Studies on Experimental Pulmonary Granulomas

I. Detection of Lymphokines in Granulomatous Lesions

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Granulomatous reactions were immunologically induced in guinea pigs by several procedures, including intravenous injections of Bacille Calmette Güerin (BCG) into animals immunized with complete Freund's Adjuvant and an intravenous injection of agarose beads linked to a specific antigen (dinitrophenylated bovine serum albumin) into immune animals. The tissue extracts obtained from lungs at various stages of granuloma formation were examined for macrophage migration inhibition (MIF) activity. The activity was found in a high incidence during the early stages of the granulomatous response. In contrast, MIF activity could be detected only rarely in granulomatous spleens and not in granulomatous livers. Chemotactic factor activity and mitogenic factor activity were only sporadically detectable. The MIF activity was associated with fractions showing chemical heterogeneity. One fraction was physicochemically indistinguishable from conventional lymphocyte-derived MIF; the other was a substance of large molecular weight. These results demonstrate the presence of biologically active mediators in immune granulomas, which may be related to early events involved in the induction or enhancement of such reactions. (Am J Pathol 95:391–406, 1979)

GRANULOMATOUS INFLAMMATION appears to be dependent on the particulate nature of the inducing agent and on its ability to induce a state of cell-mediated immunity.[1,2] Various experimental models have been employed to study the relationship of granuloma formation and cell-mediated immunity by using a variety of infectious agents including mycobacteria, fungi, and protozoa.[1-3]

Boros et al,[3] utilizing schistosome-egg–induced pulmonary granulomas, showed that the course of granuloma formation correlated with the development of delayed hypersensitivity to a soluble schistosome antigen when the hypersensitivity was evaluated by foot pad reactions in the mouse and by MIF production *in vitro*. They also showed that the diminution of granuloma in the later stage of infection paralleled the rise of circulating antibody levels against the same antigen.

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The studies of Kasdon and Schlossman [4] showed that the animals sensitized to human serum albumin (HSA) and challenged intravenously (i.v.) with HSA covalently linked to Sepharose 2B beads developed a typical granulomatous inflammatory response, whereas the unsensitized animal exhibited only a localized foreign-body–type reaction to the beads. Their observations suggested that the locally retained antigen may trigger antigen-specific T lymphocytes to release mediators which contribute to granuloma formation in an accelerated manner. Unanue and Benacerraf [5] showed that accelerated granuloma formation in a previously sensitized animal is immunologically specific and is dependent on the carrier rather than the hapten recognition. The ability to produce granulomas could be transferred to normal animals by T lymphocytes but not by serum from sensitized animals.[5]

The association of cell-mediated immunity with granuloma formation raises the possibility that lymphokines play a role in the evolution of granulomas, since these mediators are known to affect all components of inflammatory responses. *In vitro*, lymphokines attract and immobilize macrophages and stimulate them to mature.[6-8] Except for the schistosome egg granuloma,[9] however, virtually no data are available which demonstrate the production of lymphokines at the site of granuloma formation and their roles in the evolution of granulomas.

In the present study, several experimental models of pulmonary granulomas were explored. We produced granulomas by injecting Bacille Calmette Güerin (BCG) or complete Freund's adjuvant (CFA) into animals immunized with BCG or CFA and by injecting agarose beads coated with dinitrophenylated bovine serum albumin (DNP-BSA) into animals immunized with DNP-BSA. The presence of MIF in such lesions was investigated by direct assay of aqueous extracts of the various tissue preparations.

Materials and Methods

Immunization

Hartley guinea pigs weighing 300 to 500 g were used throughout the experiments. Four protocols were employed for the production of pulmonary granulomas:

1. Animals were immunized with an intramuscular (i.m.) injection of 0.5 mg BCG (a gift from NIH, Tokyo), and challenged intravenously (i.v.) 4 weeks later with 1 mg BCG (2 mg/ml). In this series of experiments, immunized animals without BCG challenge were used as controls.

2. Animals were immunized i.m. with 1 ml of complete Freund's adjuvant (CFA, Difco, Detroit) and challenged 3 weeks later with 1 mg of BCG i.v. (2 mg/ml). In this series of experiments, control animals were injected with incomplete Freund's adjuvant (IFA) and challenged with BCG in a similar manner.

