IMMUNOLOGICAL STUDIES OF EXPERIMENTAL COALWORKERS' PNEUMOCONIOSIS*

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Abstract—A comparative immunological and microbiological study of experimental coalworkers' pneumoconiosis (CWP) was made in rats and mice subjected to long-term exposures of coal-mine dust aerosols. Such aerosols were realistically prepared at a concentration equal to the maximal level of respirable dust permitted by Federal standards and animals were exposed for lengths of time equal to human work contact. Among the factors studied were the production of IgA and lung reactive antibody, lung microflora and changes in pulmonary clearance. Additional experiments were concerned with the effects of passively administered lung antibody on the pulmonary clearance.

It was found that both species responded immunologically in a similar manner to humans with CWP in that IgA levels were significantly elevated and lung reactive antibodies were stimulated. Coal-mine dust inhalation had little effect on the pulmonary inactivation of inhaled bacteria, but the concomitant occurrence of passively administered lung reactive antibody seemed to enhance the inactivation.

INTRODUCTION

Many histopathologic studies have been performed on various laboratory animals exposed in different ways to coal dust in attempts to determine the pathogenesis of coal-workers' pneumoconiosis (CWP), but little attention has been given to the immunological and microbiological aspects of such exposure. Moreover, many of these studies, which have been ably reviewed by ZAIDI (1969), are open to serious criticisms. Many employed intratracheal injections of aqueous suspensions of coal dust. KING *et al.* (1958) pointed out that such artificial means of dust administration do not lead to the same kind of tissue reactions seen in spontaneous disease due to dust inhalation.

Another criticism of some early studies concerns the use of the rat, an animal almost always subject to chronic respiratory disease due to *Mycoplasma pulmonis* (LINDSEY *et al.*, 1971) which produces pathogenic effects that often render the experimental results valueless. These effects due to chronic infection are often accentuated by other forms of pulmonary stress.

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Additional defects of some of the early work include exposing the animals to exaggerated concentrations of suspended dusts and using powdered coal instead of realistic dust samples obtained from a working mine which would include powdered sand from blasting or motoring operations, other mineral dusts produced as by-products of mining, organic matter other than coal, micro-organisms indigenous to these substrates and other materials. With modern air samplers, it has been possible to collect large quantities of all airborne "coal-mine dust" (CORN *et al.*, 1972) which can then be used more realistically in experimental studies.

The immunological consequences of silicosis, both spontaneous and experimental, are well known (VIGLIANI and PERNIS, 1963; ESBER and BURRELL, 1967; MILLER and ZARKOWER, 1974a). They include the production of lung reactive antibodies and rheumatoid factor, the deposition of globulin in silicotic lesions, depressed B cell function, but enhanced T cell responsiveness, and a decrease in phagocytic ability of alveolar macrophages. The present report describes a series of immunological and microbiological studies on two different species of laboratory animals chronically exposed to realistic respirable levels of coal-mine dust.

MATERIALS AND METHODS

Animals

Outbred Swiss-Webster albino male mice, weighing 20–22 g, were obtained from Carworth Laboratories, New York City, N.Y. Male CFN rats, weighing 150–175 g, were obtained from the same source. Neither species was pathogen free. All animals were housed in the animal room facilities of the Chronic Toxicology Laboratories, NIOSH, Cincinnati, Ohio. Although the experiments with each species were not done concurrently, each species was housed in separate facilities.

Coal-mine Dust

Coal-mine dust was obtained from a mine in Cambria County, Pennsylvania, known to be associated with a fairly high prevalence of coalworkers' pneumoconiosis among its employees. The dust No. 51408 was of respirable size (geometric mean 1.23 μm, $34\% < 1$ μm, $90\% < 2.4$ μm diameter) and contained 3.0–4.0% free crystalline SiO_2 and 17.8% ash. The dust was dispersed in chambers containing the animals by means of a Wright dust-feed mechanism at an average concentration approximately equal to the maximum Federal compliance level of 2.0 mg/m^3.

All test animals were exposed to this coal-mine dust 6 h/day, 5 days/week for periods ranging from 3 weeks to 12 months. Control animals were treated similarly except that their chamber had a by-pass so that they were not exposed to the dust.

