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An Emphysema-Like Reaction in Lungs of Adjuvant Arthritic Rats (40525)

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Introduction. Emphysema is a chronic respiratory disease in which the alveolar walls are progressively destroyed. The pathological changes may be caused by endogenous proteolytic or elastolytic enzymes which somehow escape normal control. For example, there is a relationship between chronic pulmonary emphysema and a genetic deficiency of serum α^1-antitrypsin, a circulatory protein which functions as an inhibitor of a variety of proteases (1).

Emphysema-like conditions have been induced in animals by exposure to aerosols or intratracheal injections of papain, elastase, or other noxious substances (2–7). In our own previous study chronic exposure to aerosolized solutions of papain induced emphysema-like changes in lungs of rats (8). These changes included an increase in average alveolar size and a decrease in mean linear intercept when alveolar walls were counted in histological preparations of lungs fixed *in situ* under a standard pressure. We also observed highly significant increases in biochemically determined lysosomal enzymes (β-glucuronidase and arylsulfatase) in lungs of rats which inhaled the papain aerosol.

Intradermal injection of Freund's complete adjuvant (*Mycobacterium butyricum* in heavy mineral oil) into the plantar region induces the well-known experimental disease of "adjuvant arthritis" in rats which is characterized by a progressive swelling of the fore- and hindpaws and results in destruction of cartilage and deformation of carpals, tarsals, and digits (e.g., (9)). We have now observed pathological changes in the lungs of rats treated with Freund's complete adjuvant. As these lesions superficially resembled those we had previously observed in lungs of rats exposed to papain aerosols, we decided to investigate further the lung changes occurring in adjuvant arthritic rats. In addition to a characterization of the lung pathology we have attempted to gain some insight into the specificity and mechanism(s) of its production. Thus, we have compared the lungs of rats receiving emulsions of killed *M. butyricum* with those receiving similar preparations of *Escherichia coli*, an organism which does not cause adjuvant disease. Adjuvant arthritis is an autoimmune disease. Thus, we have also investigated the possibility that an immunosuppressive agent, cyclophosphamide, might prevent development of the lung pathology as well as the arthritis. Finally, we have measured serum trypsin inhibitory capacity (TIC) to determine if the ability to neutralize proteolytic enzymes is reduced by the development of adjuvant disease. Such an effect would presumably predispose such animals to the production of obstructive pulmonary disease.

Materials and methods. In the first series of experiments, male Charles River CD rats weighing 100–130 g at the start were divided into four groups of 12 each. One group served as control and the other groups received one, two, or three subplantar injections of 0.3 mg *M. butyricum*[1] in 0.05 ml water–mineral oil emulsion on Days 0, 0 and 3, or 0, 3, and 7 of the experiment, respectively. Single injections were given in the right hindfoot. Multiple injections were alternated between the right and left hind footpads. On Day 20, all rats were killed. The lungs of 6 rats of each group were infused with formalin under standard conditions as previously described (8), for histological studies. The lungs of the remaining 6 rats of each group were weighed. A lung from each of these rats was then dried at 60° to constant weight while the other was immediately frozen at −30° for enzyme studies.

In the second experiment, similar rats were divided into groups of 12 each as follows: Group I, normal controls; Group II, *M. butyricum*, 0.3 mg/0.05 ml water–mineral oil emulsion injected into the subplantar region

[1] *M. butyricum*, Lot No. 0640-33, Difco.

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of the hindfeet as above on Days 0, 3, and 7 of the experiment; Group III, *E. coli*,[2] 0.3 mg/0.05 ml mineral oil suspension injected as in Group II; Group IV, as Group II (*M. butyricum*) plus cyclophosphamide (Cytoxan, Meade Johnson), 0.5 mg/day po in 0.5 ml 0.1% carboxymethyl cellulose for 20 days; Group V, as Group III (*E. coli*) plus cyclophosphamide as in Group IV. All rats were killed on Day 21, the lungs of 6 rats per group were fixed by formalin infusion and the lungs of the remaining rats were used for dry weight and enzyme studies as described in the first experiment. In addition, blood samples were obtained at the time of sacrifice for determination of serum trypsin inhibitory capacity (TIC) according to Ericksson (1). The volumes of the hindpaws (right and left rear) were determined by mercury displacement and averaged for each rat.

Lungs for histology were filled and fixed with 10% neutral formalin at 10 cm water pressure. Paraffin-embedded tissues were sectioned at 5 μm and stained with H & E. Alveolar counts were made at × 100 on sections of both right and left lungs using an ocular micrometer. Ten fields per lung were randomly selected and 10 counts were made per field. All alveoli touching a line 0.2 mm long were counted (mean linear intercept).