3. Animals were immunized with 0.15 ml CFA i.v. and 5 days later challenged with 1 mg

BCG i.v. (2 mg/ml). In this series of experiments, control animals were injected i.v. with 0.15 ml of IFA and challenged with 1 mg of BCG.

4. Animals were immunized with 100 mg of DNP-BSA in CFA and challenged with the 0.5 ml suspension of DNP-BSA coupled to agarose beads in the concentration of 2.5×10^4 beads/ml. As control, uncoupled beads were injected into immunized animals or DNP-BSA coupled beads were injected into nonimmunized animals. DNP-BSA was coupled to agarose beads (Sepharose 2B) as described previously.[10] Approximately 0.28 mg of DNP-BSA was bound to 2.5×10^4 beads.

Tissue Extracts

The animals were killed at various intervals 1 to 28 days after intravenous antigenic challenge in each protocol. Lungs, livers, and spleens were removed and weighed. Each 2.5 g of tissue was homogenized in 20 ml of RPMI 1640 tissue culture medium using a mechanical homogenizer (polytron, Brinkman Instruments) for 30 seconds. During these procedures, the tissue was kept in ice. The homogenized tissues were then spun at 3000 rpm (2020*g*) for 30 minutes in a refrigerated centrifuge. The supernatant was further spun at 13,000 rpm (17,300*g*) in a refrigerated centrifuge twice for 45 minutes each to remove cell debris, The samples were then sterilized with a millipore membrane (pore size, 0.45 μ). The extracts were then aliquoted and stored at −70 C until use. Aqueous lung extracts produced as described above contained approximately 18 mg/ml of protein, as measured by method of Lowry et al.[11]

Tissue sections from lungs, livers, and spleens of all these animals were prepared at a thickness of 5 μ and stained with hematoxylin and eosin.

Migration Inhibition Assay

Macrophage migration inhibition assay was performed as described previously.[12,13] Briefly, normal Hartley guinea pigs were injected with 20 ml of sterile light mineral oil 4 days prior to the harvest of peritoneal exudate cells. The cells were washed in Hanks' balanced salt solution and then packed into capillary tubes. Macrophage migration from these capillaries in Sykes-Moore chambers was assayed in media containing various concentrations of the aqueous tissue extracts supplemented with 25% normal heat-inactivated guinea pig serum. Migration area was measured after 24 hours of incubation. The percent migration inhibition was calculated as follows:

$$\% \text{ Inhibition} = 100 - \frac{\text{Migration area in medium with experimental extracts}}{\text{Migration area in medium with control extracts}} \times 100$$

More than 20% inhibition is considered significant.[12] The control extracts were prepared using lungs, spleens, or livers obtained from the various groups of animals as specified in the previous section.

Chemotaxis Assay

The chemotactic activity in the tissue extracts was evaluated by the use of Boyden chambers with micropore filters (Nucleopore: 2 μ for neutrophils and 5 μ for macrophages) as described previously.[14] Neutrophils were obtained from 18-hour guinea pig peritoneal exudates induced by injecting 20 ml of 0.1% oyster glycogen solution. Macrophages were obtained from 4-day exudates induced by injecting light mineral oil. The cells were washed twice in Hanks' balanced salt solution and suspended in RPMI 1640 medium at a cell concentration of 2.7×10^6/ml. The extracts were diluted serially from 10 to 10,000 times in RPMI 1640 medium. Such samples were injected into the lower compartment of the chambers and the cells were placed in the upper compartment. On each filter, five random fields were counted for the cells migrating from the upper toward the lower compartment.

Mitogenic Assay

The similar samples utilized for chemotaxis assays were examined for mitogenic activity to lymphocytes. Target lymphocytes were obtained from spleens of normal guinea pigs, and the assays were performed as described previously in detail.[14]

Diaflo Ultrafiltration on Lung Extracts

Lung extracts (5 ml) were subjected to ultrafiltration using XM 100, PM 30, and PM 10 membranes (Amicon Corp., Lexington, Mass) in succession to obtain estimates of molecular weight of the MIF-like substance. The retentates were concentrated to 5 ml prior to testing. They were stored at −70 C until use.

