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TOXICITY OF SULPHUR MUSTARD IN ADULT RAT LUNG ORGAN CULTURE

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Toxicity of sulphur mustard in adult rat lung organ culture

Thomas W. Sawyer*[a], Paul E. Wilde[b], Paul Rice[b], M. Tracy Weiss[a]

[a]*Medical Countermeasures Section, Defence Research Establishment Suffield, Box 4000, Medicine Hat, Alberta, Canada T1A 8K6*
[b]*Biology Division, Chemical and Biological Defence Establishment, Porton Down, Salisbury, Wilts, United Kingdom SP4 0JQ*

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Abstract

The toxicity of the chemical warfare agent sulphur mustard, (bis-(2-chloroethyl)sulphide, HD), was examined in adult rat lung organ cultures. Assessment of HD-induced damage by the MTT cytotoxicity assay indicated that the median lethal concentration (LC_{50}) of HD in these cultures was reproducible, and in the μM range. Damage to the lung slices was expressed only after a latent period of 48 h and did not increase significantly with longer expression times. Histopathological examination of HD-treated lung cultures showed that the structural changes in the lung tissue paralleled the toxicity measured biochemically, and were also similar to the damage found in animals and man exposed to HD in vivo. This in vitro model offers a useful tool with which to study the toxicity and mechanism of action of sulphur mustard.

Keywords: Sulphur mustard, bis-(2-chloroethyl)sulphide, HD; Tissue culture; Lung; MTT; Histopathology; Toxicology

1. Introduction

The respiratory system is the primary target organ for gaseous and airborne particulate toxicants, and assessment of these xenobiotics by inhalation is an expensive and complex undertaking. Interpretation of these types of studies, as well as the determination of cause-effect relationships, is clouded by the high complexity of the lung and systemic involvement. Tissue culture models have been utilized to circumvent these problems and organ culture systems, in particular, offer attractive alternatives to study lung toxicology.

Historically, peripheral lung organ culture has been limited to foetal or neonatal lung tissue (Bhatnager et al., 1979; Minor, 1979) with attempts at adult lung organ culture being considerably less successful. Typically, adult peripheral lung organ cultures degenerate both biochemically and morphologically after only 3–7 days in culture (Weinhold et al., 1979). Recently, a model has been described in which adult rodent lung can be cultured for periods of 4–6 weeks (Placke and Fisher, 1986; 1987a,b; Fisher and Placke, 1988). The lung slices are cultured in serum-free, chemically-defined medium and maintained on gelatin sponges at the air-liquid interface. Histopathological and biochemical investi-

* Corresponding author.

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gations of lung toxicants are thus possible over extended periods of time.

Bis-(2-chloroethyl)sulphide, more commonly known as sulphur mustard (HD is the military designation for the pure form of this agent) or mustard gas, was first used as a chemical warfare (CW) agent in 1917 at Ypres (Papirmeister et al., 1991). More recently its use has been documented in the Iraq-Iran war during 1984–1986 (United Nations, 1984, 1986, 1987). Although the primary intended effect of HD as a CW agent is as a skin vesicant producing large, fluid-filled blisters, it also has profound effects on the eye and on the respiratory system (Willems, 1989; Papirmeister et al., 1991; Wormser, 1991). Currently, there is no firm consensus as to the mechanism of toxic action of HD, although several theories have been proposed (Papirmeister et al., 1985, 1991; Whitfield, 1987).

The adult lung organ culture model described by Placke and Fisher (1987a) appears to offer a useful in vitro system with which to study the respiratory toxicity of HD. In this paper we describe the toxicity of HD to adult rat lung organ cultures as measured by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) cytotoxicity assay and histopathology.

2. Materials and methods

2.1. Chemicals

Medium 199, MTT, glutamine, hydrocortisone, retinyl acetate, nystatin, gentamicin, bovine insulin, agarose and isopropanol were obtained from Sigma Chemical Co. (St. Louis, MO). Penicillin, streptomycin and Fungizone® were obtained from Gibco Ltd. (Grand Island, NY). Sulphur mustard was prepared by the Chemical Biological Defence Section, Defence Research Establishment Suffield at greater than 98% purity.

2.2. Animals

Male Sprague–Dawley rats (300–400 g) were obtained from Charles River Laboratories (St. Constant, Quebec) and given food and water ad libitum. The animals were allowed to acclimate for at least 1 week prior to experimental use.