Lung β-glucuronidase was determined by the method of Gianetto and deDuve (10) after homogenizing the tissue in a 10% (w/v) Tris–acetate buffer 0.04 *M* containing 0.1% (v/v) Triton X-100, pH 7.4, as previously described (8).

Results. Experiment 1. All of the rats injected with Freund's adjuvant developed a typical polyarthritis. One, two, or three subplantar injections of *M. butyricum* induced, in proportion to the total dose, decreases in body weight gain, increases in relative (but not absolute) lung weights, and increases in lung β-glucuronidase activity (Table I). There were no significant changes in lung water contents in adjuvant arthritic rats (Table I). Alveolar counts revealed a dose-proportional decrease in alveolar septa in the adjuvant-treated rats (Fig. 1). The histological appearance of the alveoli of lungs of the polyarthritic rats was reminiscent of that induced by papain inhalation (Fig. 2). The severity of the disease, which included scarring of the alveoli, septal fibrosis, and granuloma formation, was proportional to the number of injections.

Experiment 2. Subplantar injections of *M. butyricum* but not of *E. coli* in a water–mineral oil emulsion induced typical adjuvant polyarthritic disease in rats. The paw volumes of the rats treated with *M. butyricum* were double those of control while those of *E. coli*-treated rats were similar to control (Table II). Only a moderate inflammatory response was elicited by *E. coli*. This subsided 2 days after injection. Cyclophosphamide treatment significantly reduced the paw swelling due to injection of *M. butyricum* (Table II) but did not affect paw volume in *E. coli*-treated rats.

Lung weight and β-glucuronidase activity increased significantly in *M. butyricum*-treated rats but not in *E. coli*-treated rats (Table II). This increase in lung weight and β-glucuronidase was not as great in rats which received cyclophosphamide and *M. butyricum* as in rats which received *M. butyricum* alone (Table II).

Trypsin inhibitory capacity (TIC) was significantly ($P < 0.01$) decreased in rats treated with *M. butyricum* but not in those injected with *E. coli* (Table II). The decrease in TIC was partially inhibited by cyclophosphamide ($P < 0.05$). Linear alveolar counts were significantly ($P < 0.01$) decreased by injections of *M. butyricum* but not by *E. coli* (Table II). This effect appeared to be partially counteracted by cyclophosphamide therapy (Table II). Also, cyclophosphamide reduced and, in some cases, inhibited completely the histologically observable lung granuloma formation in *M. butyricum*-treated rats.

Discussion. In addition to the granulomatous changes described by others (11, 12), the lungs of adjuvant arthritic rats exhibited histopathological features and biochemical changes characteristic of pulmonary emphysema induced by chronic inhalation of papain (8). Thus, an increase in lung β-glucuronidase activity and a decrease in linear alveolar counts were observed. It is recognized that histopathological changes cannot be construed as proof of emphysema, particularly in rats which are predisposed to such changes from a variety of causes. However, the sec-

[2] *E. coli*, stock No. EC-1, Lot 85c-6850, Sigma.

TABLE I. LUNG CHANGES IN ADJUVANT ARTHRITIC RATS (SIX/GROUP)

Group	Body wt gain (g)	Lung weight		Lung water content (%)	Lung β-glucuronidase (E at 540 nm)
		g	g/100 g		
Control	165	1.27 ± 0.03	0.46 ± 0.02	78.5 ± 0.2	0.42 ± 0.03
One inj. adjuvant[a]	106	1.34 ± 0.04	0.54 ± 0.02	78.9 ± 0.4	0.50 ± 0.02
Two inj. adjuvant[a]	99	1.33 ± 0.01	0.66 ± 0.04[b]	79.9 ± 0.5	0.53 ± 0.03[b]
Three inj. adjuvant[a]	77	1.34 ± 0.04	0.75 ± 0.03[b]	79.6 ± 0.3	0.73 ± 0.04[b]

[a] 0.3 mg *M. butyricum* in 0.05 ml water–mineral oil emulsion intradermally.
[b] $P < 0.01$ vs control.

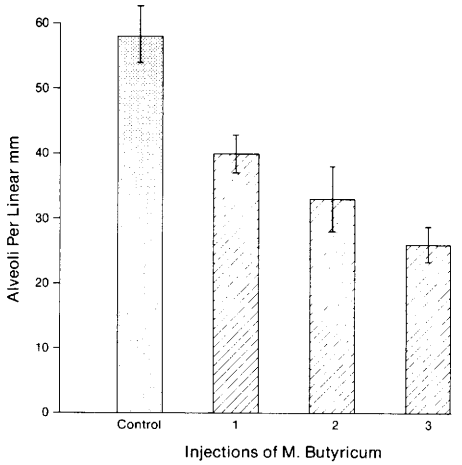

FIG. 1. Influence of number of subplantar injections of *M. butyricum* in 0.05 ml water–mineral oil emulsion on alveolar counts in rats. Injections were made on Days 0, 0 and 3, or 0, 3, and 7. Rats were killed on Day 20.

tions of lungs of the concomitantly studied controls did not show similar histopathology. Thus pending investigation of pulmonary physiology in adjuvant-treated rats it seems proper to term the lung changes as emphysema like.

