THE RESPONSE OF THE HISTIOCYTES AND MACROPHAGES IN THE LUNGS OF RABBITS INJECTED WITH FREUND'S ADJUVANT*

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WHEN Freund's adjuvant is given intravenously it elicits an inflammatory reaction in many organs and an exaggerated proliferation of cells of the reticuloendothelial system (Laufer, Tal and Behar, 1959 ; Rupp, Moore and Schoenberg, 1960 ; Steiner, Langer and Schatz, 1960). The use of Freund's adjuvant provides a model to study the characteristics of the cells of the reticuloendothelial system (Moore, Rupp, Mumaw and Schoenberg, 1961 ; Schoenberg, Gilman, Mumaw and Moore, 1961 and 1963 ; Moore and Schoenberg, 1963) and the mechanism of action of the adjuvant (Freund, 1947 ; Moore, Lamm, Lockman and Schoenberg, 1963 ; Schoenberg, Rupp and Moore, 1964).

In these experiments the cellular reaction in the lungs of rabbits treated with an intravenous injection of complete Freund's adjuvant, the combination of complete adjuvant and diphtheria toxoid, incomplete adjuvant or an intratracheal injection of Bayol F were used to study : (1) the relation of the histiocyte-macrophage response to the appearance and molecular species of circulating antibody to *Mycobacterium butyricum* and diphtheria toxoid ; (2) the presence of antigen, antibody and γ-globulin in these cells ; and (3) the development of skin sensitivity to the mycobacterium and its relation to the histiocyte-macrophage reaction in the lungs.

MATERIALS AND METHODS

Albino rabbits of both sexes weighing 2·5–3·5 kg. were maintained on a standard laboratory diet with unrestricted access to water.

Group 1.—Two rabbits were killed after receiving 1 ml. of complete Freund's adjuvant (Difco Products Co., Chicago, Ill.—8·5 ml. Bayol F ; 1·5 ml. Arlacel ; and 5 mg. *Myco. butyricum*) injected into the marginal ear vein, at each of the following times : 30 min., 1 hr., 12 hr. and 1, 2, 3, 5, 7, 10, 11, 14, 18, 21, 28, 35, 42, 49, 56, 70 and 84 days. The injection of the adjuvant did not result in respiratory difficulty due to pulmonary emboli from the oil.

Group 2.—Four rabbits were given 1 ml. of complete Freund's adjuvant intravenously followed by 70 Lf of soluble diphtheria toxoid through the same needle (diphtheria toxoid 160 Lf/ml., Lederle Laboratories Division, American Cyanamid Corp., New York). Two were killed at 7 days and 2 at 14 days.

Group 3.—Twelve rabbits were injected intravenously with 1 ml. of incomplete Freund's adjuvant (Difco Products Co.) containing all ingredients except *Myco. butyricum*. Four were killed on each of the following days after injection : 7, 14 and 21.

Group 4.—Two rabbits were injected with 1 ml. of Bayol F into the trachea below the larynx. Seven days later 4 ml. of rabbit γ-globulin labelled with fluorescein isothiocyanate was injected intravenously. The animals were killed 30 min. later. This group was con-

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trolled by 2 rabbits that received Bayol F in the same fashion, but which did not receive the labelled protein intravenously.

Group 5.—Twenty-four rabbits were prepared with 1 ml. of complete Freund's adjuvant intravenously and then skin tested with 0·3 mg. of heat killed, desiccated, finely divided *Myco. butyricum* suspended in 0·5 ml. of 0·15 M NaCl. Several animals were tested on each of the following days after intravenous immunization ; 1, 3, 5, 7, 10, 11, 14, 18, 21, 28.

The animals that received the complete adjuvant and the complete adjuvant and diphtheria toxoid, were bled at intervals from the marginal ear vein and at the time of killing by cardiac puncture to obtain sera for the determination of circulating antibody to *Myco. butyricum* and diphtheria toxoid. Antibody titres to *Myco. butyricum* were measured by complement fixation by the method described previously (Moore *et al.*, 1963), and to diphtheria toxoid by the haemagglutination method of Stavitsky (1954). Fractionation of the rabbit sera was done by diethylaminoethyl (DEAE) cellulose columns. Sucrose density gradient ultracentrifugation separation of selected sera was also done. The fractions were lyophilized and the nature of the proteins determined electrophoretically.

At the appropriate time the animals were anaesthetized with sodium pentobarbital and the heart and lungs removed as a block. A volume of 30 ml. of 5 per cent sucrose was introduced into the lungs by way of the trachea. The lungs were gently massaged and the fluid collected by inverting the specimen. A considerable number of cells could be obtained in this manner. Aliquots of the suspensions of cells were used to prepare blocks for electron microscopy. Cell spreads were made by smearing a drop of the suspension on a glass slide. Several slides of each preparation were fixed in 70 per cent alcohol for subsequent staining with haematoxylin and eosin, with toluidine blue and for the periodic acid Schiff (PAS) reaction. Additional unfixed cell spreads were stained by the immunofluorescent technique to demonstrate cells containing γ-globulin and for antibody to diphtheria toxoid in the case of animals from Group 2. Cell spreads from the animals that had been given Bayol F and then fluorescein isothiocyanate labelled γ-globulin intravenously were mounted in glycerine and examined for fluorescence without further treatment.