2.3. Lung slice preparation

All animals were anaesthetized by inhalation of ether vapour. This method of anaesthesia was judged to be least likely to affect drug metabolizing enzyme activities in those cultures treated after the day of explantation. When the animal was fully anaesthetized, the abdominal and thoracic surfaces were disinfected, the abdominal cavity was opened and the animal exsanguinated through arterial puncture of the abdominal aorta. The lungs, heart and trachea were aseptically dissected out and transferred to a sterile petri dish. The organs were rinsed with warm PBS supplemented with Fungizone® (2.5 μg/ml), penicillin (10 IU/ml) and streptomycin (10 μg/ml) and then the trachea was cannulated with ethyl vinyl acetate microtubing (0.04 inch I.D., 0.07 inch O.D.). The cannula was attached to a gravity perfusion apparatus and the lungs were infused at a constant hydrostatic pressure of 20 cm water pressure with warm (37°C) 0.5% agarose/medium M-199 for 4 min. The trachea was then tied off below the cannula and the perfused lung removed and cooled at 4°C for 30 min. After the perfused lung had solidified, the heart, trachea and fascia were removed. The lobes of the lung were then cut into 1 mm thick slices using a McIlwain tissue chopper (Mickle Laboratory Engineering Co. Ltd. Surrey, UK) and placed onto pieces of sterile gelatin sponge (Gelfoam, Upjohn Co., Kalamazoo, MI) in 35 mm dishes (Costar, Cambridge, MA). Two or three lung slices were placed onto each piece of Gelfoam®, which had been previously saturated with medium consisting of M-199 supplemented up to 1.0 μg/ml bovine insulin, 0.1 μg/ml hydrocortisone, 0.1 μg/ml retinyl acetate, 10 IU/ml penicillin, 10 μg/ml streptomycin, 0.25 μg/ml Fungizone®, 1.0 μg/ml nystatin and 5.0 μg/ml gentamicin (complete M-199). All cultures were incubated in a humidified atmosphere of CO_2/air (5:95%) at 37°C. Culture medium was changed three times a week.

2.4. Sulphur mustard exposure

Tissue samples were routinely exposed to HD for 60 min, either at the time of explantation or at various time intervals afterwards. At the time of exposure the lung slices were transferred to culture

dishes containing warm PBS, and stock solutions of HD dissolved in isopropanol were added to the medium so that the desired HD concentration was reached at 0.25% isopropanol (v/v). Control cultures were treated with solvent only. Treatment was carried out at 37°C and after the desired incubation time the lung slices were rinsed once with PBS, transferred back to the Gelfoam® and then incubated under normal culture conditions until harvested for determination of HD toxicity.

2.5. Histopathology

Lung slices were exposed to HD on the day of explantation and then collected at specified timepoints after HD exposure (2, 5, 8 and 21 days). The slices were then transferred into individual vials containing an excess of neutral buffered formalin. The slices were allowed to fix for at least 48 h at room temperature before being embedded in paraffin wax and cut at $5 \pm 2 \times 10^{-6}$ m. The subsequent histological sections were routinely stained with haemotoxylin and eosin (H + E) and examined at magnifications up to ×400 on a Zeiss Axioplan research photomicroscope. Control, isopropanol-exposed lung slices maintained in culture for 2, 5, 8 and 21 days were processed in a similar manner to provide baseline histological material with which the test slices could be compared.

2.6. Cytotoxicity

Viability of the lung slices and the effects of HD exposure were measured using the MTT cytotoxicity assay. The assay was characterized with respect to MTT substrate concentration and assay incubation time prior to routine toxicity testing. Based on the results of these experiments, lung slices were routinely incubated in an MTT solution (75 μg/ml in complete M-199) for 40 min at 37°C in a gently shaking waterbath. The slices were then removed, the MTT formazan product was extracted into 1 ml isopropanol and quantitated by measuring the absorbance at 570 nm. The lung slices were transferred into 1 ml of 1.0 N NaOH and stored overnight at 4°C. An additional 2 h incubation at 37°C completed the digestion of the lung slices. After neutralization with 1.0 N HCl, an aliquot was taken for protein determination using the Micro BCA Protein Assay (Pierce, Rockford IL).