Immunological Parameters

Mice and rat sera were serologically examined for antibodies to homologous lung connective tissue by the antiglobulin consumption test (HAGADORN and BURRELL,

1968). Mouse IgA was measured by commercially available radial diffusion kits (Meloy Laboratories, Springfield, Va.). Rat splenic lymphocytes were isolated and tested for lymphocytotoxin production upon stimulation with soluble lung connecfive tissue antigen by previously described methods (CATE and BURRELL, 1974).

Lung antibodies, reactive for only the collagen components of the lung, were prepared against homologous lung connective tissue by immunizing mice in a way to produce ascitic fluid (BURRELL *et al.*, 1974). Ascitic fluids containing at least six units of anti-globulin consumption per 0.2 ml against lung antigen were pooled. Two dozen mice to be treated were passively immunized with three 0.05 ml i.v. injections of this pooled homologous fluid per week for the duration of the experiment. An equal sized group of control mice was similarly injected with non-reactive ascitic fluid.

Microbiological Parameters

Following killing by cervical separation, mouse lungs were lavaged with 0.6 ml sterile saline and this fluid was then cultured aerobically for bacteria, fungi and mycoplasma. Mycoplasma were cultured on media specially formulated for murine strains (KOHN and KIRK, 1969) as certain media formulated for human strains may not support growth of *M. pulmonis*.

Media used for culturing fungi and mycoplasma were held for 6 weeks before calling them negative, while all others were held for 2 weeks.

Methods of Assay

Assay for the bactericidal ability of individual mouse lungs was performed according to the method of GREEN and GOLDSTEIN (1966) using aerosolized, ^{32}P radiolabelled *Staphylococcus epidermidis* as the indicator particle. These bacteria were aerosolized in a specially constructed chamber capable of exposing eight mice in individual restrainers at one time (BURRELL, 1970). Since only the snouts of the animals are exposed to aerosol and the mice may be left in the restrainers after exposure when using this device, error due to entry into the mice of radiolabel from grooming activity of contaminated fur is minimized. Approximately 3.0 ml of the radiolabelled bacterial suspension was aerosolized over a 30-min period using a no. 40 DeVilbiss glass nebulizer driven by a pump at 3 psi.

Half of the animals were removed immediately following this exposure for analysis and the remainder at 2 or 4 h afterwards. The remainder of the assay and the calculations were performed as in the Green–Goldstein method.

RESULTS

CWP in Rats

In the first experiment, 200 rats were divided into a test group of 132 animals that were exposed to the coal-mine dust regimen and the remainder served as the control group. After every 3 months, animals were removed at random from each group for

bleeding and killed for pathological examination. All bleedings were tested for the occurrence of lung antibodies. A summary of the serological findings may be found in Table 1. No evidence of lung reactivity appeared until after 9 months of exposure, while such reactivity never did appear in the controls.

TABLE 1. THE DEVELOPMENT OF LUNG REACTIVE ANTIBODIES IN RATS EXPOSED TO COAL-MINE DUST

Category	Length of exposure (months)					
	Pre-exp.	3	6	9	12	16
Treated	0/40*	0/25	0/25	6/24	5/25	6/22
Control	0/20	0/12	0/12	0/12	0/10	0/10
					↑ Exposure terminated	

* Results are expressed as the number positive/number tested. Positive samples were any sera showing two or more units of consumption in the antiglobulin consumption test using lung connective tissue as antigen.

Histological examination of the lungs from the test animals initially revealed indications of chronic inflammatory changes of the bronchi, emphysema which became pronounced after 6 months' exposure, pleural thickening and fibrosis. Except for some perivascular and peribronchiolar accumulations of mononuclear cells, the controls failed to show similar changes. However, after 9 months of the sham treatment, the controls also began to show evidence of chronic bronchitis, focal aggregates of alveolar histiocytes, and early formation of granuloma and fibrosis, characteristics of chronic respiratory disease (LINDSEY *et al.*, 1971). *Mycoplasma pulmonis* was cultured with ease in both the treated and control animals from 9 months on. The number of isolates increased as the animals aged. Often, culture of the lungs from diseased animals failed to develop characteristic mycoplasma, but cultures from the obviously caseated middle ear, the organ most affected by this infection in rats, invariably were positive.