Samples of tissue were taken from the lungs for electron microscopical examination prior to intratracheal washing. These and the cells from the lung washings were fixed in phosphate buffered osmium tetroxide (pH 7·2–7·4) containing sucrose. The specimens were gradually dehydrated in ethanol, infiltrated with methacrylate and polymerized in butyl-methyl methacrylate (9 : 1) with 1·5 per cent dibenzoyl peroxide as the catalyst. Sections were cut with a glass knife on a Porter–Blum microtome for examination in an RCA-2D electron microscope. Samples of lung tissue were also fixed in formalin, embedded in paraffin and sectioned in the usual manner. These were stained with haematoxylin and eosin, with toluidine blue and for the PAS reaction.

The immunofluorescent antibody procedure (Coons, Leduc and Connolly, 1955) for the identification of γ-globulin in the cell spreads and frozen sections of unfixed lung tissue was by the direct method. Sheep anti-rabbit γ-globulin globulin conjugated with fluorescein isothiocyanate was layered on the specimens and allowed to remain in a moist chamber at room temperature for 1 hr. The sections were then washed thoroughly with phosphate-saline buffer at pH 7·0, ionic strength 0·15, mounted in glycerine and examined for fluorescence. This was controlled by treating adjacent sections with unlabelled sheep anti-rabbit γ-globulin globulin in a moist chamber at room temperature for 1 hr., washing with phosphate-saline buffer and then staining with the labelled antibody as described.

In order to identify specific antibody (diphtheria toxoid), the cell spreads and tissue sections from the animals in Group 2 (Freund's adjuvant and diphtheria toxoid) were stained by the indirect method. The sections and cell spreads were flooded with soluble diphtheria toxoid for 30 min., washed thoroughly with phosphate-saline buffer, and then stained with fluorescein isothiocyanate labelled anti-diphtheria toxoid rabbit γ-globulin with and without prior treatment with the unlabelled antibody, as described. The antibody was prepared by ammonium sulphate fractionation of sera obtained from rabbits hyperimmunized with diphtheria toxoid without Freund's adjuvant. The antibody titre of the conjugate was 1/5120.

To identify antigenic fragments of *Myco. butyricum* in the lungs of animals in Group 1 (Complete Freund's adjuvant), anti-Freund's adjuvant rabbit γ-globulin was prepared by ammonium sulphate fractionation of sera obtained from hyperimmunized rabbits and conjugated with fluorescein isothiocyanate. The antibody titre to *Myco. butyricum* of these preparations was 1/512–1/1024 (complement fixation) after conjugation. The staining procedure was as described above.

RESULTS

Tissue and cell studies

Histology and cytology.—The histological appearances of the lungs from animals that received an intravenous injection of complete adjuvant and the adjuvant and diphtheria toxoid were similar. Within 1 hr. there was an increase in the number of cells within the alveolar walls. Histiocytes were common and there were more small lymphocytes and polymorphonuclear leucocytes than seen in normal lungs (Fig. 1). By 3 days histiocytes were numerous within the alveolar walls and macrophages were common in the alveolar spaces. Throughout the lung there were clusters of mononuclear cells forming granulomata within the alveolar walls with small lymphocytes, polymorphonuclear leucocytes and mature plasma cells at the periphery (Fig. 2). (For the purposes of this paper, those cells that are free in the alveolar spaces are called macrophages and those found in the alveolar walls histiocytes. Cytologically these cells were indistinguishable. When they are discussed in terms of the cell washings, the name macrophage is used.)

There was an increase in histiocytes, macrophages and granulomata to 28 days, after which they remained fairly constant for a short time and then began to regress. By 7 weeks there was a definite decrease. As the cellular response subsided, the remaining granulomata were concentrated in the subpleural areas and in the lymphoid tissue within the lung. Most of the cellular exudate and granulomata were gone without residual fibrosis by the 10th week.

Seven days after the intravenous injection of the incomplete adjuvant (Group 3), histiocytes, macrophages and granulomata were found. However, the response was minimal compared to that produced by the complete adjuvant and was subsiding by 21 days. Small lymphocytes and plasma cells were rare.

The sections from the lungs of animals, that received an intratracheal injection of Bayol F (Group 4) showed macrophages within some alveolar spaces. In this group there was no proliferation of histiocytes in the alveolar walls. Lymphocytes, polymorphonuclear leucocytes and granulomata were absent.

It was easy to harvest a large number of cells by intratracheal washing from all animals, except those which received the incomplete adjuvant intravenously. The majority of the cells, more than 90 per cent, were macrophages. Mitotic figures were frequently seen in these preparations. There were also a few small lymphocytes, polymorphonuclear leucocytes and bi- or multinucleated forms of the macrophages.

The macrophages and histiocytes (Fig. 3) in the tissue sections and lung washings had an abundant cytoplasm that stained poorly with eosin, when the haematoxylin and eosin stain was used. This staining characteristic was maintained throughout the experiment in all groups. Independent of the method of promotion of the cellular response, the macrophages and histiocytes during the first 2–3 weeks had a number of vacuoles in their cytoplasm, more concentrated around the nuclei. The number of vacuoles increased after injection, particularly in those animals that received complete adjuvant or the adjuvant and diphtheria toxoid. From 3–5 weeks (complete Freund's adjuvant, Group 1) the vacuoles were more uniformly distributed throughout the cytoplasm and after 6 weeks were localized at the border of the cells.

