera (COHU 4712) at the different times of the protocol, leading to the CBF measurement of 6 to 50 ciliated cells. The variations in light intensity induced by the ciliary beating were detected by a photodetector placed on the video monitor and converted into a frequency spectrum by using a fast Fourier transform software. For this spectrum, the mean CBF was calculated.

Morphological Studies

Cultures were fixed *in situ* with 2.5% glutarldehyde and post-fixed with 1% OsO_4. They were then dehydrated through graded concentrations of ethanol and embedded in epoxy resin. Plastic dishes were then sectioned by use of a punch, and thin and ultrathin sections were cut perpendicular to the surface of the culture. Thin preparations were stained with toluidin blue and observed with an Axiophot light microscope (Zeiss, Oberkochen, Germany). Ultrathin preparations were mounted on copper grids, stained with uranyl acetate and lead citrate, and examined in transmission electron microscopy (TEM).

Sulfur Mustard Preparation

Because of the low solubility of SM, the first 10 mM solution was prepared in ethanol. This solution was then diluted 100-fold in the culture medium to obtain a final concentration of 0.1 mM SM, with a final ethanol concentration of 1%. This ethanol concentration was found to be non-cytotoxic. Indeed, in control experiments where the cells were exposed to 1% ethanol, we did not observe any change in either cell morphology or in CBF measurements. The culture medium was replaced by the SM solution to carry out the intoxication. Because of the unstable properties of SM, (a half-life of 6 min in the culture medium at 37°), the time related to the dilution and application of the toxic never exceeded 2 min.

Protocol

For each experiment, 12 to 20 culture dishes were used, one for CBF measurements and the others for morphological examinations. For CBF measurements, each change of the culture medium was followed by a 15 min waiting time to allow temperature stabilization. CBF was first measured in basal conditions, and also with 1% ethanol in the culture medium. The medium was then removed and the intoxication performed. CBF was measured at 30, 60, and 90 min, and at 2, 3, 4, 5, and 6 hr after the intoxication. When the ciliary beating stopped, the corresponding exposure time was noted.

Morphological changes of the explant and outgrowth cultures were analyzed before and 1, 2, 4, 6, and 24 hr after intoxication. Videomicroscopic observations of the cultures were performed during the experiment and were complemented by both light and electron microscopic observations. All the handling of the toxic compound was carried out under specific conditions ensuring safety and security for operators (gas masks and butyl gloves) and their environment (special hood, special room).

Statistical Analysis

The CBF results were exposed as mean ± SD. Variations were tested by a Friedman test. If a significant change was shown, each experimental period was compared to the control using a Wilcoxon test.

RESULTS

Videomicroscopic Observations of Airway Epithelial Cell Cultures

The rabbit tracheal mucosa is organized in a pseudo-stratified epithelium composed of three main types of epithelial cells: basal, ciliated, and secretory cells (Figure 1). After 48 hr of culture, the explants were surrounded with an area of adherent epithelial cells, which progressively increased to form an outgrowth of differentiated as well as undifferentiated cells (Figure 2A). After addition of 0.1 mM SM, the external zones of the outgrowth became less adherent and tended to roll up (Figure 2B). This was evident from 2 hr onwards after the beginning of the experiment and progressed to the detachment of the entire culture.

Ciliary Beating Frequency

Cell cultures were exposed to SM solutions of various concentrations. Ten mM and 1 mM solutions were drastically destructive and led to a rapid arrest of the ciliary beating, within 10 and 30 min of the experiments, respectively. No significant difference of the CBF could be detected in the basal culture medium supplemented with 1% ethanol (12.3±1.4 Hz) compared with the medium that was not supplemented with 1% ethanol (11.9±1.5 Hz). After intoxication with 0.1 mM SM, the mean CBF was maintained in the range of 11.3-12 Hz until the end of the observation time as defined in the protocol (Figure 3). No statistical difference could be observed between the different periods of the experiments and the control. Nevertheless, in two of the six experiments, the ciliary beating of the ciliated cells in cellular fields randomly selected at the periphery of the outgrowths suddenly stopped after 90 min of intoxication. On

the other hand, in the four other experiments, CBF was measured even up to and beyond 6 hr without any modification. In these latter cases, the cellular fields corresponded to areas randomly selected at the borders of the explant.