The exposure was terminated after 12 months, but immunological and pathological monitoring continued. Sixteen months after initiation of the experiment, the spleens from 14 treated rats, 4 control rats and 7 new, young rats purchased from the same source were evaluated for the presence of lymphocytes reactive with soluble lung connective tissue antigen by means of lymphocytotoxin assays. None of the lymphocytes cultured from either control group demonstrated antigen induced lymphocytotoxin, but 8 of the 14 spleens from rats exposed to the coal-mine dust contained lymphocytes producing sufficient lymphocytotoxin upon antigen stimulation to cause 20% or more cytopathogenic effects on monolayers of rat embryo fibroblast target cell cultures.

CWP in Mice

Since it was impossible to differentiate between coal-mine dust and mycoplasma-induced pathology in rats, the experiment was repeated using mice as the experimental

animal, a species which does not exhibit the extensive pathology due to chronic *Mycoplasma* infection. In this experiment, more attention was paid to the infectious aspects of the disease. The mice were divided into a group of 120 that were exposed to the coal-mine dust regimen and 60 controls that received the sham treatment. Bleedings and pulmonary lavages were performed at monthly intervals. The results of the lung reactive antibody studies are given in Table 2. Although the number of

TABLE 2. THE DEVELOPMENT OF LUNG REACTIVE ANTIBODIES IN MICE EXPOSED TO COAL-MINE DUST

Category	Length of exposure (months)				
	Pre-exp.	3	4	5	6
Treated	0/5*	1/6	1/4	2/8	4/7
Control		0/2	0/2	0/4	1/5

* Results are expressed as the number positive/number tested. Positive samples were any sera showing two or more units of consumption in the antiglobulin consumption test using mouse lung connective tissue as antigen.

samples tested was admittedly small because of the limitations in serum sample size, the same general trend in serologic reactivity seen in the treated rats prevailed. Most of the mouse serum samples were used to measure serum IgA to determine if mice reacted in a manner similar to humans (HAGADORN and BURRELL, 1968).

The results, shown in Fig. 1, show that there occurred an increase in IgA in the

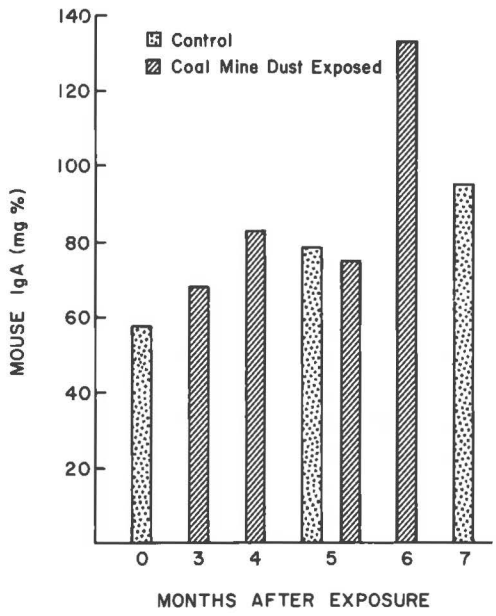

FIG. 1.—Comparison of serum IgA levels between mice exposed to coal-mine dust and sham treated controls.

group exposed for 6 months to the coal-mine dust when compared to the control value even though the latter value was obtained 1 month later.

Lavages from six control and twelve treated mice were cultured at 3, 4, 5 and 6 months after beginning exposure. Of the controls cultured, only one 3-month animal and two each from each of the other sampling periods yielded bacterial growth. *Staphylococcus aureus* was the usual isolate, but there was no pattern among the other isolates. At least three to six mice of each treated sample yielded positive cultures and although there again was no discernible pattern, *Neisseria* spp., alpha haemolytic streptococci, and *Streptomyces* spp. predominated. At no time were fungi or *Mycoplasma* recovered from the pulmonary lavages.