The cells from the different groups of animals had the ultrastructural features

described for histiocytes and macrophages (Fig. 4). Moderately electron dense inclusions, either completely or partially surrounded by a limiting membrane, were present and tended to parallel the number and distribution of vacuoles in the fixed and stained preparations and the distribution of γ-globulin found by immunofluorescence.

When the tissue sections and cell preparations were examined for gross carbohydrate content and cytoplasmic basophilia, the macrophages and histiocytes from each of the groups of animals had a faint to moderate PAS reaction and faint basophilia (toluidine blue staining) of the cytoplasm in the intervacuolar areas. In no case did the vacuoles display either the PAS reaction or basophilia.

Immunofluorescent staining.—When the preparations were examined for γ-globulin with fluorescein isothiocyanate labelled sheep anti-rabbit γ-globulin globulin, packets of fluorescent material of varying intensity corresponding to the areas of vacuolization were found in the cytoplasm of the macrophages and histiocytes from all groups of animals. (Figs. 5*a* and *b*). The fluorescent staining could be inhibited by using unlabelled sheep anti-rabbit γ-globulin globulin prior to the application of the labelled antibody. It was possible to identify rabbit γ-globulin in the cytoplasm of a few of the macrophages and histiocytes as early as 12 hr. after the injection of the complete adjuvant. With time, and generally paralleling the degree of vacuolization of the cytoplasm, there was an increase in the amount of γ-globulin in individual cells and in the number of cells that contained γ-globulin. This was most apparent in the animals that received the complete adjuvant and the adjuvant and diphtheria toxoid. The accumulation of γ-globulin in the cells from animals receiving only complete Freund's adjuvant reached a maximum at 4 weeks (Figs. 5*a* and *b*), persisted at a high level for an additional week, and then gradually declined, so that only a few of the remaining macrophages and histiocytes contained a small amount of γ-globulin at the end of the experiment (Table).

The amount and intensity of the fluorescent staining in the immunized animals tended to parallel the serum antibody titres. For example, during the interval

TABLE.—*Antibody Titres and Skin Sensitivity to* Myco. butyricum *in Relation to the Cellular Response in the Lungs*

Days after adjuvant	AB. titres *M. b.**	19S AB. titres *M. b.** (per cent)	7S AB. titres *M. b.** (per cent)	Histiocyte macrophage response	Granuloma formation	γ-globulin in histiocyte and macrophage	Plasma cells with γ-globulin	Skin test with *M. b.**
1–4	0	—	—	+	±	±	—	—
5	0	—	—	+	+	+	—	—
7	2–4	†	—	2+	2+	+	—	—
10	16–24	†	—	3+	3+	2+	±	±
14	36	100	—	4+	4+	2+	+	2+
18	192	100	—	4+	4+	3+	2+	2+
21	192	100	—	4+	4+	3+	3+	4+
28	192	75	25	4+	4+	4+	+	4+
35	192	50	50	4+	4+	4+	±	
42	128	25	75	3+	3+	3+	±	
51	80	25	75	+	2+	2+	±	
63	80	25	75	±	2+	+	±	
71	80	†	100	±	+	—	±	
84	24	†	†	Normal population	+	—	±	

* *M. b.* = *Myco. butyricum*. † Too low to measure.

of peak antibody synthesis to the toxoid, the cells obtained from animals that received the combination of the complete adjuvant and diphtheria toxoid contained more fluorescent material than those that received the adjuvant alone.

A small amount of specific antibody to diphtheria toxoid (Group 2) was present at 7 days in the same general location in the cytoplasm of the macrophages and histiocytes as the total γ-globulin (Fig. 6), and was considerably greater by 14 days. However, antibody to diphtheria toxoid represented a small portion of the fluorescent material (intensity and amount) compared to the γ-globulin demonstrated by the direct method (sheep anti-rabbit γ-globulin globulin). Using the immunofluorescent technique, Askonas (1959) has also described cells in the lungs of hyperimmunized animals containing antibody in discrete packets, and suggested that in this form the antibody is not readily excreted.

A few mature plasma cells, particularly at the periphery of the granulomata and in the lymphoid aggregates had a uniform fluorescent staining of their cytoplasm for γ-globulin 10–14 days after injection of the adjuvant (Fig. 7). The number of plasma cells that contained γ-globulin and the intensity of fluorescence of these cells was maximal by 21 days, after which, there was a rapid decay, so that by 28 days only a few plasma cells contained γ-globulin. This corresponded to the sequence in which antibody could be demonstrated in plasma cells in the spleen of these animals and others treated in the same way (Schoenberg *et al.*, 1964).

EXPLANATION OF PLATES

FIG. 1.—Section of a lung one day after the animal received complete Freund's adjuvant. The alveolar walls contain numerous histiocytes, lymphocytes and polymorphonuclear leucocytes. H. and E. ×245.

FIG. 2.—Granulomata composed of clusters of mononuclear cells in the lung 7 days after the injection of complete adjuvant. The cells at the periphery of the granulomata are largely lymphocytes, polymorphonuclear leucocytes and plasma cells. H. and E. ×120.