Ammonium Sulfate Salt Precipitation

Salt precipitation on organ extracts was performed by using saturated solution of ammonium sulfate at pH 7.8. Ammonium sulfate was added slowly to 5 ml of the lung extracts with positive MIF activity or their controls until 33% saturation was reached. This was stirred for 1 hour in cold and then centrifuged in a refrigerated centrifuge at 10,000 rpm (3400*g*) for 10 minutes. The precipitate was resuspended in PBS. The above procedure was continued by bringing the ammonium sulfate concentration to 40%, 50%, and 60%, and the final supernatant was concentrated on an Amicon PM10 membrane. This was then dialyzed with a large volume of water, PBS, and RPMI 1640 medium, in this order, for 24 hours. The specimens were then millipore-filtered (pore size, 0.45 μ) and stored at −70 C until use.

Sephacryl Gel Filtration

The aqueous lung extract (5 ml) with the positive MIF activity and the corresponding lung extract from control animals were applied on a Sephacryl S-200 column (2.5 × 80 cm), which was equibriated and eluted with phosphate-buffered saline (PBS, 0.01 M, pH 7.4). Five-milliliter fractions were collected and pooled into four larger fractions. Fraction I contains the eluate from void volume corresponding to blue dextran marker; Fraction II contains eluates from the tubes eluting with IgG; Fraction III contains eluates from the tubes eluting with BSA; and Fraction IV contains eluates from the tubes eluting with egg albumin marker. The pooled fractions were concentrated to 5 ml on an Amicon PM 10 membrane (molecular weight cut, 10,000 daltons). MIF assays were performed on each fraction and were compared with the corresponding fraction from control extracts.

Results

Granuloma Formation in Lungs and MIF Activity in Their Extracts

In the first series of experiments, Hartley albino guinea pigs were immunized with BCG i.m. and challenged with BCG i.v. 4 weeks later, as described in *Materials and Methods*. The histologic sections of these lungs showed diffuse histiocytic infiltrates at approximately Days 2 to 3. Granulomas began to appear on Days 10 to 12 and then increased in size. As described in *Materials and Methods*, extracts of these granulomatous lungs were assayed for MIF activity. Controls consisted of extracts of lungs from animals that had not received the second i.v. injection of BCG. The MIF activity was only found sporadically (2 of 10 samples) in the

granulomatous lung extract (Table 1). The activity was detected only in the early stage of granuloma formation.

In the second series of experiments, guinea pigs were immunized with foot pad injections of CFA instead of BCG i.m. and then challenged with intravenous injection of BCG. Histologic sections from these lungs revealed granulomas approximately 6 to 8 days after the BCG i.v. challenge; thus, the inflammatory lesions appeared earlier than in the first series of experiments. When extracts from these lungs were assayed for MIF, activity was found more often than in the previous series of experiments; the MIF activity in the extracts was detected on Days 4, 12, 14, and 17. The range of activity found was 22 to 27% inhibition, and 6 of 12 samples showed MIF activity (Table 1). The activity was noted before the appearance of typical granulomas but also persisted even after granulomas were formed.

In the third series of experiments, the animals were immunized with i.v. injection of CFA and then challenged with BCG i.v. In this series, animals injected with IFA and challenged with BCG were used as controls. Histologic sections from this series of experiments showed diffuse histiocytic infiltrates 24 hours after the BCG challenge in CFA-injected animals. By the third day there was extensive replacement of lung parenchyma with microgranulomas composed of epithelioid cells and giant cells. Granulomas continued to grow, and the well-developed granulomas with organization were noted by Day 7. The histologic appearance is shown in Figures A and B. This is similar to the patterns obtained in the first two series, although the timing differs. In contrast, the IFA-injected lung showed diffuse histiocytic infiltrates on Day 7 and only a rare discrete granuloma was identified around Day 7 through 14, as shown in Figure 2.

Table 1—MIF Activity in Granulomatous Lungs

Experiments*	No. of lung extracts assayed	No. of lung extracts with positive MIF activity	% Positive†
Series 1	10	2	20
Series 2	12	6	50
Series 3	33	16	57
Series 4	16	15	94
Controls‡	41	0	0

* See text for the protocol for each series of experiments.