Of greater value was the number of bacterial colonies isolated per 0.1 ml sample of each lavage. Table 3 shows a comparison of the counts of each isolate obtained from

TABLE 3. NUMBER OF MICROORGANISMS PER LUNGS OF COAL-DUST-TREATED MICE COMPARED WITH CONTROLS

Months of exposure	No. of animals cultured	Category	No. of animals showing quantities of bacteria/0.1 ml lavage		
			1–100	10^2–10^3	10^3–10^4
3	12	Treated	2	1	0
	6	Control	0	1	0
4	12	Treated	5	0	1
	6	Control	0	1	0
5	12	Treated	3	3	0
	6	Control	2	0	0
6	12	Treated	4	0	0
	6	Control	1	1	0

the two groups. Although more coal-mine-dust-treated mice yielded quantifiable cultures, the data do not begin to reflect the entire picture. During the last 2 months of exposure, the mortality among the exposed mice (51.7%) was much greater than the controls (8.3%) and it can be assumed that most of the deaths were due to severe pulmonary infections. Since the laboratory studies were being conducted in one location (Morgantown, WV) while the animals were housed and exposed elsewhere (Cincinnati, OH), it was not possible to culture the dead or dying mice.

Effects of Coal-mine Dust on Pulmonary Inactivation of Inhaled Bacteria

A second group of eighty new mice was divided into equal control and test groups for the third experiment. These mice were assayed at 1, 2 and 3 months of exposure for

pulmonary inactivation of inhaled, radiolabelled bacteria. A comparison of clearances between treated and control mice tested at each sample period is presented in Table 4.

TABLE 4. EFFECT OF COAL-MINE DUST ON PULMONARY INACTIVATION OF INHALED BACTERIA

Months of treatment	N=	Category	% Bacteria inactivated (4 h after aerosolization)	S.D.*	Average number of bacteria inhaled/mouse
1	10	Treated	98.24	1.23	1.5×10^5
	3	Control	99.09	0.23	1.7×10^5
2	10	Treated	95.56	3.86	2.9×10^4
	5	Control	96.14	2.34	2.6×10^4
3	9	Treated	94.94	3.46	2.9×10^3
	5	Control	96.66	2.85	3.1×10^3

* S.D.—standard deviation.

The percentage of bacteria inactivated 4 h after aerosolization was very uniform, however, no significant differences were seen between the two groups of mice at each sample period. There was a tendency for a greater disparity between control and treated mice as exposure lengthened. The method of assay had the further advantage of allowing the quantity of bacteria inhaled to be measured in each group and such observation indicated that both groups inhaled the same amount of labelled bacteria throughout the exposure period. Although there was a logarithmic decrease in number at each sample period, there was no difference between each group. The decrease noted was due to technical differences in sample preparation.

Effect of Lung Antibody on Pulmonary Inactivation of Inhaled Bacteria

One final experiment was conducted to determine the effect of lung antibody seen in spontaneous CWP on the pulmonary inactivation process. This was accomplished by passively immunizing normal mice with highly reactive lung antibody fluids for 45 days, during which time sample treated and untreated groups of mice were removed for pulmonary inactivation assays. The comparison of percentage inactivation and numbers of bacteria inhaled are shown in Table 5. The results show that after 31 days of passive immunization treatment, the treated mice killed a greater percentage of bacteria than the controls. The difference was about one standard deviation and although the *t*-test for independent samples shows this not to be a significant difference, it was borderline at the 95% confidence level. Of further note was that the control mice inhaled more bacteria throughout the course of the experiment and this difference was statistically significant by the 45th day at the 95% confidence level.

TABSE 5. EFFECT OF LUNG ANTIBODY ON PULMONARY INACTIVATION

Days of treatment	N=	Category	% Bacteria inactivated (2 h after aerosolization)	S.D.[a]	Average number of bacteria inhaled per mouse
17	2	Treated	98.91	N.D.[b]	1.9×10^{3}[c]
	2	Control	98.22	N.D.	2.0×10^{3}[c]
31	5	Treated	94.45	2.87	5.1×10^{4}[d]
	5	Control	90.86	3.99	6.0×10^{4}[d]
45	5	Treated	94.37	2.70	1.0×10^{5}[d]
	5	Control	91.89	3.45	1.3×10^{5}[d]

[a] S.D.—standard deviation.
[b] N.D.—Not done.
[c] Average of counts from four animals.
[d] Average of counts from eight animals.