The severity of the arthritis and the emphysema-like changes were proportional to the number of administrations of the adjuvant. Neither the polyarthritis nor the lung changes appeared when lyophilized *E. coli* was substituted for *M. butyricum*. From this we infer that the specific mycobacterial antigens required to induce the autoimmune adjuvant disease are likewise necessary for the development of the emphysema-like syndrome. This inference is supported by the observation that an immunosuppressive agent, cyclophosphamide, partially reduced the severity of both the polyarthritis and of the lung reactions. A decrease in TIC has been reported previously in adjuvant polyarthritis (13). The dramatic reduction in serum trypsin inhibitory capacity is consonant with the increased susceptibility of the polyarthritic rats to emphysema. Although others have shown that iv injection of bacterial enzymes may induce emphysema in rats (14), it is doubtful that such a mechanism is responsible for the lung changes observed in the present study. *E. coli* cells contain proteolytic enzymes, yet these did not induce the emphysema-like changes. It is also generally accepted that only proteinases with elastolytic properties are capable of inducing emphysema, and we are unaware of studies which indicate that either *M. butyricum* or *E. coli* preparations used in the present study contain elastase activity. Furthermore, an immunosuppressive agent such as cyclophosphamide would not be expected to antagonize proteolytic enzymes.

Infection has been postulated as a cause of emphysema (15); therefore, it is possible that bacteria or other infectious agents may in fact cause emphysema indirectly through a systemic disease, perhaps by stimulation of the immune system. The mechanisms whereby the alveoli are destroyed and TIC is depressed in our experiments with killed organisms obviously need elucidation. Further studies are also required to determine if the lung changes observed are reversible. However, the parallel development of polyarthritic and emphysema-like symptoms in our rats suggests the possibility that these two seemingly different types of connective tissue diseases may have analogous etiologies.

Summary. Subplantar injections of Freund's complete adjuvant (*M. butyricum*) were used to induce a typical adjuvant polyarthritis in rats. The lungs of the arthritic rats exhibited histological and biochemical changes characteristic of an emphysema-like condition. The lung pathology included