FIG. 3.—Macrophages harvested from the lungs 3 weeks after the injection of complete adjuvant. The cytoplasm is abundant and shows varying numbers of vacuoles. H. and E. ×660.

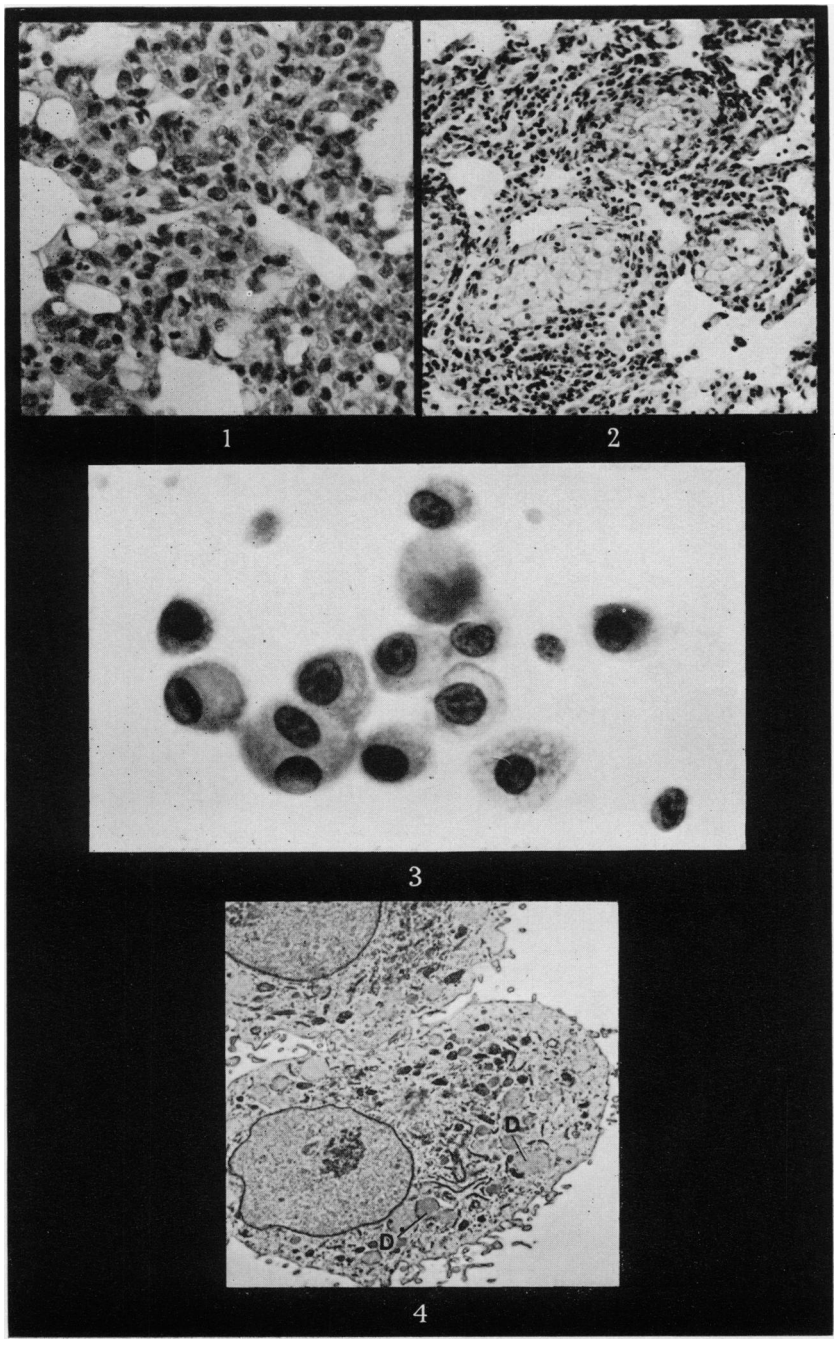
FIG. 4.—Electron micrograph representative of the macrophages obtained in the lung washings. Note the numerous moderately electron dense structures (D) with limiting membranes. These correspond to the distribution of vacuoles in the cells stained with H. and E. (Fig. 3) and the fluorescent material (γ-globulin) demonstrated with immunofluorescence (Fig. 5 and 6). ×3130.

FIG. 5.—(A) γ-globulin in the cytoplasm of macrophages obtained from lung washings one week after the injection of adjuvant; (B) the increased accumulation of the γ-globulin 4 weeks after the injection of adjuvant. Fluorescein isothiocyanate labelled sheep anti-rabbit γ-globulin globulin. Direct method. ×400.

FIG. 6.—Specific antibody to diphtheria toxoid is shown in the cytoplasm of the macrophages from the lung washings obtained 7 days after immunization. When compared with the cells shown in Fig. 5, there is far less fluorescent material. Fluorescein isothiocyanate labelled rabbit anti-diphtheria toxoid. Indirect method. ×400.

FIG. 7.—Representative clusters of plasma cells in the lung from an animal that received complete adjuvant and diphtheria toxoid 14 days before. Some of these plasma cells will have antibody in their cytoplasm at this time. H. and E. ×425.

