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A GERM-FREE STATUS DOES NOT PROTECT FROM THE LETHAL EFFECTS OF ACUTE LUNG DAMAGE CAUSED BY *O,S,S,*-TRIMETHYL PHOSPHORODITHIOATE

(Germ-free rats; lung toxicity; trialkyl phosphorothioates; pesticides)

B. NEMERY[a]*, D.K. TUCKER[b] and S. SPARROW[a]

[a]*Toxicology Unit and* [b]*Experimental Embryology and Teratology Unit, Medical Research Council Laboratories, Carshalton, Surrey, SM5 4EF (U.K.)*

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SUMMARY

To investigate whether a normal resident microbiological flora of conventional rats influences the lethality of chemical-induced lung damage, the pneumotoxin *O,S,S*-trimethyl phosphorodithioate (OSSMe, 75 or 100 mg/kg, s.c.) was administered to age-matched conventional and germ-free male F344 rats. Microbiological and serological examinations confirmed the germ-free state of the germ-free rats and showed that no specific lung pathogens were present in the conventional rats. As in conventional rats, clinical symptoms and death of OSSMe-treated germ-free rats resulted from respiratory failure. The germ-free rats were not more resistant, but rather more susceptible to OSSMe than conventional rats. Increases in lung weight and histological examination of lung tissue 3 days after dosing with OSSMe (75 mg/kg, s.c.) showed no differences between germ-free and conventional rats. Despite alterations in their nasopharyngeal flora, death in the conventional rats was probably not caused by bacterial superinfection. The higher susceptibility of germ-free rats to OSSMe can be partly attributed to pharmacokinetic differences, since plasma levels of OSSMe decreased more slowly in germ-free than in conventional rats. It is concluded that germ-free rats are not protected from the lethal consequences of acute chemical-induced lung damage.

INTRODUCTION

Bronchopulmonary infection is probably the single greatest threat to the patient in acute respiratory failure. The occurrence of such infections reduces the likelihood

*To whom correspondence and reprint requests should be sent at: Industrial Toxicology and Occupational Medicine, U.C. Louvain, Clos Chapelle-aux-Champs 30–54, 1200 Brussels (Belgium).

Abbreviations: ARDS, adult respiratory distress syndrome; OSSMe, *O,S,S*-trimethyl phosphorodithioate.

of survival and increases morbidity [1–3]. In patients with ARDS pulmonary sepsis probably results from an increased susceptibility of their upper respiratory tract to bacterial colonization, mainly by Gram-negative bacilli from the hospital environment or from the endogenous intestinal flora [1].

Several animal models have been developed to study ARDS. Recently, Campbell et al. [4] demonstrated the devastating effects of nosocomial superinfection with Gram-negative bacteria on the outcome of oleic acid-induced lung injury in anaesthetised, intubated and multi-catheterized baboons. It is not known whether prognostically significant superinfection occurs when pneumotoxins are given to intact animals. This question is particularly relevant to those compounds that lead to respiratory insufficiency and death after a few days of illness, with general debilitation and weight loss, a situation shown to be associated with diminished antibacterial defence [5].

OSSMe is a potential impurity in several organophosphorus insecticides [6] which, mainly in the rat, causes respiratory distress and death 3 to 5 days after a single oral or parenteral administration [7,8]. Morphologically, the lung lesion consists of early injury to the alveolar type I cells, resulting in pulmonary oedema and inflammation, and subsequent proliferation of type II cells [9]. It has been suggested that delayed deaths following treatment with an analogous compound, *O,O,S*-trimethyl phosphorothioate, were due to or associated with bacterial bronchopneumonia [10]. To investigate the possibility that the relation between dose and mortality was dependent on the occurrence of bacterial or viral superinfection, OSSMe was given to germ-free rats, i.e., animals free from any known living organism [11].

ANIMALS, MATERIALS AND METHODS

Inbred male F344 rats of 7 to 12 weeks (165 to 250 g body weight) were used.

Microbiological examinations were carried out at the Pathology Laboratory of the Medical Research Council, Carshalton, according to standard techniques and recommendations of the (former) Accreditation Microbiological Advisory Committee [12].

Germ-free rats were reared in isolators according to standard procedures [13] and with regular sterility checks [14]. The latter consisted of weekly screening of faecal samples (direct microscopical examination by Wayson's vital stain and cultures in media and incubation conditions appropriate for aerobic and anaerobic bacteria, mycoplasma and fungi), and less frequent screening of whole animals (including serological tests for the detection of viral contamination).