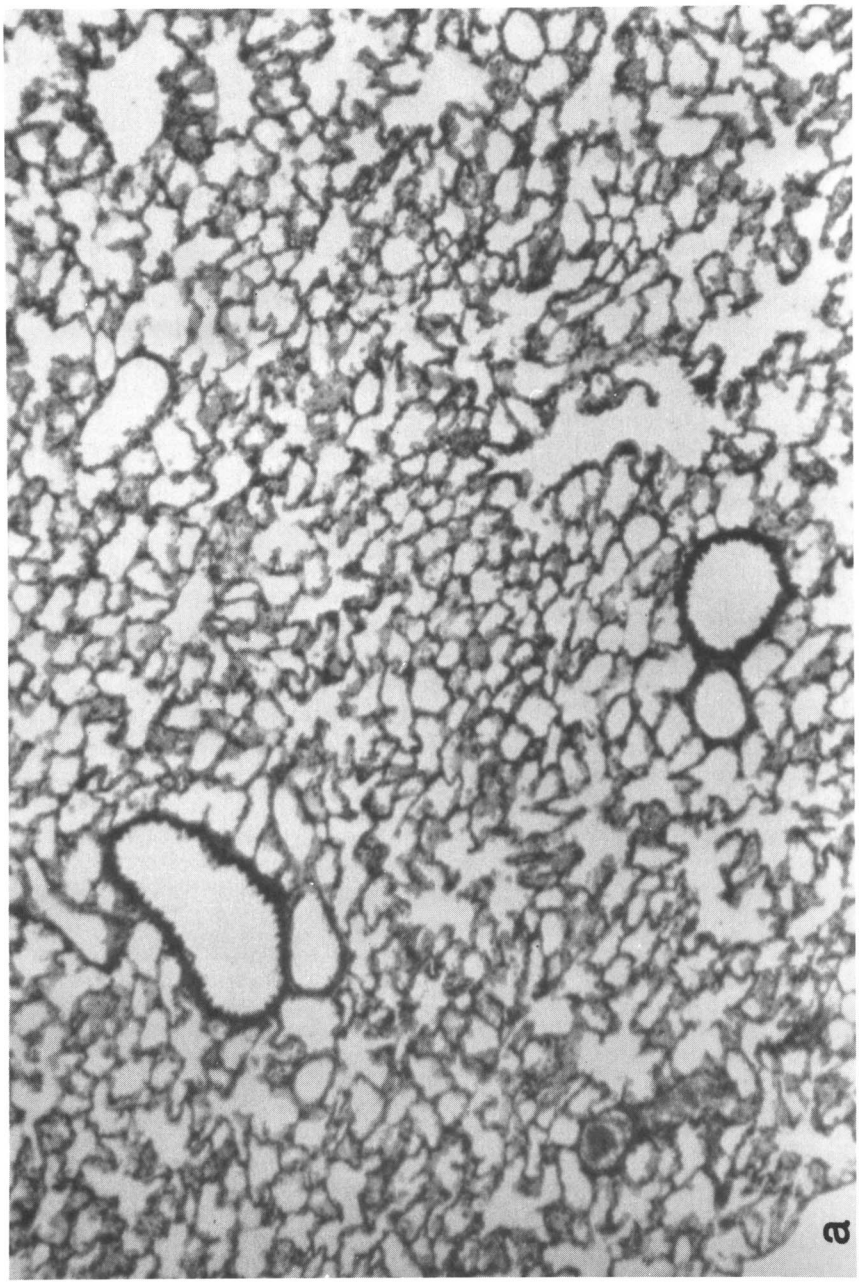

FIG. 2. Granulomata and emphysematous lesions in adjuvant arthritic rats. Lungs were fixed in 10% neutral formalin at 10 cm water pressure, paraffin embedded, sectioned at 5 μm, and stained with H&E. (a) Lung from control rat (× 252). (b) Lung from adjuvant arthritic rat. Note broken alveolar septa and granulomatous masses (× 252). (c) Lung from adjuvant arthritic rat. Emphysema-like condition of alveoli at sites distant from granulomatous masses (× 252). (d) Lung from control rat (× 1600). (e) Lung from adjuvant arthritic rat (× 1600). (f) Granuloma in lung of adjuvant arthritic rat (× 1600).