3. Results

3.1. MTT cytotoxicity assay

The results of the experiments characterizing the MTT cytotoxicity assay with the lung slices are shown in Fig. 1. The MTT substrate was rapidly metabolized by the lung slices and the specific activity (expressed as μg of MTT formazan product produced per mg of protein) was directly related to the initial MTT substrate concentration and incubation time.

Treatment of the lung slices with HD produced a reproducible, concentration-dependent decrease in the specific activity of MTT dehydrogenase. However, this toxicity expressed itself only after a pronounced latent period, regardless of when the cultures were treated. When the cultures were treated on the day of explantation (Fig. 2, Table 1), only minimal toxicity was detectable by 24 h post-treatment, but near maximal toxicity could be measured by 48 h post-treatment. Maximal toxic-

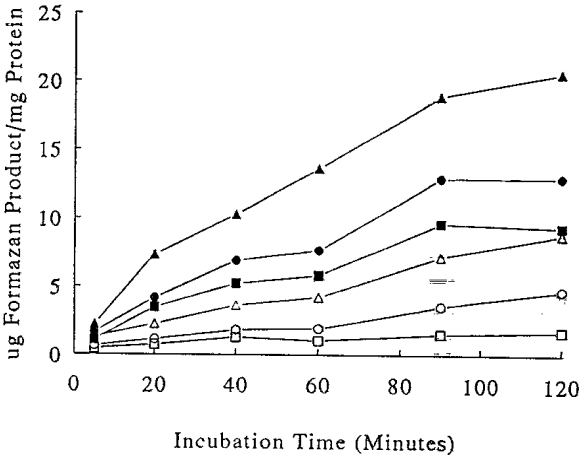

Fig. 1. Characterization of the MTT cytotoxicity assay in rat lung slices. Rat lung slices were used at the time of explantation to investigate the effect of MTT substrate concentration (10 μg/ml, □; 25 μg/ml ○; 50 μg/ml, △; 75 μg/ml, ■; 100 μg/ml, ●; 250 μg/ml, ▲) and incubation time (5, 20, 40, 60, 90 or 120 min) on MTT dehydrogenase activity. Values are the means of two representative experiments utilizing three slices per data point.

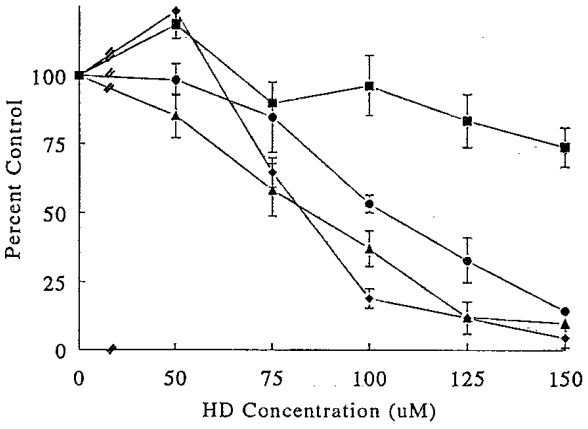

Fig. 2. Toxicity of HD in lung organ cultures treated on the day of explantation. Lung cultures were treated with HD on day zero and harvested 1 day (■), 2 days (●), 3 days (▲), or 7 days later (◆) to determine viability using the MTT cytotoxicity assay. Values represent the means ± S.E. of the mean of three experiments using three lung slices per concentration.

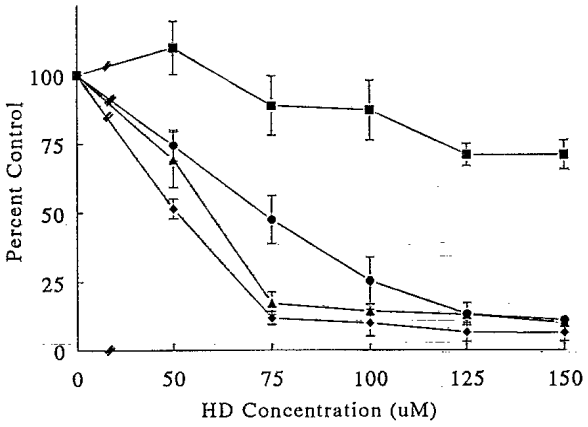

Fig. 3. Toxicity of HD in lung organ cultures treated after 7 days in culture. Lung organ cultures were treated after 7 days in culture and harvested at 1 day (■), 2 days (●), 3 days (▲), or 7 days later (◆) to determine viability using the MTT cytotoxicity assay. Values represent the mean ± S.E. of the mean of three experiments, using three lung slices per concentration.

ity of HD was expressed by 3 days post-treatment and no further significant increases in toxicity were evident over longer time periods. A similar latent period before the expression of HD toxicity was also demonstrated when the cultures were treated with HD after 7 days in culture (Fig. 3, Table 2).