COMMENT

Although the rat has been a popular laboratory animal for experimental pathologists in the study of silicosis and other pneumoconioses, it has become increasingly clear that it is a poor choice for pulmonary pathology studies because of the almost universal prevalence of chronic *Mycoplasma* infections. Unless investigators employ pathogen-free rats, maintain strict quarantine during the experiments, and carry out a rigid microbiological monitoring programme, their results will be inconclusive. The rat is also a poor choice immunologically since rat IgA exists in such small amounts that it cannot be technically isolated or quantitated readily (BISTANY and TOMASI, 1970). The disadvantages of rats aside, the coal-mine-dust-treated rats did produce lung-reactive antibodies and some evidence of cell-mediated immunity to appropriate antigens that were not found in the controls. In these respects, rats have responded immunologically in a similar manner to coal miners.

The mice proved to be a better immunological and microbiological model of CWP up to a point. They produced lung antibody and exhibited rises in IgA, as do humans, if exposed long enough. However, exposed mice displayed a severe mortality at about the same time these immunological findings became apparent. Since an occasional living mouse sampled at this time exhibited a severe pneumonia, it is assumed that this was responsible for the mortality.

The study was designed primarily to examine the microbiological and immunological aspects of CWP in mice, and although histopathologic studies were not performed on the mice, gross examination of their lungs at time of sacrifice revealed well-developed coal macules on the pleura of most of the exposed mice.

GOLDSTEIN *et al.* (1969) studied the effect of intratracheal injections of aqueous silica suspensions on the pulmonary inactivation of aerosolized bacteria. Although

this treatment produced extraordinarily severe silicosis, it failed to alter the bactericidal activity of the lungs. Since the primary defence against inhaled organisms is by alveolar macrophages (GREEN and KASS, 1964) and since it is well known that silica is extremely toxic to such cells (KESSEL *et al.*, 1963), one might have expected a marked decrease in bacterial inactivation. The authors concluded that their results indicated that these defences can incur severe anatomic injury without functional derangement.

In the present experiments, natural exposure to realistic levels of coal-mine dust exhibited surprisingly little effect on bacterial inactivation by the lungs in view of the high mortality seen after 6 months' exposure. Since the inactivation studies only extended for 3 months of exposure, it is quite possible that an indication of functional impairment might have been obtained if such observations could have been made on animals exposed to the coal-mine dust for longer periods. As with humans who must work many years underground before showing evidence of CWP (MORGAN *et al.*, 1973), animals must be subjected to long periods of exposure to coal-mine dust inhalation before developing external evidence of disease.

ZARKOWER and MORGES (1972) have shown that carbon dust alone when administered by inhalation brought about measurable depressions of certain humoral immune responses. The methods used demonstrated almost certainly only IgM responses whereas in CWP the major immunoglobulin class stimulated appears to be IgA (HAGADORN and BURRELL, 1968). In further studies, MILLER and ZARKOWER (1974b) were able to show that carbon dust inhalation enhanced T cell mediated responses systemically, but depressed them in the mediastinal nodes. Additional experiments with silica dust inhalation (MILLER and ZARKOWER, 1974a) concluded with similar results. Thus, although these dusts do not seem to alter *in-vivo* phagocytosis, they conceivably could have an effect on other cells of the afferent limb of the immune response.

Since it is known that patients with CWP produce lung antibodies (BURRELL *et al.*, 1964), especially if their disease is of the complicated types (unpublished observations) and since a variety of data indicates these antibodies to be pathogenic (BURRELL *et al.*, 1974), an experiment was conducted to determine if the presence of these antibodies over an extended period of time had any effect on the bacterial inactivation of the lungs. Although statistical analysis suggested that such antibodies did enhance bacterial inactivation, it was not dramatic. Our methods employed the more easily phagocytized *S. epidermidis*, but had we used the more phagocytic resistant *S. aureus*, the disparity might have been greater. Also, if observations could have been extended beyond 45 days, the difference between treated and control animals might have been even more apparent. Previous studies showed a similar enhancement of clearance of bacteria from the internal circulation (BURRELL *et al.*, 1974).