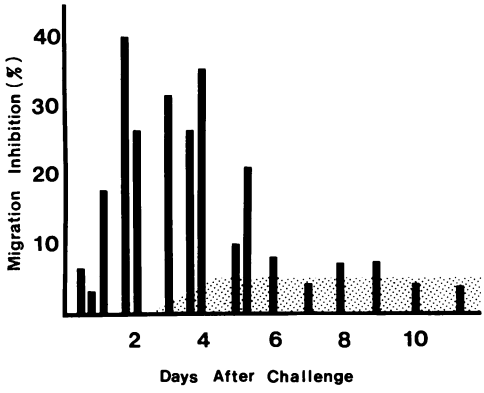
† The ratio of lung extracts with the MIF activity to the total number of the extracts examined

‡ The corresponding controls were utilized in each series of experiments as specified *Materials and Methods.* A total of 41 control lungs were examined, including 5 for Series 1, 6 for Series 2, 15 for Series 3, and 15 for Series 4.

In this series the MIF activity was detected as early as 24 hours after BCG challenge in the animals immunized with CFA. The activity was detectable through Day 5. The temporal relationship between the appearance of granulomas in the lung and the MIF activity recovered from its extract is summarized in Text-figure 1. Sixteen of thirty-three lung extracts examined showed MIF activity (Table 1).

In contrast, when MIF assays were performed on spleen and liver extracts, only 2 of 28 splenic extracts and none of the total of 35 liver extracts had detectable MIF activity, although by Day 7 both spleen and liver showed well-formed granulomas.

In the last series of experiments, the animals were immunized with DNP-BSA in CFA and challenged with DNP-BSA coupled to agarose beads. Two groups of control animals were used in this series: one group was immunized with DNP-BSA in CFA and challenged with plain beads and the second was not immunized but injected with beads coated with DNP-BSA. Histologic examination of lungs from guinea pigs of the experimental group showed the formation of granulomas around the beads; these were composed of epithelioid cells and giant cells, as shown in Figure 3. In addition, a few neutrophils were observed around the beads. These changes were seen in the animals within 2 days after the injection of the beads. Animals killed on the fourth day showed further increase in the size of granulomas; most of the lung parenchyma was completely replaced by granulomatous inflammation. In contrast, the control groups of immunized animals injected with plain beads and of nonimmunized animals injected with DNP-BSA–coupled beads showed slight histiocytic collection around beads, although an occasional small foreign body granuloma was noted by 7 days after the injection of beads (Figure 4). In this series, MIF activity was noted in 15 of 16 lung extracts from experimental



TEXT-FIGURE 1—MIF activity detected in lung extracts obtained at various stages of granulomatous lesions. *Bars* indicate MIF activity in percent inhibition (see text for definition); *dotted area* indicates the approximate histologic development of pulmonary granuloma lesions.

animals, but no activity was recovered from control lungs. The activity was found 2 days after challenge and persisted through Day 5. The average percentage inhibition of macrophage migration was 37%, with a range of 21 to 71%.

Other Lymphokine Activities in the Extracts

Extracts obtained in the course of the above experiments were also examined for lymphokine activities other than MIF. In spite of extensive studies utilizing various dilutions or concentrations of the extracts, chemotactic activity for macrophages (MCF) was found only sporadically (in 4 of 24 samples) and no consistent trend was observed except that the activity was always found in 100- or 1000-fold dilutions of the extract. This is in contrast to the situation for MIF, in which undiluted extract was necessary for detecting activity, as described above. Similar results were obtained for neutrophil chemotactic activity (NCF). Because of the sporadic nature of these results, no attempt was made to further characterize the chemotactic factors.

In addition, the extracts were examined for the mitogenic activity (MF) to lymphocytes. After many experiments utilizing various dilutions of the extracts, we found only minimal activity (stimulation index, 1.5 to 2.3) in some of the extracts; the reproducibility of such results was very poor.

Thus, in contrast to the situation for MIF, no significant or consistent detection of MCF, NCF, or MF lymphokines was achieved.