The toxicity of HD was slightly greater in lung cultures when they were treated seven days after the day of explantation (Tables 1, 2, Figs. 2, 3). However, no further significant increases in toxicity were observed even when the cultures were treated after 21 days in vitro and assayed 7 days later (data not shown).

3.2. Histopathology

3.2.1. Control lung slices. At 2 days, the lung slices exhibited an essentially normal alveolar architecture with occasional observations of focal degeneration of the endothelium lining of the large vessels at the hilum of the lung. At 5 days, focal disruption of the alveolar walls was evident and some samples showed several individual alveolar spaces lined with hyperplastic (proliferating) type

Table 1
Toxicity of sulphur mustard in lung organ cultures treated on the day of explantation

Median lethal concentration (LC_{50}, μM)[a]						
Harvest days after treatment						
1	2	3	7	14	21	28
—	106 ± 11	85 ± 13	81 ± 6	83 ± 14	79 ± 16	75 ± 10

[a]Lung cultures were treated on the day of explantation and harvested at selected time-points to assess toxicity using the MTT cytotoxicity assay.

Values represent the mean ± S.D. of three concentration-response experiments.

The LC_{50} data obtained on day 2 was significantly different than data obtained on days 7, 14, 21 and 28 (Duncan's Multiple Range Test, $P \leq 0.05$). No other data points were significantly different from each other.

Table 2
Toxicity of sulphur mustard in lung organ cultures treated at 7 days in vitro

Median lethal concentration (LC_{50}, μM)[a]			
Harvest days after treatment			
1	2	3	7
—	72 ± 11	58 ± 8	50 ± 5

[a]Lung cultures were treated after 7 days in culture and harvested at selected time-points to assess toxicity using the MTT cytotoxicity assay. Values represent the mean ± S.D. of three concentration-response experiments. The LC_{50} data obtained 2 days after treatment was significantly different from that obtained 7 days after treatment (Duncan's Multiple Range Test, $P \leq 0.05$). No other data points were significantly different from each other.

II pneumocytes. This hyperplasia became generalized by 8 days (Fig. 4a) By 21 days, the samples showed obvious type II cell hyperplasia but no evidence of any additional significant abnormality.

3.2.2. Lung slices exposed to 10 μM HD. At 2 days post-exposure, the lung slices showed advanced degeneration of the epithelium lining small segmental bronchioles. The alveolar walls appeared to be lined by intact epithelium and there was no evidence of type II hyperplasia. The endothelium lining the small vessels that accompany the bronchioles appeared focally swollen and vacuolated. At 5 days post-exposure (Fig. 4b,c), all samples exhibited remarkably consistent features that included diffuse, advanced degeneration of bronchiolar epithelium and vascular endothelium, pyknosis (shrinkage) of alveolar epithelial cells and focal, florid type II pneumocyte hyperplasia. Focally, large vacuolated macrophages containing ingested cellular debris were present. Diffuse disruption of alveolar capillaries with leakage of red blood cells into the alveolar lumina and pulmonary interstitium was also noted in some samples. These observations became more diffuse and severe in character by 8 days post-exposure, with hyperplasia of type II pneumocytes being a particularly striking feature at this time point. At 21 days after HD exposure, the histological picture was dominated by florid type II pneumocyte hyperplasia, but there was also evidence of regenerative atypia in the bronchiolar epithelium, i.e. pseudostratification of epithilial cells, increased mitotic activity and cellular pleomorphism, and disruption of alveolar walls leading to a coalescence of individual alveolar spaces. In some cases there was evidence of relatively florid dystrophic calcification towards the edges of the slices.