Conventional rats were reared in unbarriered animal facilities. Routine microbiological screening of this conventional F344 colony revealed a low positive antibody titre against Sendai virus in the first batch of rats used (preliminary experi-

ment), and the presence of *Staphylococcus aureus* in the nasopharyngeal flora at the end of the study. Otherwise the tests were negative for other bacterial pathogens, viruses (including sialodacryoadenitis virus, rat coronavirus, reovirus III, and Kilham virus), fungi, mycoplasma, and parasites (except *Syphacia oblevata*).

Two main experiments were conducted; experiment 1 involved 12 conventional and 12 germ-free rats and experiment 2 involved 8 conventional and 8 germ-free rats. In both experiments, the conventional rats (born in the same week as the germ-free rats) had been placed, 1 week before dosing, in an isolator in the germ-free unit and treated in the same way as germ-free rats (sterilised food and water, vitamin K supplementation, peracetic acid exposure, etc.).

OSSMe (synthetised by Dr. P. Farmer, MRC Toxicology Unit, and 99.7% pure as assessed by gas chromatography/mass spectrometry) was dissolved in saline (75 to 100 mg/ml), sterilised by γ-irradiation and kept at 4°C for a maximum of 2 weeks until used. Sterilisation did not affect the purity of the compound.

Animals were dosed subcutaneously in the nape of the neck; control animals were left untreated. The germ-free rats were weighed with a spring balance within the isolator, but for convenience the conventional rats were weighed and dosed outside their isolator.

For the microbiological investigations blood for serological studies was drawn by transthoracic cardiac puncture after CO_2 euthanasia and swabs from the nasopharynx, lung sections (conventional rats only), and caecal contents (germ-free rats only) were cultured.

For the histological studies, rats were anaesthetised with ether, their abdominal vessels were transected, the thoracic organs removed en bloc, the left bronchus clamped and the left lung dissected for estimating lung wet and dry weights (the left lung consistently represents 35% of the total lung weight, and was dried 48 h at 108°C). Phosphate-buffered formalin (pH 7.2) was slowly instilled into the trachea with a syringe until the right lung lobes appeared fully distended. After fixation and cutting, the tissue was embedded in paraffin and 5-μm sections were stained with haematoxylin and eosin.

RESULTS

Initially the lethality of OSSMe was assessed when given subcutaneously to conventional male F344 rats, kept in a normal animal room. This was necessary because LD_{50} values for rats of this sex and strain and for this route of administration were not available [8]. Four groups of 4 rats each were given 25.0, 39.6, 62.7 or 99.4 mg/kg. The resulting lethality figures were 0/4, 1/4, 1/4, and 3/4, respectively, yielding an estimated LD_{50} of 74 mg/kg (95% confidence limits: 44 and 103 mg/kg) as calculated by probit analysis.

In experiment 1 the clinical evolution and lethality following OSSMe were com-

pared between germ-free and conventional rats. In each category 4 rats served as controls, 4 rats received 75 mg/kg and 4 rats received 150 mg/kg. All treated rats showed transient mild to severe signs of cholinergic poisoning as previously described [8]. They lost weight and on the second day after dosing began to show signs of laboured breathing. In the conventional animals, only 3 out of 4 rats from the 150 mg/kg group died (60–72 h, 72–80 h and 84–96 h after dosing). In contrast all treated germ-free rats died (1 around 48 h and 3 between 60 and 72 h after dosing in the 150 mg/kg group; all 4 between 72 and 80 h after dosing in the 75 mg/kg group). The lungs of these dead rats, which were only removed from the isolator

TABLE I

EFFECTS OF *O,S,S*-TRIMETHYL PHOSPHORODITHIOATE (75 mg/kg, s.c.) ON BODY WEIGHT AND LUNG WEIGHT IN GERM-FREE AND CONVENTIONAL RATS