Physicochemical Characteristic of MIF in the Lung Extracts

Basic Characteristics, Including Heat Stability and Dialyzability

It is important to demonstrate that the extracts' migration inhibitory properties are not merely due to cytotoxic effects. To examine this, macrophage migration in the media containing the experimental extract was observed after prolonged incubation (48 to 72 hours). Under these conditions the inhibited migration observed at 24 hours was found to be reversible, demonstrating viability of the target cells. Trypan blue dye exclusion test on the cells incubated (24 hours) with various organ extracts showed no difference of cellular viability (75 to 85%) between experimental and control extracts. These results indicate that the MIF activity in the extract is not due to a cytotoxic factor. The material was found to be heat-stable at 56 C for 30 minutes. On dialysis of the lung extracts against a large volume (1:100) of RPMI 1640 medium at 4 C for 24 hours, the activity was retained.

Ammonium Sulfate Precipitation

The pulmonary extracts were sequentially precipitated by saturated ammonium sulfate solution. As shown in Table 2, the fraction precipitated by the 40% saturated solution had MIF activity.

Sephacryl Gel Filtration

Two-fold–concentrated extract equivalent to 10 ml of the original extract was applied on Sephacryl S-200 gel column and eluted with pH 7.4 PBS. The elution pattern measured at 280 μ is shown in Text-figure 2. Four pooled fractions were obtained as described in *Materials and Methods* and were assayed for MIF activity. When migration in each fraction was compared with that in the corresponding fraction from control extracts, the MIF activity was detected in two fractions, one corresponding to void volume with a molecular weight greater than 100,000 daltons and the other in the fraction which has a mean molecular weight of 67,000 daltons, as shown in Text-figure 2.

Ultrafiltration by Amicon Membranes

The lung extracts were fractionated by ultrafiltration with Amicon membranes. The MIF activity was retained by PM30. The activity was partially lost by the filtration through XM100. These results agree with the result of a gel filtration study, indicating that most of the MIF activity belongs to the substance with a molecular weight between 30,000 and 100,000.

Discussion

The present studies have clearly shown that MIF activity can be detected in the extracts obtained from granulomatous lungs. The physicochemical analysis of the substance has revealed that it is heat-stable at 56 C for 30 minutes and that it is nondialyzable. Studies with gel filtration and ultrafiltration membranes have indicated that at least two heterolo-

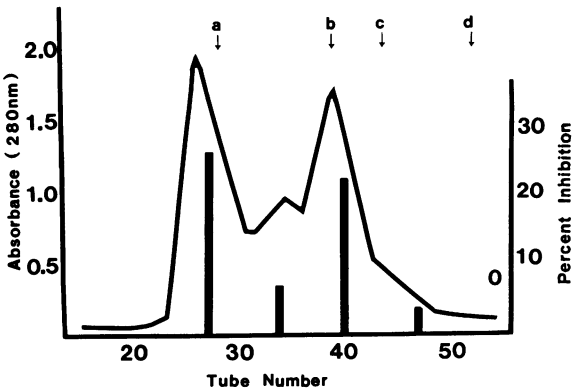
Table 2—Ammonium Sulfate Salt Precipitation of Lung Extracts

Salt concentration (%)	% Migration inhibition*
33	2.2 ± 7.4
40	21.4 ± 4.0
50	1.9 ± 6.9
60	−1.5 ± 4.5
>60	−7.0 ± 2.1

* Data represent a mean with standard error of results obtained from three independent experiments.

gous fractions are responsible for the MIF activity: one has a molecular weight larger than 100,000 and the other has a mean molecular weight of 67,000. Thus, the smaller substance with MIF activity found in the lung extract is similar to conventional, lymphocyte-derived guinea pig MIF produced *in vitro*.[15,16] Further studies on this molecule with anti-lymphokine antibody [17] and sugar-binding characteristics [18] would provide further evidence relating the identity or nonidentity of the MIF activity observed to that of conventional MIF. The significance of the larger molecular weight substance with MIF activity found in the granulomatous lung extracts is unknown. Myrvik et al [19] found an increased amount of hyaluronic acid in lavage fluids obtained from the granuloma-induced lungs of rabbits. They have shown strong macrophage aggregating activity of hyaluronic acid, indicating that the substance may be important in granuloma formation in rabbits. Since hyaluronic acid has a large molecular weight (>100,000), we looked for hyaluronic acid in the larger molecular weight substance with MIF activity. A preliminary study, however, has revealed no significant increase in hyaluronic acid in our preparations compared with negative controls.