FIG. 8.—(A) Electron micrograph showing fragments of *Myco. butyricum* within the lung tissue. Heat killed, desiccated, finely divided *Myco. butyricum* did not show any ultrastructure suggestive of bacteria. There are a number of vascular channels that contain various sized fragments of bacteria (B). Similar type material can be seen coating the endothelial surfaces. Polymorphonuclear leucocytes (P) and red blood cells (R) are present within the alveolar walls. Many of the red blood cells show a less dense centre. This change is common in the red cells after the intravenous administration of adjuvant and is unexplained. Histiocyte (H), Macrophage (M), Alveolar lining cell (A). ×2400. (B) Higher magnification showing the fragments of *Myco. butyricum* (B) coating the luminal surface of the endothelial cell (E), passing through the capillary wall (C) and within the alveolar space (S). ×15,000.



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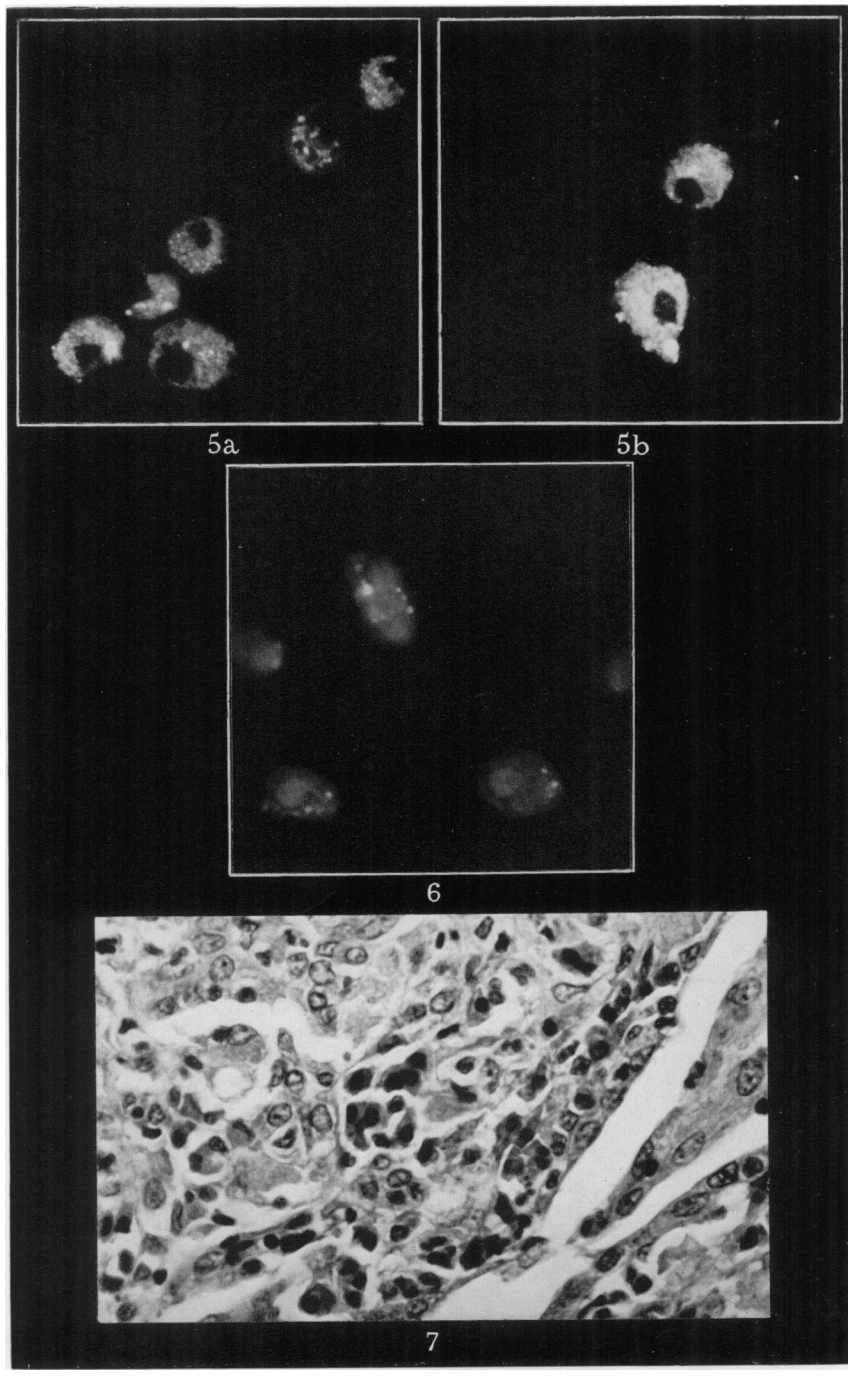