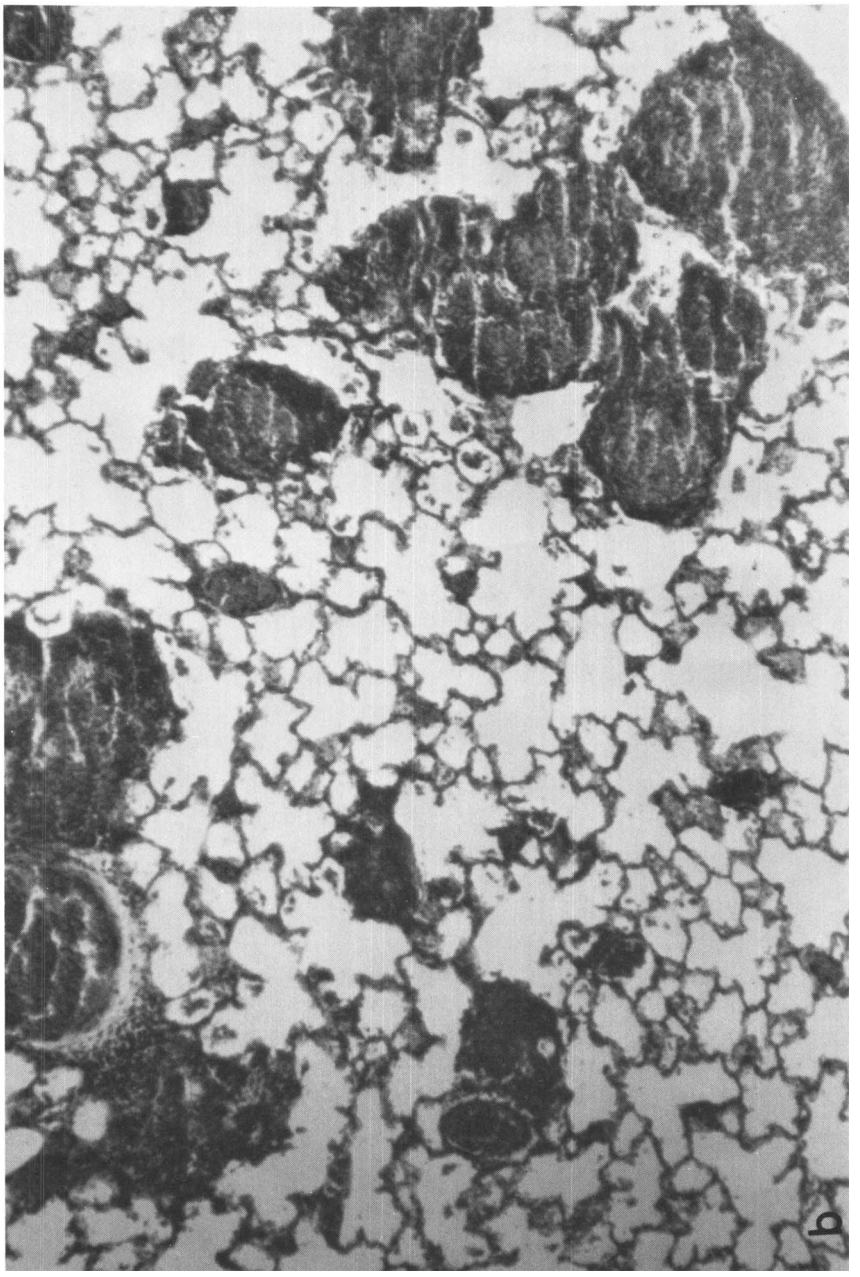

FIGURE 2b

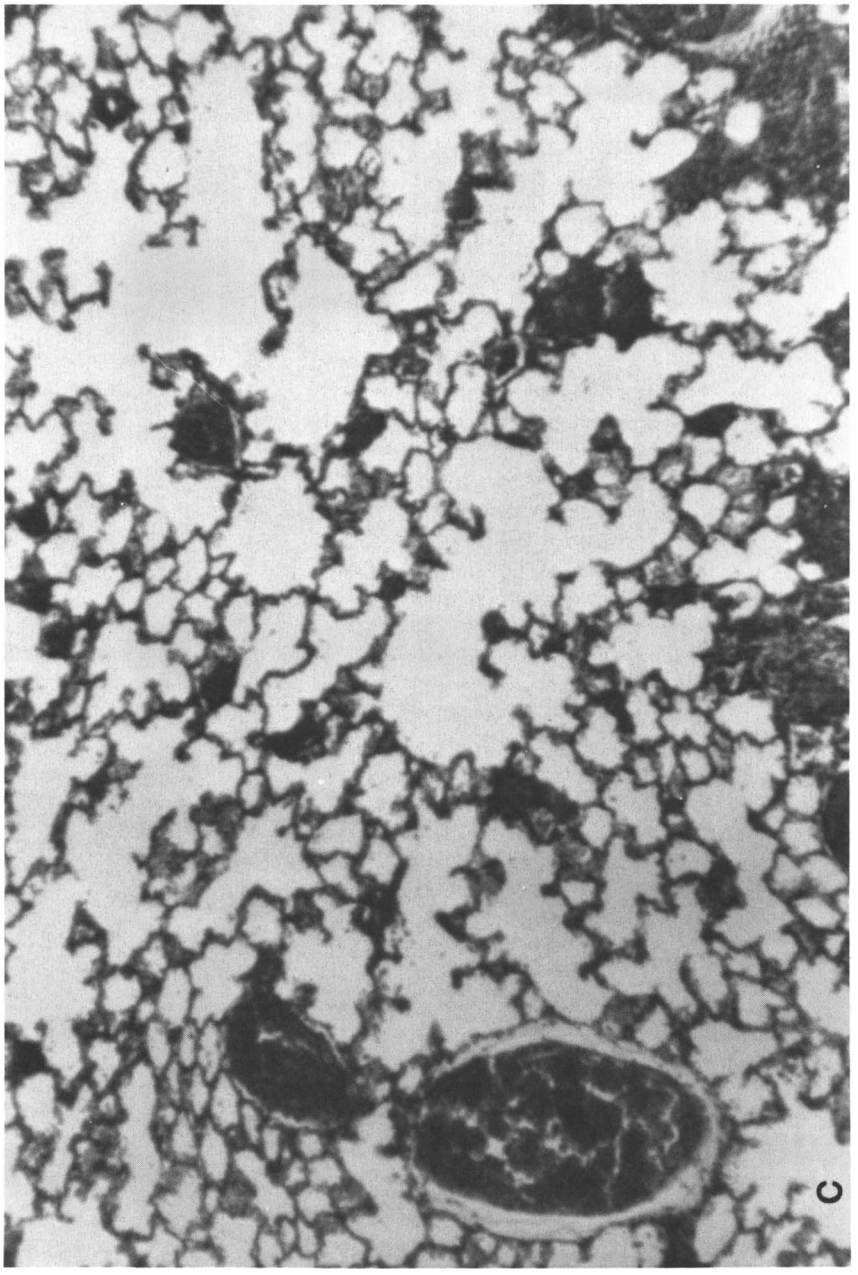

FIGURE 2c

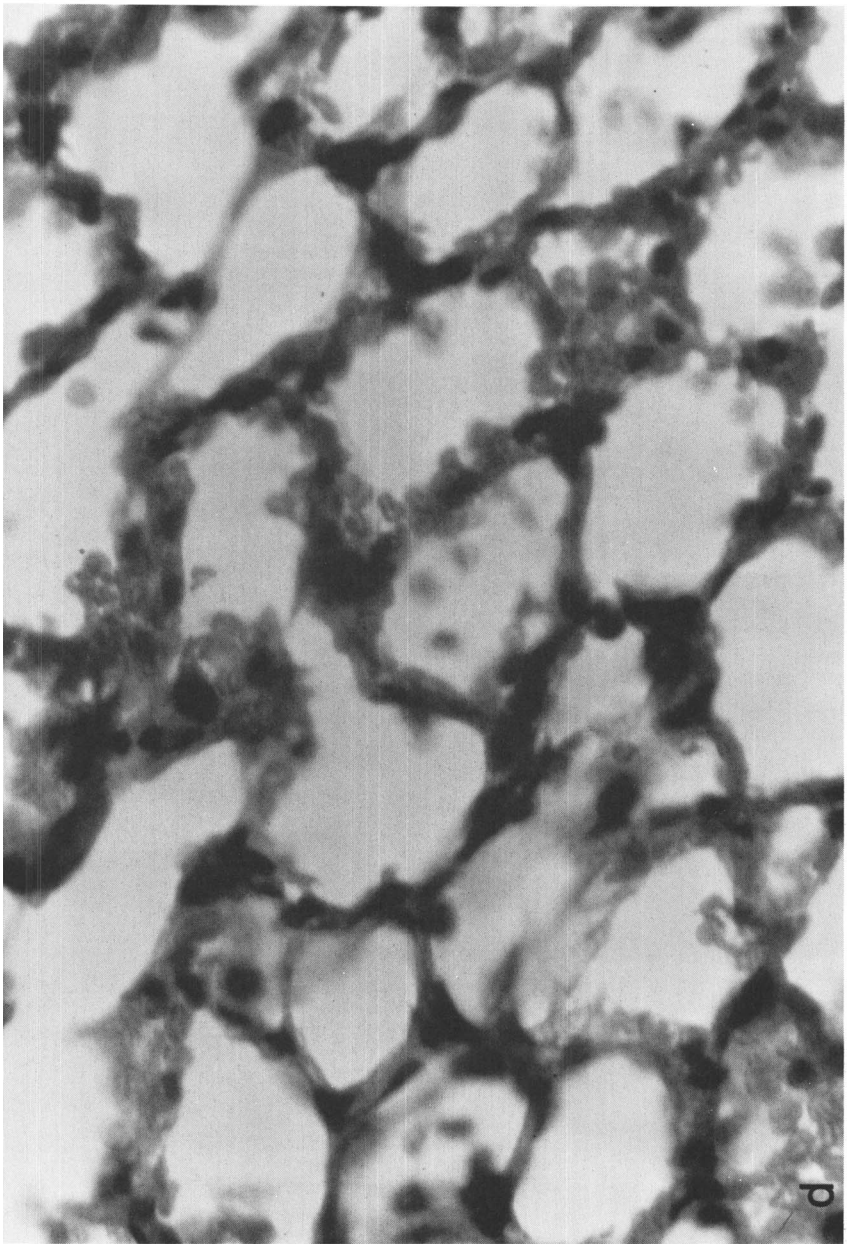

FIGURE 2d

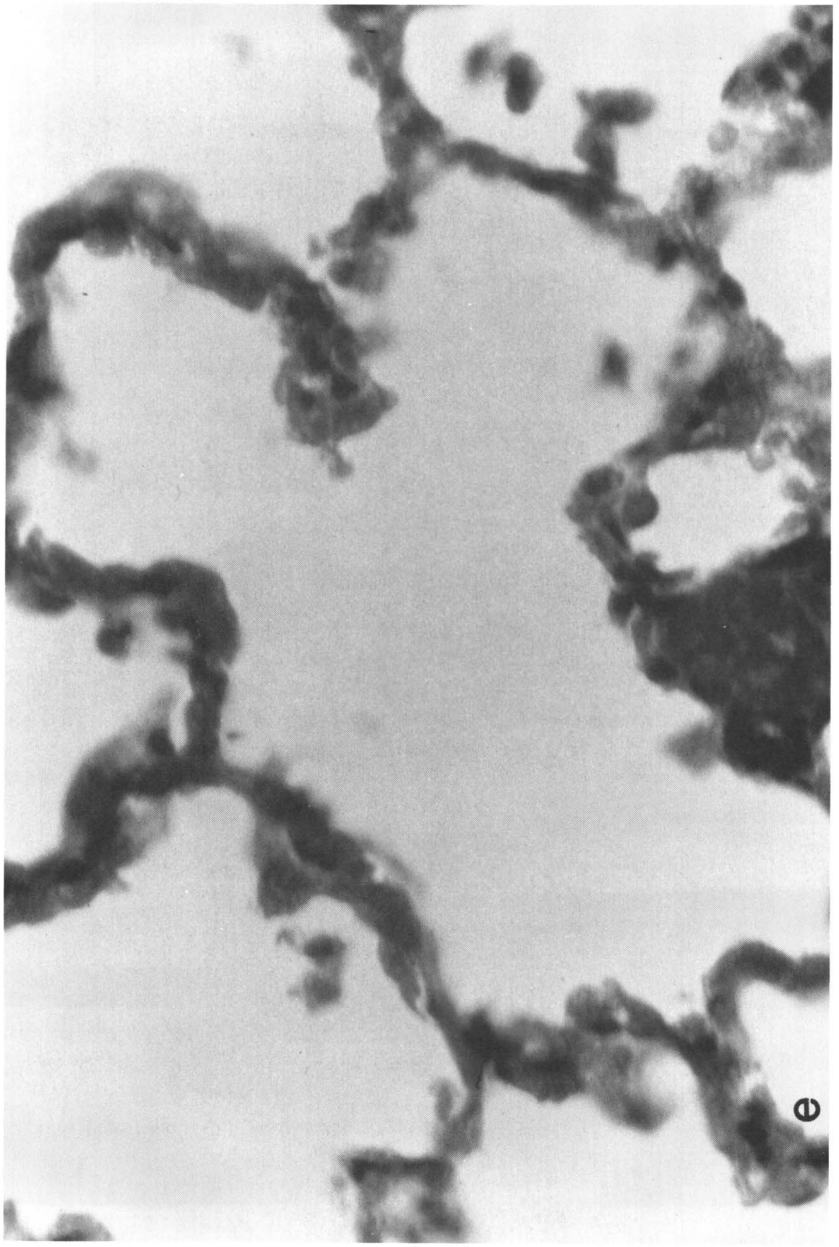

FIGURE 2e

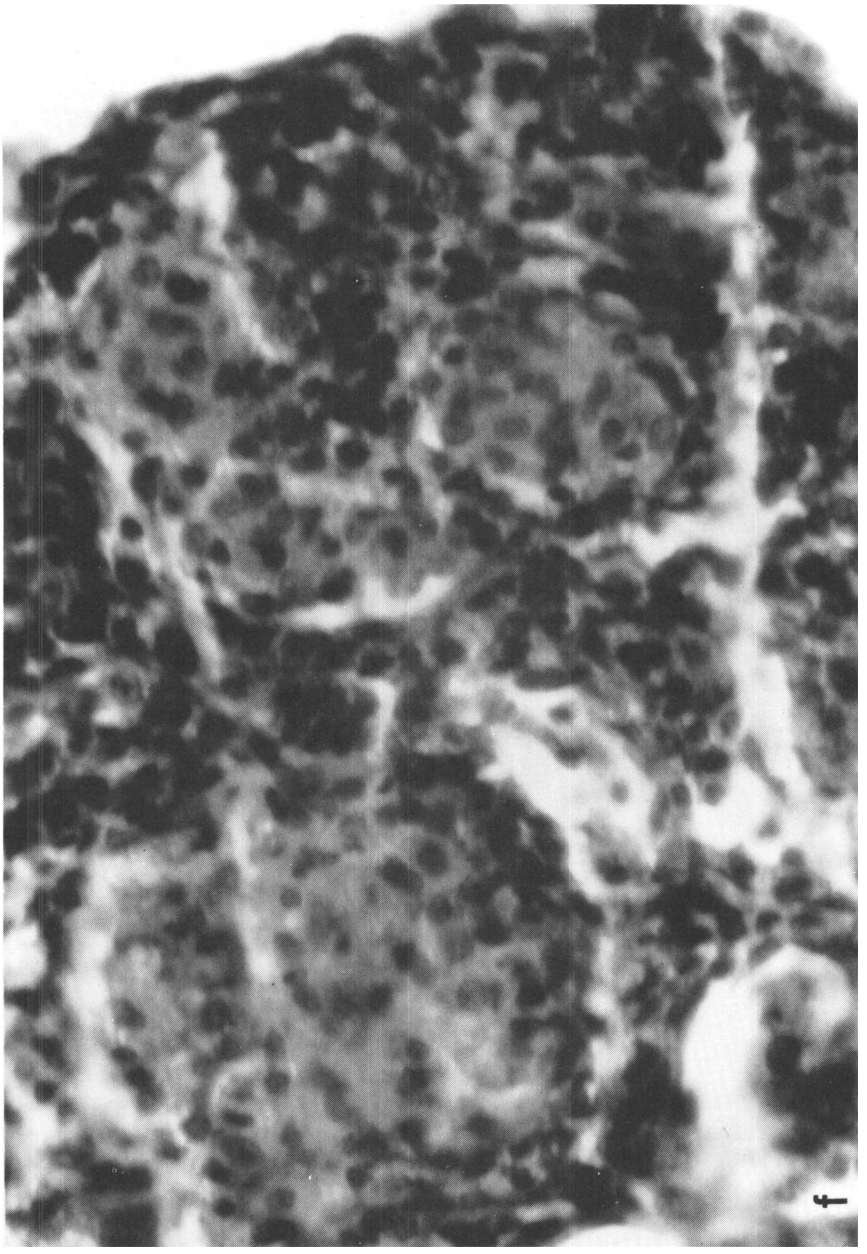

FIGURE 2f

TABLE II. COMPARISON OF EFFECTS OF *M. butyricum* AND *E. coli* IN FREUND'S ADJUVANT IN RATS AND INHIBITION BY CYCLOPHOSPHAMIDE

Group (six rats each)	Body wt gain (g)	Mean paw volume (mm Hg displaced)	Lung wt (g)	Lung β-glucuronidase (*E* at 540 nm)	TIC (mg trypsin inhib./ml)	Alveolar count (alveoli/mm)
Control	165	9.8 ± 0.2	1.20 ± 0.04	0.47 ± 0.02	2.80 ± 0.05	59 ± 2
M. butyricum	62	19.8 ± 1.5[a]	2.00 ± 0.03[a]	1.00 ± 0.09[a]	1.85 ± 0.08[a]	42 ± 2[a]
E. coli	150	9.8 ± 0.2	1.20 ± 0.04	0.49 ± 0.04	2.48 ± 0.20	52 ± 3