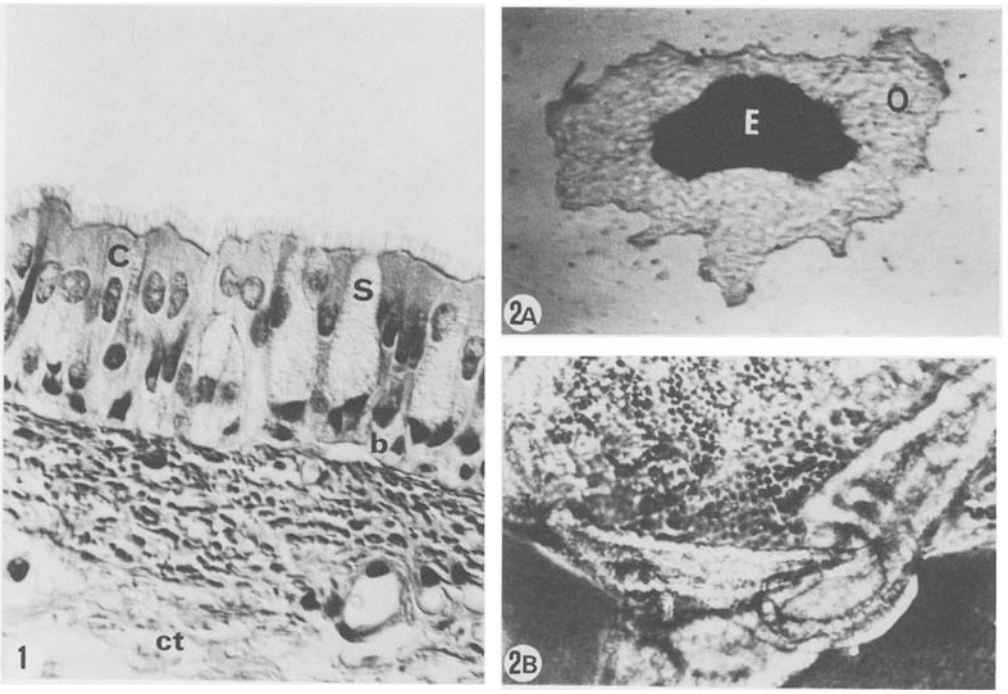

FIGURE 1. Histological section of columnar pseudo-stratified epithelium from rabbit trachea. C, ciliated cell; S, secretory cell; b, basal cell; ct, connective tissue. (x 650)

FIGURE 2. Phase-contrast microscope observations of 5-day-old primary cultures from rabbit trachea epithelial cells.

A) Control culture shows the explant (E) surrounded by the outgrowth (O). (x 16)

B) After 2 hr of 0.1 mM SM exposure, detachment of the outgrowth periphery floating in the medium. (x 90)

Light Microscopy

After 5 days of culture, the cellular organization of the explant was closely identical to that of trachea, whereas the outgrowth cells were organized into a double layer of ciliated and nonciliated cells (Figures 4A and 4B). One hour after intoxication, vacuolisation of epithelial cells of the explant was observed, whereas control explants did not show any abnormality (Figure 4C). The vacuoles were preferentially located in the ciliated cells of the explant and were never observed in the outgrowth cells. After 1 hr, vacuoles were numerous but small, and they became large after 24 hr. After 2 hr, the progressive cell layer detachment from the substrate, already observed by videomicroscopy, was confirmed (Figure 4D). The cells remained well-organized among themselves, and the tight-junctions were preserved. The epithelial cell detachment also occurred in the explant after 4 hr, with the whole surface epithelium tending

to separate from the underlying connective tissue (Figure 4E). After 6 hr, a marked increase in the intercellular spaces was observed in the outgrowth, and preferentially in the basal rather than in the apical cells (Figure 4F).