3.2.3. Lung slices exposed to 100 μmM and 1000 μM HD. Histological examination of the lung slices from these two groups revealed a very similar pattern of pathological damage to that described above for the 10 μM exposure group. There was, however, a very distinct dose-related response with all features showing maximum severity in the highest dose group and some evidence that the time-scale of individual events had become accelerated at 1000 μM HD (Fig. 4d,e).

4. Discussion

During the 75 years since HD was first used at Ypres, interest in the toxicology of this CW agent has been largely determined by its perceived likelihood of use in war. There is thus a renewed interest in this compound due to its use during the Iran/Iraq war (United Nations, 1984, 1986, 1987), and more recently, due to the findings of large stockpiles of HD in post-war Iraq (P.A. Lockwood, Inspector, UNSCOM 17, pers. comm.). Despite several decades of research, the mechanism of toxic action of HD and antidotes against this toxicity are unknown.

The majority of studies that have examined HD have focused on its vesicant action, and although the HD injury is well documented, both grossly and ultrastructurally, research has been hampered by the lack of a good animal model for HD vesication. This has led to a number of cellular models which utilize cell cultures (Meier et al., 1987; Cowan et al., 1991; Mol et al., 1991; Smith et al., 1991; Meier and Johnson, 1992), most notably primary keratinocyte cultures (Mol et al., 1989; Smith et al., 1990; Mol and de Vries van de Ruit, 1992). Sulphur mustard induced damage to the respiratory tract is less well documented and those few studies that have systematically examined res-

piratory tract injuries were conducted prior to 1925 in a limited number of animal species (Papirmeister et al., 1991). The availability of an organ culture model in which explants of adult peripheral lung can be maintained for several weeks, thus offers a unique opportunity to study the respiratory toxicity of HD. Previous work with this culture system utilizing a variety of pulmonary