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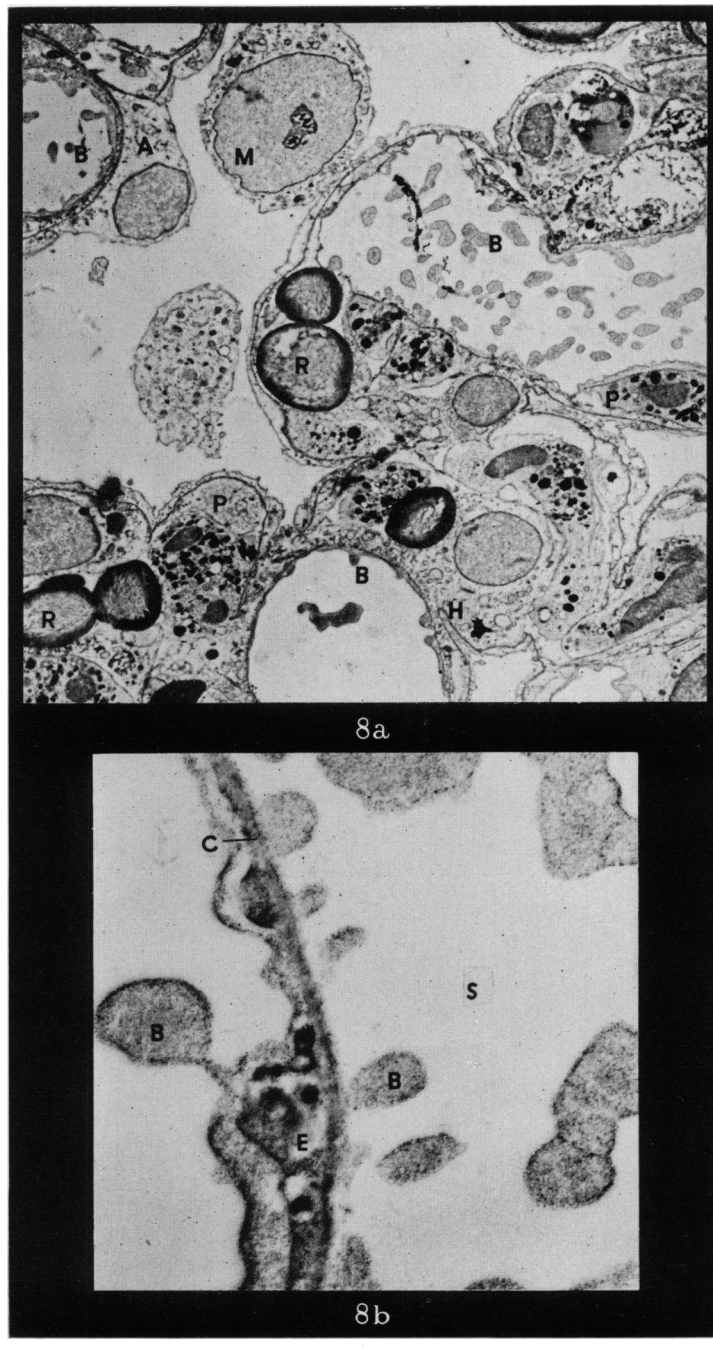
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The fluorescence of the plasma cells could be markedly attenuated by prior treatment of the sections with unlabelled antibody.

Though plasma cells were present in the histological sections of the lungs at 7 days, specific antibody to diphtheria toxoid was not identified in these cells until 14 days after immunization.

The macrophages obtained from the lungs of the rabbits, that had been given Bayol F by way of the trachea and fluorescein isothiocyanate labelled rabbit γ-globulin intravenously (Group 4) and then examined without further treatment, showed many aggregates of flourescent material in their cytoplasm within 30 min. This material could not be completely eluted from the cells by repeated washing in phosphate buffer (pH 7·0) containing 5 per cent sucrose.

Mycobacterial fragments.—Fragments of mycobacteria could be demonstrated as small fluoresent particles within vascular spaces, macrophages, histiocytes and extra-cellularly only in the first 3–4 days after injection, using fluorescein isothiocyanate labelled antibody to *Myco. butyricum.* The fluorescent staining could be inhibited by first incubating the sections with unlabelled antibody or by absorption of the labelled antisera with *Myco. butyricum* before staining.

Electron microscopic examination of the tissue sections showed fragments of mycobacteria adhering to the surface of endothelial cells and between the endothelial cells and the basement membranes of small blood vessels, and in the alveolar spaces within 30 min. after injection of the adjuvant (Figs. 8*a* and *b*). Fragments of *Myco. butyricum* could also be seen within the cytoplasm of some of the macrophages and histiocytes by 24 hr. Similar to the results obtained from the fluorescent procedure, fragments of mycobacteria could no longer be identified either free in the tissue or within cells 3–4 days after the injection of the adjuvant. Electron microscopic examination of heat killed, desiccated, finely divided *Myco. butyricum* placed on carbon coated grids showed material that was morphologically the same as that interpreted to be fragments of *Myco. butyricum* in the tissue sections. Intact mycobacteria or definitive substructures other than those illustrated could not be identified in these preparations.

Granulomata.—The large mononuclear cells forming the granulomata were different from the macrophages and isolated histiocytes. Their cytoplasm showed minimal staining with either eosin or toluidine blue and did not contain material that reacted with the PAS procedure. γ-globulin, antibody to diphtheria toxoid and fragments of mycobacteria could not be demonstrated in these cells with the immunofluorescent procedures. Electron microscopic examination showed numbers of small granules of varying size and electron density in the cytoplasm. There were occasional vacuoles and lipid bodies. Mitochondria were numerous and small aggregates of endoplasmic reticulum were found. The margins of these cells frequently interdigitated with neighbouring cells by long cytoplasmic processes. Mycobacterial fragments were not identified in these cells by electron microscopy.