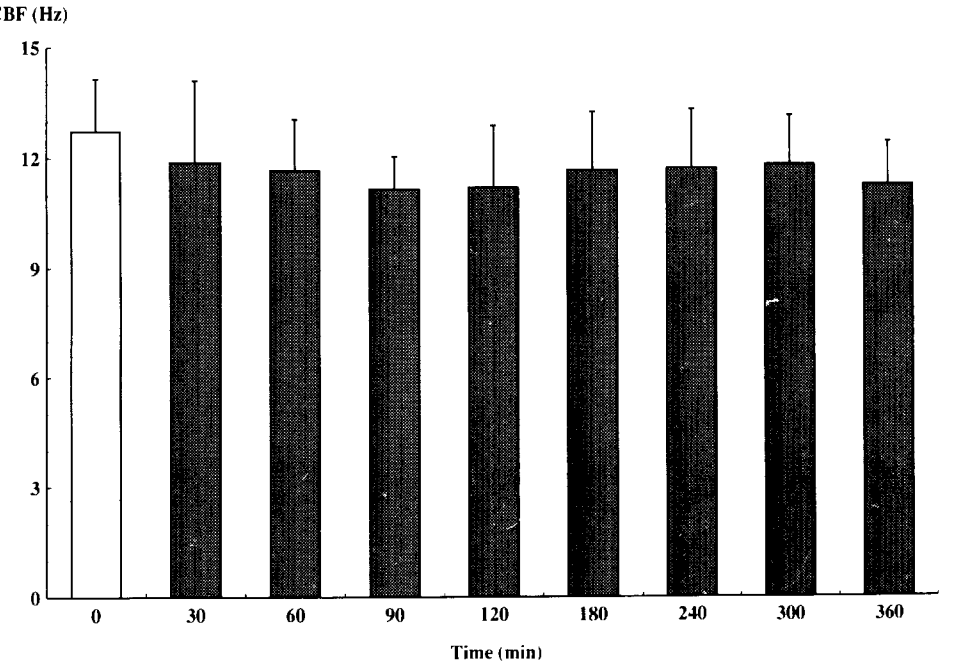

FIGURE 3. CBF variations of ciliated cells according to the time of intoxication by 0.1 mM SM. Bars represent the mean and standard deviation of six experiments up to 90 min and of four experiments from 120 to 360 min after time of intoxication.

Electron Microscopy

In addition to the numerous intracytoplasmic vacuoles, the first ultrastructural modifications affected mitochondria. One hour after intoxication, they appeared swollen in the epithelial cell explant (Figure 5A) and remained altered. After 6 hr, several cytoplasmic abnormalities were noticed (Figure 5B), including an increase in the number of intermediate filaments and the assembling of the cell organelles close to the nucleus, leaving large organelle-free zones at the periphery of the cell. In the nucleus, a disorganization of the chromatin with the appearance of large osmiophilic granules were observed. These morphological changes were noticed in the cells of the explant as well as in the cells of the outgrowth. In contrast, we did not detect any ciliary abnormality in either the explant or the outgrowth cells.

DISCUSSION

We have evaluated the toxicity of SM on respiratory tract cells in an *in vitro* cell culture model from rabbit surface tracheal cells. The explant-outgrowth technique, used by Kennedy