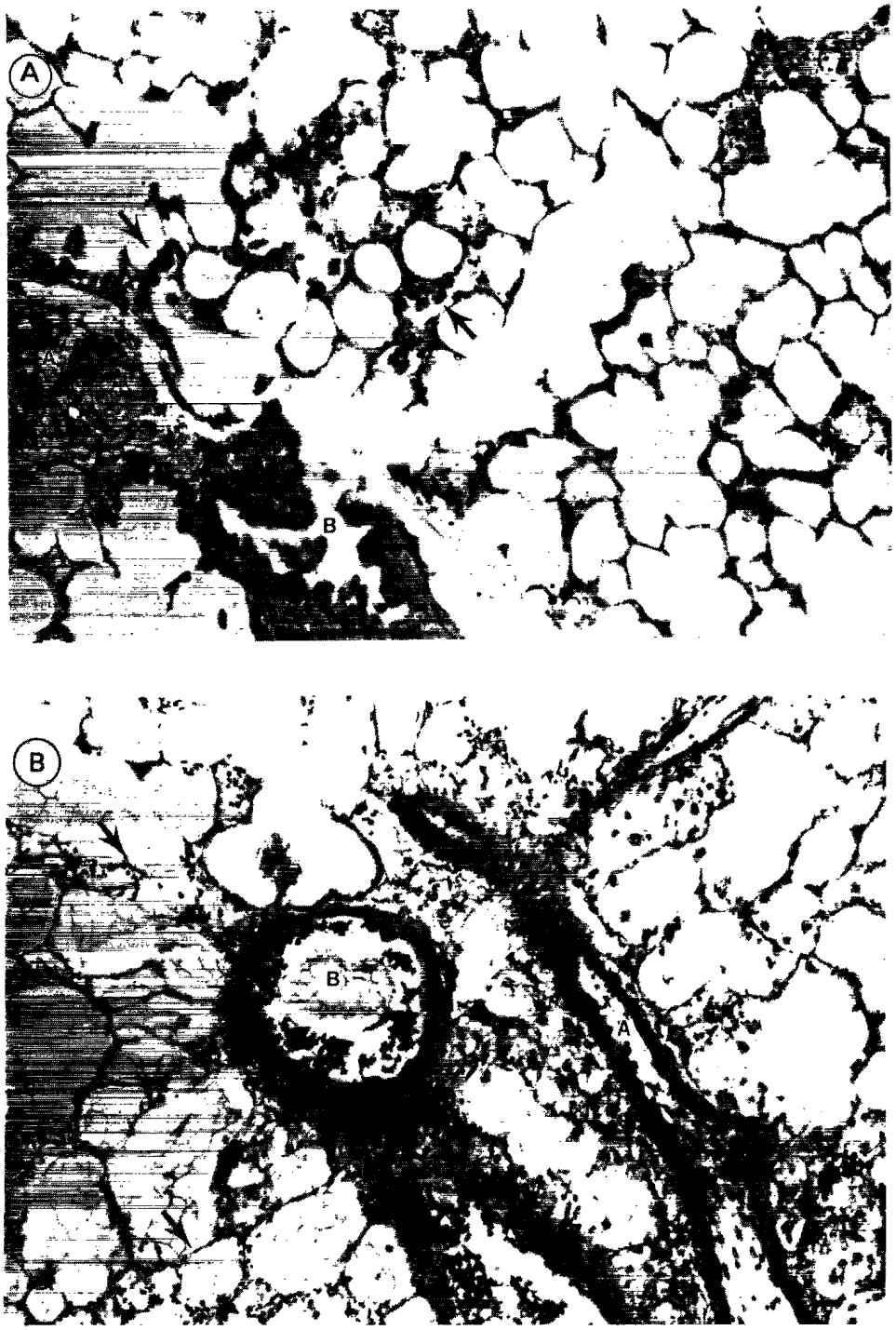

Fig. 4 (a,b).

toxicants have reproduced in vitro tissue lesions that correspond closely to those observed in whole animals and humans after inhalation exposure of each compound (Placke and Fisher, 1986, 1987a,b; Fisher and Placke, 1988).

To quantitate HD-induced damage in the lung cultures we used the well known tetrazolium MTT cytotoxicity assay. This assay assesses cell viability by measuring the reduction of the soluble MTT tetrazolium salt to a blue insoluble formazan prod-