Various evidence, including transfer experiments by T cells,[5] carrier specificity of granuloma formation,[5] and temporal correlation between granuloma formation and development of delayed hypersensitivity,[3] has pointed to an essential role of the cell-mediated immunity in immunologic granuloma formation. The present results support these contentions since we could find MIF activity in the granulomatous lung extracts. It should be stressed that this does not imply that MIF is involved in the mechanism of granuloma induction; MIF represents a marker for the presence of lymphokine activity and demonstrates that functionally active T cells involved in cellular immunity are present. Thus, it remains unclear



TEXT-FIGURE 2—Elution profile of Sephacryl S-200 gel filtration of lung extracts. MIF activity (*bars*) was found in the two pooled fractions. Molecular markers are indicated by *a*, blue dextran; *b*, bovine serum; *c*, ovalbumin; and *d*, cytochrome c albumin.

whether MIF or some other lymphokine is playing a prime role in the induction of pulmonary granuloma. Another possibility is that lymphokine production is not causal but is a consequence of granuloma formation. The determination of the definite cause and effect relationship between any lymphokines and granuloma formation will have to await results from experiments in which one attempts to induce granulomas in an accelerated manner by injecting lymphokines into nonimmune animals. In any case, it should be noted that the MIF activity was usually recovered in lung extracts prior to the appearance of granulomas, as indicated by light microscopy (Text-figure 1). This temporal relationship suggests that lymphokines do play a role in the induction of granulomas.

In the present experiments, we have employed four protocols to induce pulmonary granulomas. Although all four procedures produced similar granulomatous lesions in the lung by 2 weeks after the intravenous injection of BCG or antigen-coated agarose beads, the kinetics of granuloma formation differed according to the procedure employed in each series of experiments. Thus, the procedures used in the second series of experiments led to more rapid induction of granulomatous lesions than did the first series of experiments; the third was faster than the second; and the fourth was the fastest. As shown in Table 1, the sooner the granuloma developed, the more frequently the MIF activity was recovered in their extracts. These results, therefore, seem to indicate that immunologic events accelerate the formation of pulmonary granulomas. In the first series of experiments, immunologic reaction *in situ* may have been minimal; this may be the reason for slower development of granulomatous lesion and for poor recovery of the MIF-like substance in the extract.

Boros et al [9] reported that the MIF activity was recovered from culture supernatants when the shistosome-egg–induced granuloma was cultured *in vitro* without additional stimulation. Since the lung tissue in their study contained fully developed granulomas, the results seem to indicate that the cells within granuloma-containing tissue had the ability to produce MIF. In contrast, the present studies have shown that the extracts from lungs at an early stage of granuloma formation contained MIF while the extract from lungs with fully developed granulomas had the activity only occasionally. This suggests that *in vivo* the capacity for mediator production is lost due to suppressive or regulatory events which are not duplicated in *in vitro* settings. Another important point that emerged from these studies is that the cellular and humoral factors may have to be examined in terms of their effect on the initial induction as well as on the continuation of granulomatous inflammatory reactions.

Another interesting but puzzling finding has emerged from the present study: Although spleens and livers from these experimental animals have shown granuloma formation as extensive as that in the lungs, it was almost impossible (2 of 28 spleens and none in livers) to find MIF activity in those extracts. One possibility may be that MIF produced in those organs is subject to immediate destruction by various enzymes in the organs. A slower rate of destruction may have enabled us to detect it in the lung extracts. Another possibility is that different sets of lymphokines are operative in various cell-mediated immune reactions at different reaction sites. Thus, it has been shown that chemotactic factors for macrophages and lymphocytes, but not MIF, were recovered from delayed-type skin reaction sites,[20] while MIF appeared to be the main lymphokine involved in the induction of macrophage disappearance reactions in the peritoneal cavity.[21] Therefore, it is possible that different mixtures of lymphokines may be responsible for the induction of granulomatous lesions in different organs affected.