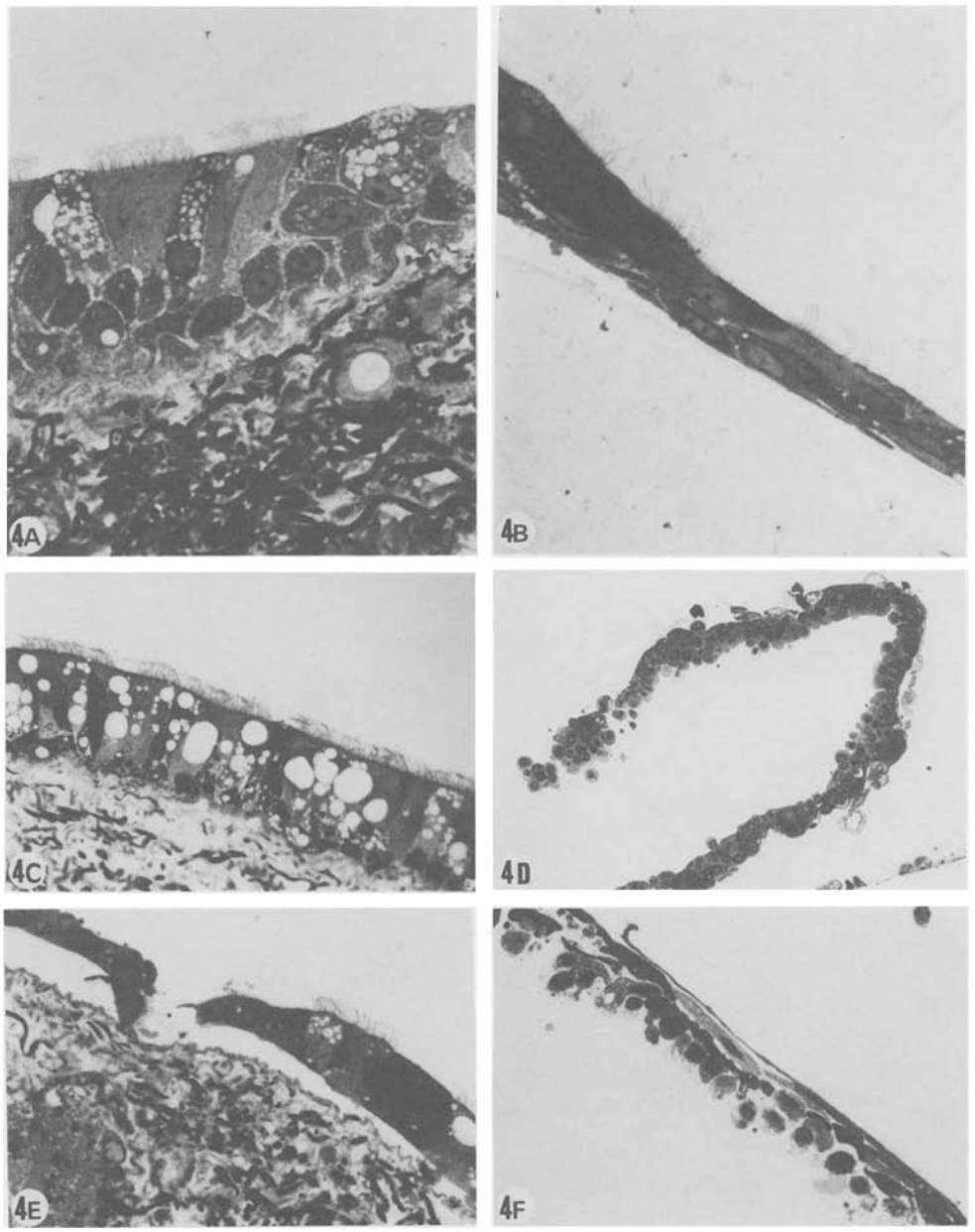

FIGURE 4. Light microscopic observations of 5-day-old culture from rabbit trachea epithelial cells in the explant (A, C, E) and in the outgrowth (B, D, F). A, B, control culture; C, 1 hr; D, 2 hr; E, 4 hr; F, 6 hr after intoxication by 0.1 mM SM. (x 625)