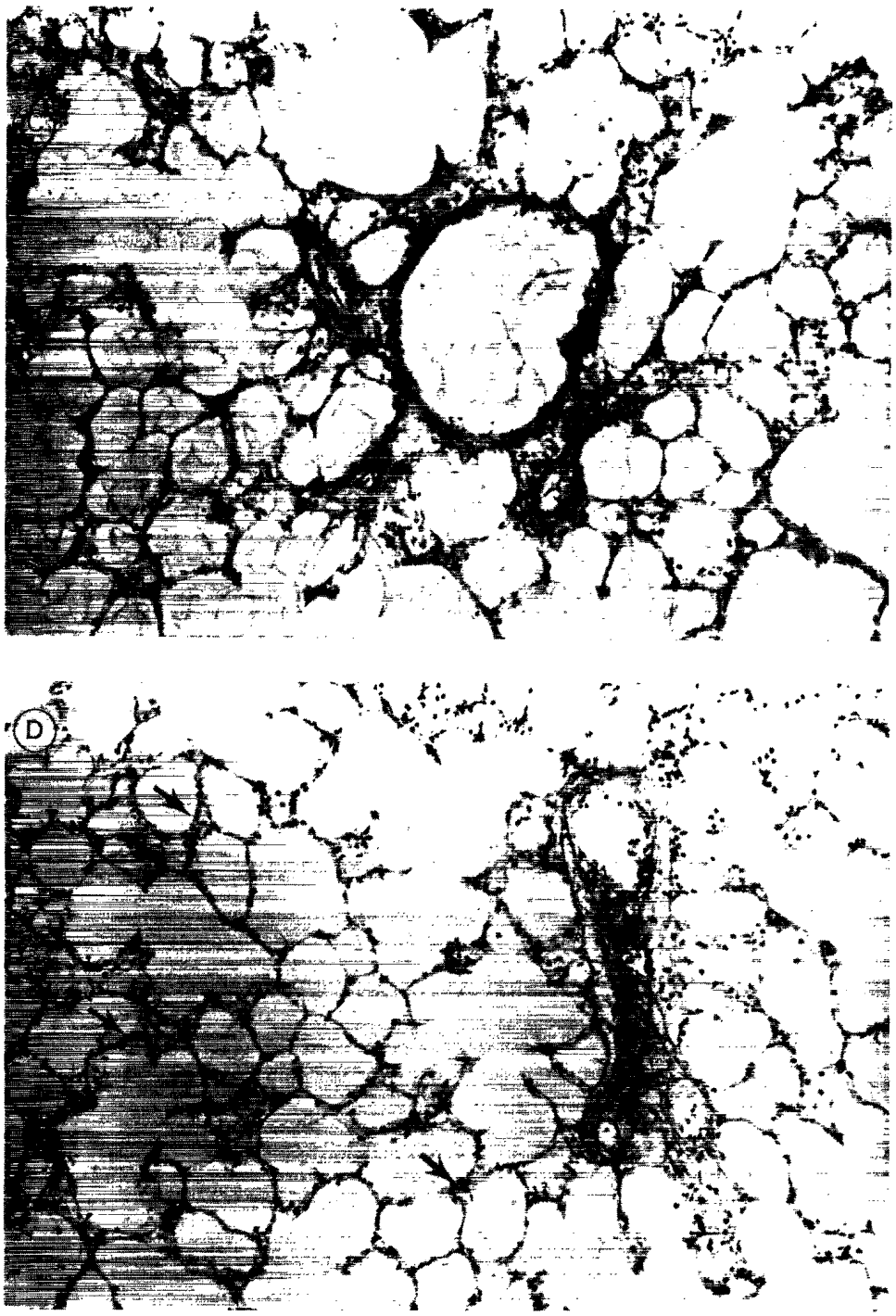

Fig. 4 (c,d).

Fig. 4. Histopathology of cultured rat lung slices. Haematoxylin and eosin. (a) Control lung slice after 8 days in culture. Slice shows a well-preserved microscopic architecture but prominent type II pneumocyte hyperplasia (arrows) and focal alveolar epithelial degeneration. A = small segmental arteriole. B = terminal bronchiole. ×250. (b) Lung slice exposed to 0.01 mM HD and cultured for 5 days. The lung slice shows complete necrosis of the endothelial lining of a segmental arteriole (A) and the epithelial lining of a small bronchiole (B). The alveolar lining epithelium is also focally degenerate (arrows). ×250. (c) Lung slice exposed to 0.01 mM HD and cultured for 5 days. The slice shows diffuse degeneration of the alveolar lining with constituent cells exhibiting nuclear pyknosis and cytoplasmic condensation. ×250. (d) Lung slice exposed to 0.1 mM HD and cultured for 2 days. Although the microscopic architecture of the slice is preserved, there is advanced degeneration of the alveolar lining epithelium (arrows). ×250. (e) Lung slice exposed to 0.1 mM HD and cultured for 2 days. High-power view of a large, peribronchial artery showing complete necrosis of the intima and lining endothelium (arrows). The muscular media of the artery appears to be intact. I = fibroelastic intima. M = muscular media. ×250.

uct by mitochondrial succinic dehydrogenase and has been well documented in monolayer tissue cultures as being very sensitive and reproducible (Mosmann, 1983; Green et al., 1984; Denizot and Lang, 1986). Preliminary work with the lung cultures indicated that the lung slices were indeed very active with respect to this enzyme and further characterization of this assay showed that the amount of enzyme product produced by the lung slices was directly related to the initial MTT substrate concentration and incubation time. On the basis of these experiments the lung slices were routinely assayed for cytotoxicity for 40 min at a substrate concentration of 75 μg/ml of MTT. These conditions allowed us to use an initial MTT substrate concentration that did not appear to be rate limiting, but which still yielded enough formazan product to be easily measured with a conveniently short incubation time.

Exposure of the lung cultures to HD resulted in a concentration-related decrease in the specific activity of the MTT dehydrogenase that expressed itself after a latent period of 2 days. When the cultures were treated on the day of isolation, only minimal decreases in MTT dehydrogenase activity were observed 24 h after exposure. By 2 days post-exposure, however, a distinct, concentration-dependent decrease in activity was observed. This concentration-response was fully expressed by 3 days with no additional changes detectable even

when measured at 28 days. The existence of a latent period prior to the expression of toxicity is a hallmark of HD toxicity in vivo, and the appearance of lesions after exposure is characterized by a latent period of variable duration dependent upon the dose (Papirmeister et al., 1991; Smith and Dunn, 1991; Wormser, 1991). Studies in our laboratory using lewisite, an arsenical vesicant, suggested that the latent period found after HD treatment in vitro was not an artifact of the lung organ culture model. Lewisite produces pain and injury soon after exposure in vivo (Smith and Dunn, 1991) and a parallel to this was found in vitro, where evidence of cytotoxicity was detected within hours of lewisite treatment (unpublished observations). No differences were detected in the toxicity of HD to the lung cultures whether the explants were exposed to HD on the day of explantation for 1 h (LC_{50} = 81 ± 6 μM, n = 3) or for 3 h (LC_{50} = 86 ± 10 μM, n = 3). This data is consistent with the short half-life of HD in aqueous solution (6 min at room temperature, Meier and Johnson, 1992).