	Body weight (g)				Mode and time of death	Wet lung weight (mg)	Wet/dry lung weight	
	day 0	day 1	day 2	day 3				
Germ-free rats (69 days old)								
Treated								
1	260	230	220	216	died 52–68 h	2585	4.99	microbiology[a]
2	255	225	210	211	died 70–72 h	2858	5.78	
3	250	230	215	205	died 72–74 h	1931	6.06	lung histology
4	210	195	195	176	ether 74 h	1380	5.51	lung histology
5	240	220	200	196	died 72–74 h	1786	5.50	lung histology
m ± sd	243 ± 20	220 ± 15	208 ± 10	201 ± 16				
Control								
6	245	240	235	244	ether 74 h	883	4.98	
7	205	220	220	223	ether 74 h	746	4.85	lung histology
8	230	245	225	235	CO_2 74 h	827	4.71	microbiology[a]
m ± sd	227 ± 20	235 ± 13	227 ± 8	234 ± 11		819 ± 69	4.85 ± 0.13	
Conventional rats (65 days old)								
Treated								
11	210	192	174	161	ether 74 h	1600	5.29	lung histology
12	212	195	180	167	died 70–72 h	2260	5.13	microbiology[b]
13	230	208	193	178	died 70–72 h	3481	5.85	
14	205	187	172	160	ether 70 h	1574	5.25	lung histology
15	224	204	184	175	CO_2 74 h	1737	5.27	microbiology[b]
m ± sd	216 ± 10	197 ± 9	181 ± 8	168 ± 8				
Control								
16	203	204	206	209	ether 74 h	811	4.84	lung histology
17	205	226	228	229	CO_2 74 h	976	4.91	microbiology[b]
18	200	202	205	205	ether 74 h	855	4.77	
m ± sd	209 ± 14	211 ± 13	213 ± 13	214 ± 13		881 ± 85	4.84 ± 0.07	

[a] Nasopharynx + caecal contents.

[b] Nasopharynx + lungs.

and autopsied 96 h after dosing, were all massively enlarged and showed signs of haemorrhage and oedema. It was concluded from this experiment that the cause of death in the germ-free animals was probably respiratory failure, and that these animals were not more resistant to chemical-induced lung damage than conventional rats.

In experiment 2, 5 conventional rats and 5 germ-free rats were given 75 mg/kg OSSMe with 3 rats serving as controls in each category. Three days after dosing the lungs from all animals were weighed. In some of these animals histological and microbiological examination was also performed (Table I). All treated animals had lost weight and their lungs were enlarged and oedematous. It is apparent from the table that the rats that had died had heavier lungs than those that were killed. This reflects the severity of the lung damage and/or the effects of agonal and post-mortem changes [15,16].

The bacteriological and serological studies of the germ-free rats confirmed their germ-free status. In the conventional rats the nasopharyngeal swabs gave positive cultures of *S. aureus*, β-haemolytic *Streptococcus* spp. and *Haemophilus parainfluenzae* in all 3 rats examined; in the 2 treated rats *Escherichia coli* was also found in the nasopharynx. The lungs of the control rat and the surviving treated rat (No. 15) were free of bacteria; however, the rat that had died several hours before the lung samples were taken (No. 12) had the same bacteria growing from the lungs as in the nasopharynx, except for *S. aureus*. The histological appearance of the lungs of the control rats was normal in conventional and germ-free rats (Fig. 1, A and B), but there were somewhat more alveolar macrophages and larger areas of 'bronchus-associated lymphoid tissue' in the conventional rat's lung. In the treated animals there was alveolitis with mixed interstitial cellular infiltrate (mainly macrophages and neutrophils), oedema and type 2 cell proliferation as well as bronchiolar epithelial cell proliferation. These changes were qualitatively similar in both categories (Fig. 1, C and D).

DISCUSSION

To our knowledge, no studies of chemical-induced lung damage have been performed on germ-free animals [17], except for a few studies on oxygen toxicity [18] and inhaled pollutants [13]. Germ-free animals are mainly used in studies of nutrition, immunology and host–parasite relations, radiobiology and metabolism of foreign and endogenous compounds by the gut microflora [11,13,17]. The respiratory system of germ-free animals has not received much attention. Germ-free rats have smaller lungs on a body weight basis than conventional rats [19] and this was also found in the present study. Histologically, the lungs from germ-free animals have fewer alveolar macrophages and lymphoid aggregates [20,21]. Functionally, the alveolar macrophages from germ-free rats have been shown to be less