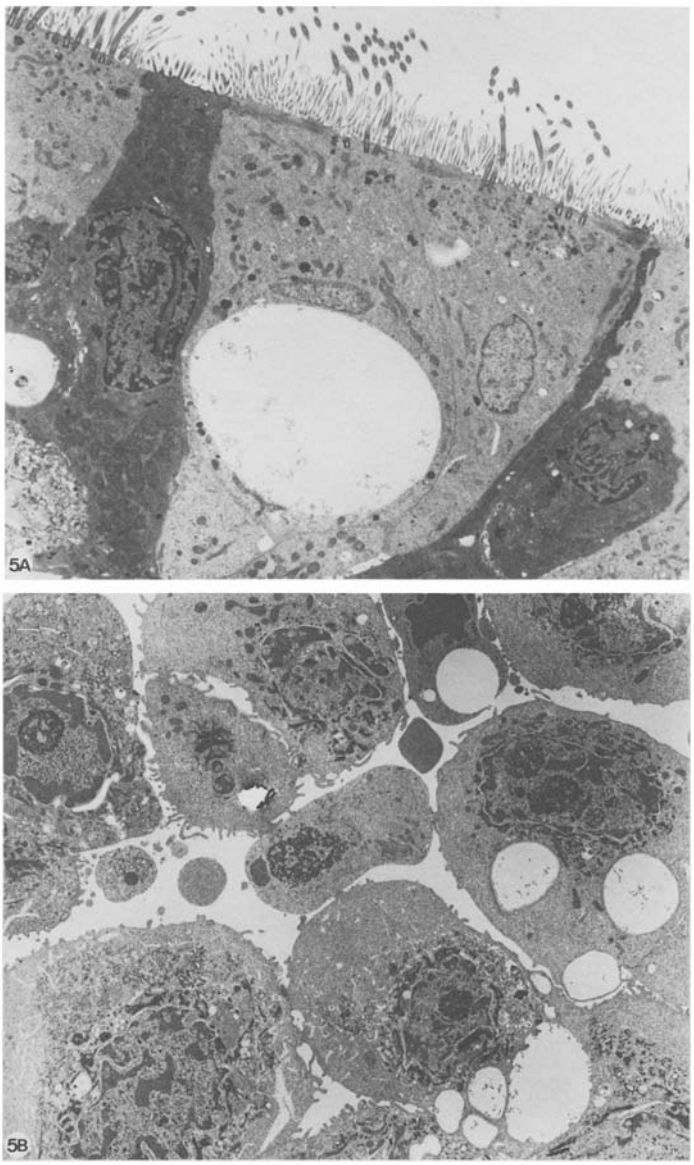

FIGURE 5. TEM observations of epithelial cells from rabbit trachea in primary culture after exposure to 0.1 mM SM: A) after 1 hr exposure at the explant level and B) after 6 hr exposure in the outgrowth. (x 3000)

and Ranyard (1983) and by Baeza-Squiban et al. (1991), provides a well-defined system for evaluating morphological changes (Fischer and Placke, 1988) or disorders of the ciliary activity (Duckett et al., 1986; Romet et al., 1990) associated with respiratory toxic exposure. Rabbit surface epithelium cells present the same cellular histological characteristics and orga-

nization as the human airway epithelium. *In vitro*, the CBF of rabbit tracheal cells was not significantly different from that human nasal and tracheal cells cultured under the same conditions (Chevillard et al., 1991; Zahm et al., 1990b). Furthermore, the culture conditions used in the present study were similar to those that have been validated for the human surface upper airway epithelial cell cultures (Chevillard et al., 1991), and allowed us to perform cell cultures in standardized and repetitive working conditions. After 5 days of culture, the explants were surrounded by outgrowths composed of well-differentiated ciliated and secretory cells and undifferentiated cells. For the evaluation of SM cytotoxicity, we performed analysis of the outgrowth cells as well as of the explant cells.

SM produced sudden and irreversible inhibition of the CBF after various times (between 1.5 hr and 48 hr), depending on the cellular field position randomly selected in the outgrowth. This phenomenon was correlated with the observation of the cellular desquamation and then with the death of the cells. Exfoliation of the cell sheets began at the periphery of the outgrowth, and these areas were the first to show inactive ciliated cells. TEM observations showed that morphological damages were mainly expressed by intense vacuolisation and disorganization of cytoplasmic and nuclear structures, without any change in the cilia structure. As no study relating to the effects of SM on the respiratory epithelial cells has been carried out until now, our morphological results can only be compared to those concerning the effects of SM at skin level. Mustard vesication has been studied *in vivo* in the skin of various animals and has demonstrated a selectivity of damage in the basal and lower cell layers and the blister formation between the epidermis and the dermis (McAdams, 1956). Pycnotic nuclei and swollen mitochondria have also been described by Vogt et al. (1984) in the basal epidermal cells of rabbit skin after application of SM. Papermeister et al. (1984) described the morphological changes in pathology produced by SM in human skin grafts on athymic nude mice; the presence of large cytoplasmic vacuoles was also noted. Concerning the *in vitro* studies performed on human epidermal keratinocytes, Smith et al. (1990) demonstrated that the cells lost 40% of viability 24 hr after exposure to 0.3 mM SM. The effects of SM on the respiratory epithelial cells in culture seem to be similar to the SM toxicity observed *in vivo* on skin cells.