The development of additional sensitivity to the toxic effects of HD by the lung slices with time in culture is unusual. Typically, primary cultures are most susceptible to toxic insult at the time of culture, presumably due to the physical trauma of isolation. However, the increased sensitivity does not appear to reflect a decrease in the health of the cultures. No parallel histopathology was observed during the first few days in culture when these changes were occurring and no further changes in sensitivity were observed up to five weeks in culture. Although the reasons for this phenomenon are unclear, it is possible that a decline in the phase II conjugating enzyme or glutathione levels may be responsible for the increase in HD toxicity in older cultures. Alternatively, a particularly susceptible cell type may be selected for in culture, although these explanations seem unlikely since this increase in sensitivity is essentially complete by 7 days in culture. Treatment of the lung cultures on the day of isolation should circumvent any problems potentially associated with this phenomena, especially since the event that leads to the primary lesion occurs within the first hour of HD exposure. Histological examination of the cultured lung slices showed that hyperplasia of type II pneumocytes was a focal occurrence in control (isopropanol-exposed) lung slices as well as being a far more obvious, diffuse and sometimes florid feature of HD exposed slices. Hyperplasia of type II cells is a recognized feature of non-exposed, lung organ cultures (Davis, 1967; Adamson and Bowden, 1975; Stoner et al., 1978; Bhatnagar et al., 1979; Placke and Fisher, 1987) and in the most recent studies has been shown to be due to an acceleration of the normal alveolar epithelial cell turnover rate associated with increased protein and DNA levels (Bowden, 1983; Placke and Fisher, 1987). The appearance of this lesion as a severe and diffuse feature in those slices exposed to HD is a reflection of real damage inflicted by the agent on type I pneumocytes and is a recognized histological finding in the reparative phase of lung damage induced by a variety of agents that target the alveolar surface (Bowden and Adamson, 1971; Adamson and Bowden, 1974). In the highest two exposure groups, it was noted that the small type I cells were becoming pyknotic and desquamating into the alveolar lumen before the type II cells showed any evidence of hyperplasia.

All three test groups also showed the presence towards the periphery of the slice, of small areas of dystrophic calcification. This is a well recognized feature in tissues which have undergone necrosis. The pathogenesis of the process leading to the deposition of calcium salts is only beginning to be unraveled (Anderson, 1983; Shoen et al., 1988), but is thought to involve local changes in tissue pH (calcium is more readily deposited at alkaline pH), the action of local enzymes in generating negatively charged moieties on the surface of denatured proteins and the formation of a nidus for calcium salt precipitation by the breakdown of cells and their connective tissue matrix.

The histological evaluation of the lung slices showed that their integrity in culture remained relatively high even after 21 days and that, on morphological grounds at least, the model represents an ideal way of studying both the biochemical and structural changes in lung tissue following challenge with a number of possible lung-damaging agents. The overall pattern of pathology observed as a consequence of exposure to a range

of HD doses was also consistent with the changes reported previously in both experimental animals and man. The pattern is one of diffuse alveolar damage with oedema and haemorrhage (not present in this current study for obvious reasons) and air-way epithelial damage. It is interesting to note that at 21 days in all exposure groups there was evidence of epithelial regeneration; this feature has been noted in previous studies (Placke and Fisher, 1987), and implies that the model is a dynamic one capable of showing a wide range of responses to chemical-induced damage.

In summary, this tissue culture system appears to be a valuable tool with which to study, not only the toxicity of sulphur mustard, but also its mechanism of action. Histopathological evaluation of HD-treated lung slices showed that lesions were produced in vitro similar to those found in animals exposed to this CW agent in vivo. In addition, the cytotoxicity of this compound was detected only after a latent period of approximately 2 days. The preservation in vitro of this unique characteristic of HD toxicity indicates that the effect of HD on this model may well parallel the toxic mechanism of action of HD in vivo.

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