Antibody titres

Complement fixing circulating antibody to *Myco. butyricum* was detected in the serum of some of the animals between the 7th and 10th day after the injection of complete adjuvant, and in all animals by the 10th day (see Table). The first measurable titres were low ($\frac{1}{2}$–$\frac{1}{4}$). They gradually increased for the next few days but still remained at low levels ($\frac{1}{16}$–$\frac{1}{24}$). Following this, there was a sharp

rise that reached average values of 1/128–1/256 between the 18th–21st day depending on the animal. The titres remained unchanged for an additional 3 weeks after which there was a steady but slow decline.

Sera obtained from animals at various times after the injection of the adjuvant were fractionated on DEAE cellulose columns. The fractions were examined by zone and immuno-electrophoresis. In the initial stages, up to 21–28 days, complement fixing antibody was confined to a fraction that migrated as a γ_{1M} globulin. The chromatographic fraction also contained some α_2 macroglobulin. Absorption with *Myco. butyricum* substantially reduced the γ_{1M} component and did not appreciably affect the α_{2M} component. There was no evidence of antibody associated with the γ_2 (7S) globulin fraction up to 21–28 days by the method used. Sucrose density gradient determinations on selected samples confirmed the observation that the antibody to *Myco. butyricum* found at this time was of the macroglobulin species.

From approximately 28 days onward complement fixing antibody was found in the fraction that corresponded electrophoretically to γ_2 (7S) globulin as well as in the γ_{1M} fraction (Table). From 5 weeks onward there was a distinct transition in circulating antibody from the 19S macroglobulin species to the 7S type.

Haemagglutinating antibody titres to diphtheria toxoid were 160–320 at 7 days and 1280–2560 by 14 days. At 7 days the circulating antibody was all of 19S-γ_{1M} type and at 14 days a mixture of 19S and γ_2 7S antibody.

It should be pointed out that the estimates of the percentages of 19S and 7S antibody do not necessarily reflect the actual values present in the serum, but are based on recoverable antibody from the sucrose density gradient and chromatographic procedures.

The largest accumulation of γ-globulin in the cytoplasm of the macrophages and histiocytes was found when both 19S and 7S antibody to *Myco. butyricum* were found in the serum. The 7S antibody persisted in the serum during the period when the γ-globulin in the cytoplasm of the macrophages and histiocytes decreased.

Antibody to diphtheria toxoid was not observed in the plasma cells until after the appearance of 19S antibody in the circulation, but was found before 7S antibody could be measured. This relation of the fluorescent staining of plasma cells to the species of antibody (19S *v.* 7S), in the circulation was similar to that reported for plasma cells in the spleens of rabbits treated in a similar manner (Schoenberg *et al.*, 1964). In the latter experiments as the titre of 7S antibody in the serum rose, the intensity of fluorescence and the number of plasma cells that showed fluorescence decreased.

Skin sensitivity

Positive skin tests of small size were obtained with *Myco. butyricum* from the 8–10th day (Table). By the 14th day the reaction was of moderate intensity. The test areas were raised, red, indurated and measured 5–6 mm. in greatest dimension. Skin tests at 21–28 days averaged 10–12 mm. 48 hr. after injection. The sites were raised, dark red and indurated with foci of superficial necrosis.

The appearance and increase in intensity of the tests for skin sensitivity paralleled the appearance and rise in the antibody titres to *Myco. butyricum*. This is not intended to imply a cause and effect relation between the skin reaction and the circulating antibody, but to indicate the immunological state of the animal

to *Myco. butyricum* at the time of appearance of skin sensitivity. The response of the histiocyte and macrophage and the formation of granulomata in the lung was well established before the appearance of skin sensitivity. There was no relationship between the progression of the cellular response in the lungs and skin sensitivity.

DISCUSSION

In the early stages of the experiment the proliferation of histiocytes in the alveolar walls, the accumulation of macrophages in the alveolar spaces and the formation of granulomata in the lungs of the animals in these experiments do not appear to be related to a recognizable immunological reaction. The histiocyte and macrophage population is substantially increased in the lung tissue within 1 hr. after the intravenous injection of the complete adjuvant, and extensive by 3 days. This phase would appear to be caused by some non-immunological effect of the adjuvant. Fragments of mycobacteria adhere to the endothelial surface of the capillaries, accumulate beneath endothelial cells and are found in alveolar spaces within 30 min.–1 hr. after the injection of the complete adjuvant. The combination of the mycobacterial fragments, Bayol F and Arlacel apparently results in a rapid and sustained increase in the proliferative capacity of some cells and vascular permeability. Innocuous particulate material is not ordinarily passed out of the vascular bed in the normal lung of the rabbit (Moore *et al.*, 1961 ; Schoenberg *et al.*, 1961, 1963). Incomplete adjuvant or heat killed, desiccated, finely divided *Myco. butyricum* suspended in saline do not produce the same effect when injected intravenously.

The γ-globulin found in the cells in the first few days after injection is probably derived from the serum proteins and does not represent a specific accumulation of antibody. Antibody to *Myco. butyricum* or diphtheria toxoid could not be demonstrated in the serum or cells during this interval, and antibody synthesis could not be detected this early in cultures of fragments of spleen (unpublished).