Some authors have studied the biochemical mechanisms by which SM induced vesication. The depletion of intracellular NAD^+ seems to be the most important event that could lead to cellular death (Gross et al., 1985; Meier et al., 1987; Smith et al., 1990). Papermeister et al. (1985) proposed that SM induced vesication through direct damage to DNA and subsequent alterations of the cellular metabolism. Because we observed the same morphological changes on the respiratory epithelial cells as on cutaneous cells, we can hypothesize that similar mechanisms could be involved in our cell cultures. The specific presence of damaged mitochondria could indicate an oxidative effect, mediated by a glutathion depletion (Chipman, 1989). Also, because of the specific desquamation observed during our experiments, we can assume that one of the SM targets could be the receptors of the basal cells for the extracellular matrix components. However, this potential action might not be the only toxic effect, since

SM dramatically affects the viability of respiratory epithelial cells in culture. The sudden CBF inhibition that we observed was more likely due to the death of the ciliated cells than to a specific ciliotoxic effect of SM. The detachment of the epithelial cells from the matrix represents the first obvious toxic effect of this chemical compound, and it should be an interesting parameter by which to evaluate the degree of the cellular intoxication. In addition, the culture system of respiratory epithelial cells seems to be a useful model for studying the mechanisms of SM-induced respiratory injury and for testing potentially antagonistic molecules.

ACKNOWLEDGMENTS

The authors thank C. Fuchey and D. Pierrot for their excellent technical assistance, and J.-M. Zahm for his helpful advice. This work was supported by INSERM/CEB grant no. 25056.

REFERENCES

BAEZA-SQUIBAN, A., ROMET, S., MOREAU, A., and MARANO, F. (1991). "Progress in outgrowth culture from rabbit tracheal explants: Balance between proliferation and maintenance of differentiated state in epithelial cells." *In Vitro* Cell. Dev. Biol. 27A: 453-460.

BALALI, M. (1984). "Clinical and laboratory findings in Iranian fighters with chemical gas poisoning." Arch. Belg., Suppl.: 254-259.

BLANQUART, C., ROMET, S., BAEZA, A., GUENNOU, C., and MARANO, F. (1991). "Primary cultures of tracheal epithelial cells for the evaluation of respiratory toxicity." Toxic. *In Vitro* 5: 499-502.

CHEVILLARD, M., HINNRASKY, J., ZAHM, J.M., PLOTKOWSKI, M.C., and PUCHELLE, E. (1991). "Proliferation, differentiation and ciliary beating of human respiratory ciliated cells in primary culture." Cell Tissue Res. 264: 49-55.

CHIPMAN, J.K. (1989). "Mechanisms of cell toxicity." Current Opinion in Cell Biology 1: 231-235.

COLARDYN, F., DEKEYSER, H., RINGIOIR, S., and DEBERSAQUES, J. (1986). "Clinical observation and therapy of injuries with vesicants." J. Tox. Clin. Exp. 6: 237-246.

DANNENBERG, A.M., PULA, P.J., LIU, L.H., HARADA, S., TANAKA, F., VOGT, R.F., KAJIKI, A., and HIGUCHI, K. (1985). "Inflammatory mediators and modulators released in organ culture from rabbit skin lesions produced *in vivo* by sulfur mustard." Am. J. Pathol. 121: 15-27.

DUCKETT, K.E., SCHILLER, S.L., GIRARD, P.R., and KENNEDY, J.R. (1986). "The effects of gossypol on the ultrastructure and function of tracheal ciliated cells." J. Submicrosc. Cytol. 18(1): 117-125.