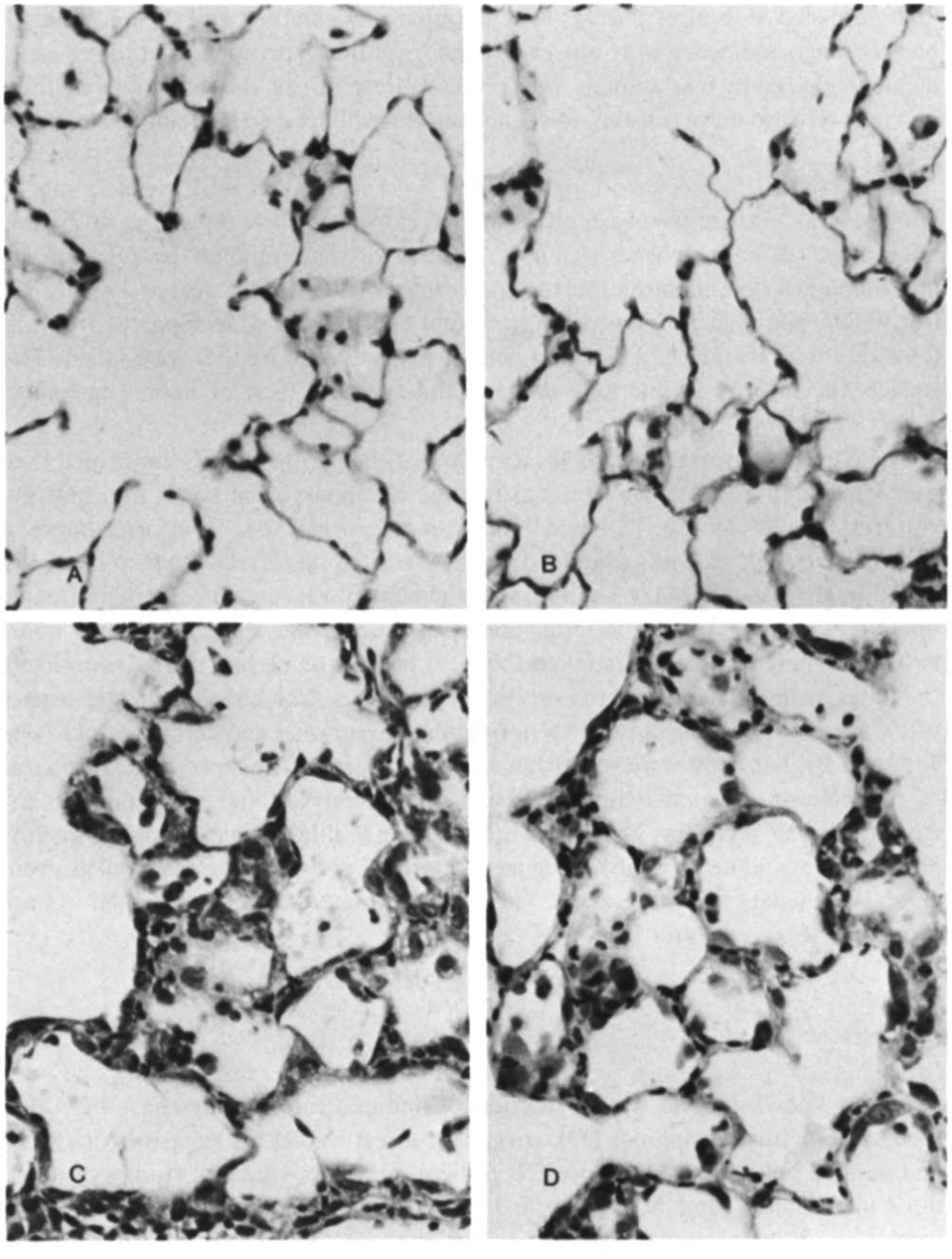

Fig. 1. Histological appearance of lung parenchyma from an untreated conventional rat (A), an untreated germ-free rat (B), a conventional rat (C) and a germ-free rat (D) 3 days after OSSMe (75 mg/kg, s.c.). The thickening of the alveolar walls with inflammatory cells and the increase in alveolar macrophages were similar in conventional and germ-free rats (haematoxylin and eosin-stained 5-μm paraffin section, × 200).

competent in terms of phagocytosis, chemotactic responsiveness, lysosomal activity, and cytotoxicity [20,22]. It was therefore conceivable that the inflammatory response to lung cell damage might differ between germ-free and conventional animals. This could in turn influence the prognosis of such damage, regardless of the occurrence of superinfection. It is indeed believed that inflammatory cells, particularly polymorphonuclear leukocytes and macrophages, play an essential role in the development of adult respiratory distress syndrome [23].