M. butyricum + cyclophosphamide	98	13.2 ± 0.5[a, b]	1.70 ± 0.02[a, b]	0.82 ± 0.07[a]	2.20 ± 0.12[a, c]	50 ± 5
E. coli + cyclophosphamide	132	9.5 ± 0.5	1.44 ± 0.02	0.61 ± 0.04	2.80 ± 0.15	51 ± 4

[a] $P < 0.01$ vs control.
[b] $P < 0.01$ vs *M. butyricum* alone.
[c] $P < 0.05$ vs *M. butyricum* alone.

markedly enlarged alveoli and septal fibrosis as well as numerous granulomatous foci. Serum trypsin inhibitory capacity was reduced. An adjuvant prepared with *E. coli* in place of *M. butyricum* failed to induce either polyarthritis or emphysema-like changes. Neither the polyarthritis nor the lung pathology was as severe in rats treated simultaneously with *M. butyricum* and the immunosuppressant, cyclophosphamide, as in rats treated with *M. butyricum* alone. The data thus suggest that inappropriate immunological responses to *M. butyricum* antigens may be related to the production of the pulmonary disease as well as the polyarthritis.

1. Eriksson, S., Acta Med. Scand. Suppl. **432**, 1 (1965).
2. Ito, H., and Aviado, D. M., J. Pharmacol. Exp. Ther. **161**, 197 (1968).
3. Gross, P., Pfizer, E. A., Tolker, E., Babyak, M. A., and Kaschak, M., Arch. Environ. Health **11**, 50 (1965).
4. Giles, R. E., Finkel, M. P., and Williams, F. D., Proc. Soc. Exp. Biol. Med. **147**, 489 (1974).
5. Marco, V., Mass, B., Meranze, D. R., Weinbaum, G., and Kimbel, P., Amer. Rev. Resp. Dis. **104**, 595 (1971).
6. Mass, B., Ikeda, T., Meranze, D., Weinbaum, G., and Kimbel, P., Amer. Rev. Resp. Dis. **103**, 907 (1971).
7. Boren, H. G., Amer. Rev. Resp. Dis. **92**, 1 (1965).
8. Colombo, C., and Steinetz, B., Arch. Int. Pharmacodyn. **216**, 86 (1975).
9. Perper, R. J., Alvarez, B., Colombo, C., and Schroeder, H., Proc. Soc. Exp. Biol. Med. **137**, 506 (1971).
10. Gianetto, R., and deDuve, C., Biochem. J. **59**, 423 (1955).
11. Pearson, C. M., and Wood, F. D., Arthritis Rheum. **2**, 440 (1959).
12. Nagai, H., Arai, H., Ariji, F., Asoo, N., Ishikawa, T., Sato, H., Yokosawa, A., Motomiya, M., and Konno, K., Lung **154**, 113 (1977).
13. Parrott, D. D., and Lewis, D. A., Ann. Rheum. Dis. **36**, 166 (1977).
14. Blackwood, C. E., Hosannah, Y., Perman, E., Keller, S., and Mandl, I., Proc. Soc. Exp. Biol. Med. **144**, 450 (1973).
15. Hallett, W. Y., Med. Clin. North Amer. **57**, 735 (1973).

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