While it is unlikely that histiocytes and macrophages produce antibody, they constitute a reservoir of serum proteins. For example, the cells harvested by intratracheal washing after the injection of incomplete adjuvant or Bayol F without antigenic material showed a substantial accumulation of γ-globulin. The avidity with which these cells may accumulate serum proteins in their cytoplasm is illustrated by the rapid passage of fluorescein isothiocyanate labelled γ-globulin into the histiocytes and macrophages of the lung, that proliferated in response to Bayol F.

The short period in which antigenic fragments of mycobacteria could be demonstrated using fluorescein isothiocyanate labelled antibody to *Myco. butyricum* or by electron microscopic examination cannot be easily explained. It is possible that a small amount of antibody, that could not be detected by the techniques used, had been synthesized elsewhere and phagocytosed by these cells. The interaction of the antibody with antigenic fragments of the mycobacteria in the cells could then act as an *in vivo* " block ", similar to the use of unconjugated antibody as a control in the fluorescent antibody procedure. This is comparable to the proposal by Westwater (1940), who suggested that a local antigenic depot of tubercle bacilli may withdraw antibody from the serum that has been formed elsewhere. Non-specific adherence of protein to the fragments of mycobacteria cannot be excluded, and this could accomplish the same effect on the fluorescent

staining. On the other hand, the fact that fragments of *Myco. butyricum* could not be seen with the electron microscope after 3 or 4 days suggests that significant degradation of the fragments occurred.

It is important to recognize that while the initial proliferative reaction may not be related to an immune mechanism, it is entirely possible that immune phenomena may play a role in the later stages of the cellular reaction. However, no direct relation could be established either histologically or cytologically between the appearance of antibody and the cellular response. Once antibody is detected in the circulation it is apparently phagocytosed with the same avidity as normally circulating γ-globulin. There is a parallel between the rise and fall of γ-globulin and specific antibody within the cytoplasm of the histiocytes and macrophages and the circulating antibody titre. When considered in terms of the type of circulating antibody, the first antibody phagocytosed by the histiocytes and macrophages is probably of the 19S species. Histiocytes and macrophages contain specific antibody to diphtheria toxoid at 7 days, when only the 19S species of antibody to diphtheria toxoid can be measured in the circulation or found in tissue culture of splenic fragments (unpublished). Later, when both molecular species of antibody are found in the circulation, it is quite likely that both species are phagocytosed.

The mononuclear cells in the granulomata were quite different in their behaviour from the histiocytes and macrophages. The absence of *Myco. butyricum*, γ-globulin and antibody to diphtheria toxoid in their cytoplasms was unexpected The cells are capable of phagocytosis and accumulation of certain particles (Moore *et al.*, 1961 ; Schoenberg *et al.*, 1961, 1963). No explanation can be offered at the present time. These cells apparently differ both functionally and structurally from the histiocytes and macrophages.

The occurrence of a histological reaction of the type described here, in experimental hypersensitivity diseases can be initiated by the adjuvant, though it may be augmented by added antigens (Askonas and Humphrey, 1958*a* and *b*). The early cellular alterations in the lungs illustrate the potential of the complete adjuvant to promote a florid cellular response and the non-specific phagocytosis of γ-globulin and antibody by macrophages and histiocytes. When Freund's adjuvant is combined with certain antigens for the production of experimental hypersensitivity diseases, there is an independent contribution of the adjuvant. The cellular response to the adjuvant cannot be explained by the development of sensitivity to the mycobacterium. There is no temporal relationship between the appearance of skin sensitivity to the mycobacterium and the appearance of histiocytes, macrophages and granulomata. The character of the histological reaction is not changed at the time when skin sensitivity occurs. This follows the proposals of Rich (1951), who argued that the initial tissue reaction is due to the mycobacterial organism or its fractions and not a function of a hypersensitivity state, even though previous sensitization with the micro-organism results in a more rapid cellular response.

The question arises concerning the possibility of the synthesis of antibody by cells in the lung. The synthesis of antibody in the lung of hyperimmunized animals has been demonstrated (Askonas and Humphrey, 1958*a* and *b* ; and Thorbecke, 1959). In the present experiments the response of the plasma cells in the lung is consistent with the production of some circulating antibody. The close temporal relationship between circulating 7S γ-globulin antibody to diphtheria toxoid and

the immunofluorescent behaviour of the plasma cells suggests that these cells, as in the spleen, are synthesizing 7S γ-globulin antibody, rather than the 19S species (Schoenberg *et al.*, 1964).

SUMMARY

The cellular response in the lungs of rabbits given complete Freund's adjuvant intravenously was considered in relation to the appearance and kind of circulating antibody, the presence of antigen and antibody in the cells, and the development of tissue sensitivity to the mycobacteria. The proliferation of histiocytes in the alveolar walls, the accumulation of macrophages in the alveolar spaces, the appearance of polymorphonuclear leucocytes, lymphocytes and plasma cells, and the formation of granulomata in the lungs occurred before any specific immunological reaction was recognized. The large mononuclear cells of the granulomata differed from the macrophages and histiocytes both structurally and functionally. Circulating antibody and skin sensitivity were not detected until the cellular reaction was well established. The presence of plasma cells containing γ-globulin at a time when 7S antibody appears in the circulation is described.

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