This study does not support the hypothesis of Hammond et al. [10] that delayed deaths following trialkyl phosphorothioate administration are related to bacterial bronchopneumonia. More generally, this study has shown that severe acute chemical-induced lung damage was no less lethal in germ-free rats than in conventional rats. As a corollary, it may be concluded that in conventional, but 'reasonably clean' rats the lethality of chemical-induced lung injury is not markedly affected by their resident nasopharyngeal or enteral flora. The end-result of the administration of OSSMe was indeed comparable in germ-free and conventional rats, despite the occurrence of changes in anti-bacterial defences, as evidenced by the presence of *E. coli* in the nasopharyngeal swabs of the treated conventional rats, and as suggested by the findings of a decreased immune function following this type of organophosphorus compound [24].

However, the absence of a significant effect of the normal flora on the lethality of the pneumotoxin OSSMe does not mean that bacterial superinfection, particularly by nosocomial bacteria, is of no consequence for the prognosis of acute lung damage. The devastating effects of such superinfection have been well demonstrated in both clinical [1–3] and experimental cases of respiratory distress syndrome [4]. Also, concomitant infection of rats by more specific respiratory viruses (e.g. Sendai virus) or pneumotropic pathogens such as mycoplasma or *Pasteurella pneumotropica* would presumably act synergistically with pneumotoxins. However, establishing this was not the purpose of the present study.

The present study also suggests that the inflammatory cell infiltration following lung damage is not primarily dependent on past infectious processes. This was also concluded by Wright et al. [18] who showed that germ-free rats and mice were more uniformly susceptible to the effects of pure oxygen than conventional animals.

Although more precise figures for comparisons of lethality would have required larger numbers of animals, which was neither feasible, nor desirable for our purpose, our results suggest that the germ-free rats were in fact more susceptible to OSSMe than the conventional rats. This may in part be due to pharmacokinetic differences between the two groups. Indeed, germ-free rats were found to have a slower rate of disposal of OSSMe than conventional rats (Fig. 2). The lungs of germ-free rats are therefore exposed to a higher integrated dose of OSSMe (germ-free rats: 885 $nmol.ml^{-1}.h$; conventional rats: 667 $nmol.ml^{-1}.h$). The reason for this slower clearance of OSSMe may simply be due to the smaller liver of germ-free rats compared to conventional rats [19], since we did not find in other experiments any

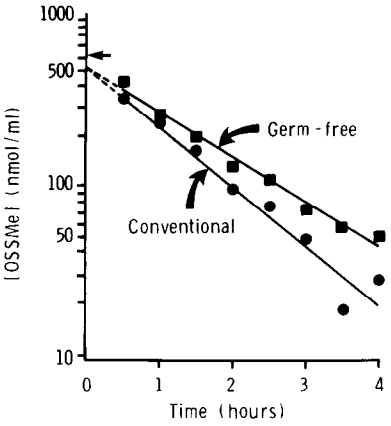

Fig. 2. Plasma and lung concentration of *O,S,S*-trimethyl phosphorodithioate (OSSMe) in germ-free and conventional rats. Eight conventional rats (80–84 days old, 230 ± 13 g) and 8 germ-free rats (83 days old, 258 ± 15 g) were given OSSMe, 75 mg/kg s.c., and killed at half-hourly intervals. OSSMe was determined in the plasma and lung homogenate as described by Aldridge et al. [25]. Values in plasma and lung were similar and coincide on the graph. The first-order rate constant of disposal of OSSMe determined from the 16 data points during the 4 h is 0.81 ± 0.06 h^{-1} in conventional and 0.61 ± 0.02 h^{-1} in germ-free rats.

significant differences between the in vitro metabolism of OSSMe by lung and liver slices or microsomes from germ-free and conventional rats (unpublished). Although liver microsomal fractions from germ-free and conventional animals metabolise steroids at different rates [26], the rate of metabolism of some foreign compounds is the same [27]. Surprisingly, this area has not been investigated very extensively.

It is concluded that germ-free rats are not protected from the lethal consequences of acute chemical-induced lung damage. Other experiments are required to investigate whether the reparative processes and long-term consequences, such as fibrosis, are also unaffected by a germ-free status.

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