Perhaps of more importance was the observation that lung antibody-treated mice inhaled significantly smaller amounts of aerosolized bacteria, suggesting that the ventilatory patterns of the treated mice had been changed. Tachypnoea and shallow breathing are associated with fewer bacteria being deposited in more proximal regions of the bronchial tree (G. M. Green, personal communication). It appears that the action of the antibody may have exerted its effects by this mechanism.

Thus, the combined effects of inhaled coal-mine dust and the production and presence of lung antibodies over extended periods of time may lead to anatomic and

functional changes in the lung that allow more micro-organisms to be deposited, both from the internal and external environments. Chronic irritation by such micro-organisms might in certain individuals be an important factor in developing complicated types of pneumoconiosis.

As with rats and mice, natural human CWP disease is most likely complicated by infection as well as other environmental stresses. Although it might be argued that there is no human parallel to the *Mycoplasma*-induced chronic respiratory disease, we feel compelled to offer the reminder that rats do not smoke. With individual species differences aside, it is also worth noting that rodents chronically exposed to levels equal to the current American compliance levels of respirable dust concentrations still produce evidence of disease and tissue alteration not seen in aged matched, sham treated controls.

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DISCUSSION

H. SMITH: I would agree that the rat is not a suitable animal because of the common occurrence of CRD. Therefore would you suggest a more suitable species?

Dr BURRELL: Dr Weller yesterday presented an excellent paper on work using the monkey. Although it is very expensive for long-term inhalational studies, you can almost do everything on a monkey that you can on a human and were I to have the proper facilities and funds that's what I would use.

J. C. WAGNER: The majority of British pneumoconiosis experiments are done with SPF animals, as a reading of the literature will show.

Dr BURRELL: The papers I have been reading do not say that specific pathogen-free rats were used. In addition, a pathological study recently published shows that even these animals are not free of *Mycoplasma* disease (LAMB, D. *Lab. Animals*, 1975, **9**, 1–8). The mouse does not show the same kinds of lesions according to Lindsey's paper that the rat does and besides, our mice were cultured for *Mycoplasma*. They were always *Mycoplasma* negative.

A. G. HEPPLESTON: How do you justify the use of the term "experimental coalworkers' pneumoconiosis" to compare with either the simple or the complicated form of the human disease in the absence of any histological evidence on the response of your rat and mouse lungs to the presence of coal dust? Subpleural black spots seen by naked eye only in the mice hardly constitute pneumoconiosis. Furthermore, the microbiological and immunological observations are open to serious question since the animals used were not specific pathogen free, and hence bore their own spontaneous microflora in their respiratory tracts to the extent that many had severe pulmonary infection.

Dr BURRELL: Well that's the point I made and why I am here today is to point out to those people who are not familiar with it, what can happen when you do not use specific pathogen-free rats. We did do histological examinations in the rats, but not in the mice. I am an immunologist and I neglected the pathology and, I thought on good grounds, because that was not my main point.

P. COLE: Even reputedly SPF mice and rats cause problems as regards *Mycoplasma* in the respiratory tract. The hamster, however, is remarkably clean with regard to this organism in the respiratory tract.

Dr BURRELL: I don't believe I can measure hamster IgA which was really my reason for using mice.

F. F. HAHN: I would agree that the lung of the Syrian hamster is very clean, but the other organs are not.

J. M. G. DAVIS: Is it really justifiable to use results from SPF animals in studies relating to disease in non-SPF human lungs? I suggest results from both SPF and non-SPF animals should be compared to determine the importance of infections in some human conditions basically caused by dust inhalation.

Dr BURRELL: Of course the scientist tries to separate things into components to find out what the individual contribution is, and that would be the justification for using SPF animals. There are microbiological factors that I didn't consider, for instance, the animals should have been kept at the temperature and humidity of the working mine because these affect the naso-pharyngeal flora, and that is just one more complicated variable. Cigarette smoking is another.

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IV

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