FISCHER, G.L. and PLACKE, M.E. (1988). "Cell and organ culture models of respiratory toxicity." In: Toxicology of the Lung (D.E. Gardner, J.D. Crapo, and E.J. Massaro, eds.). Raven Press, New York. pp. 285-314.

FOX, M. and SCOTT, D. (1980). "The genetic toxicity of nitrogen and sulphur mustard." Mutat. Res. 75: 131-168.

GROSS, C.L., MEIER, H.L., PAPERMEISTER, B., BRINKLEY, F.B., and JOHNSON, J.B. (1985). "Sulfur mustard lowers nicotinamide adenine dinucleotide concentrations in human skin grafted to athymic mice." Toxicol. Appl. Pharmacol. 81: 85-90.

KENNEDY, J.R. and RANYARD, J.R. (1983). "Morphology and quantitation of ciliated outgrowths from culture rabbit tracheal explants." Eur. J. Cell Biol. 29: 200-208.

LEIPNER, N., DEWES, W., SCHÜLLER, H., and HELM, U. (1988). "Lungenparenchymveränderungen nach Schwefellost-vergiftung." Fortschr. Röntgenschr. 147(2): 147.

McADAMS, A.J. (1956). "A study of mustard vesication." J. Invest. Dermat. 26: 317-325.

MEIER, H.L., GROSS, C.L., and PAPERMEISTER, B. (1987). "2,2'-dichlorodiethyl sulfide (sulfur mustard) decreases NAD^+ levels in human leukocytes." Toxicol. Lett. 39: 109-122.

PAPERMEISTER, B., GROSS, C.L., PETRALI, J.P., and HIXSON, C.J. (1984). "Pathology produced by sulfur mustard in human skin grafts on athymic nude mice. I. Gross and light microscopic changes." J. Toxicol.-Cut. & Ocular Toxicol. 3: 371-391.

PAPERMEISTER, B., GROSS, C.L., MEIER, H.L., PETRALI, J.P., and JOHNSON, J.B. (1985). "Molecular basis for mustard-induced vesication." Fundament. Appl. Toxicol. 5: S134-149.

ROMET, S., DUBREUIL, A., BAEZA, A., MOREAU, A., SCHOEVAERT, D., and MARANO, F. (1990). "Respiratory tract epithelium in primary culture: Effects of ciliotoxic compounds." Toxicol. *In Vitro* 4: 399-402.

SMITH, W.J., CLARK, L., GROSS, C.L. CHAN, P.C., and MEIER, H.L. (1990). "The use of human epidermal keratinocytes in culture as a model for studying the biochemical mechanisms of sulfur mustard toxicity." Cell Biol. Toxicol. 6(3): 285-291.

SOHRABPOUR, H. (1984). "Clinical manifestations of chemical agents on Iranian combatants during Iran-Iraq conflict." Arch. Belg., Suppl.: 291-297.

VOGT, F.R., DANNENBERG, A.M., SCHOFIELD, B.H., HYNES, N.A., and PAPERMEISTER, B. (1984). "Pathogenesis of skin lesions caused by sulfur mustard." Fundament. Appl. Toxicol. 4: S71-S83.

WADA, S., NISHIMOTO, Y., MIYANISHI, M. KAMBE, S., and MILLER, R.W. (1968). "Mustard gas as a cause of respiratory neoplasia in man." Lancet 1: 1161-1163.

ZAHM, J.-M., DIONISIUS, J.-P., PIERROT, D., CHEVILLARD, M., and PUCHELLE, E. (1990a). "Mesure de la fréquence de battement ciliaire sur écran vidéo." Innov. Tech. Biol. Med. 11(2): 118-129.

ZAHM, J.-M., PIERROT, D., HINNRASKY, J., FUCHEY, C., CHEVILLARD, M., GAILLARD, D., and PUCHELLE, E. (1990b). "Functional activity of ciliated outgrowths from cultured human nasal and tracheal epithelia." Biorheology 27: 559-565.

received: 4/28/92
accepted: 6/15/92