The present results demonstrate that one may detect at least one lymphokine, ie, MIF, in granulomas induced by immunologic means. Since it was preferentially found in the early phase of granulomatous inflammation, the role of lymphokines may be to enhance or potentiate granulomas in the stage of induction and to focus the inflammatory response into relatively compact masses, perhaps by interfering with inflammatory cell migration.

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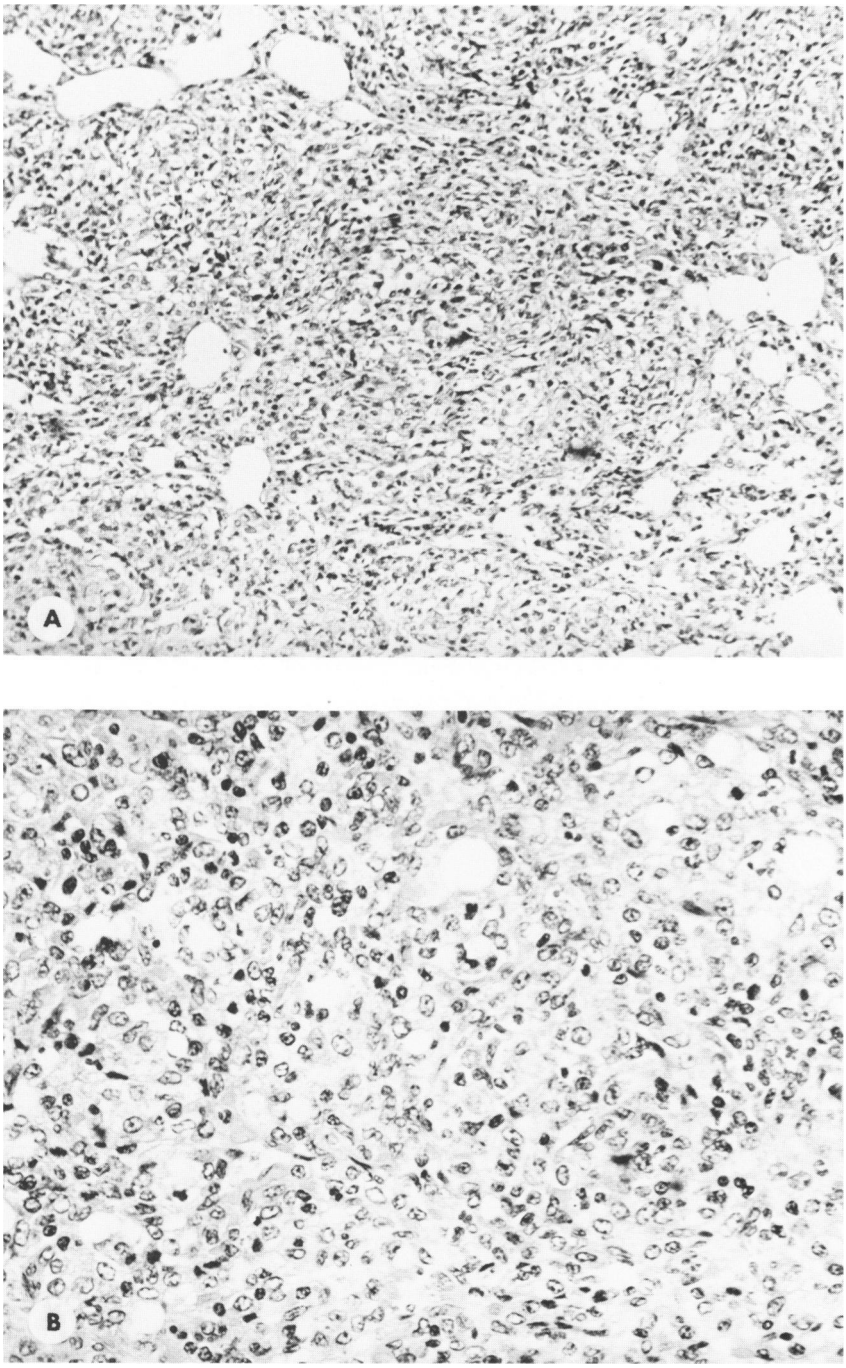
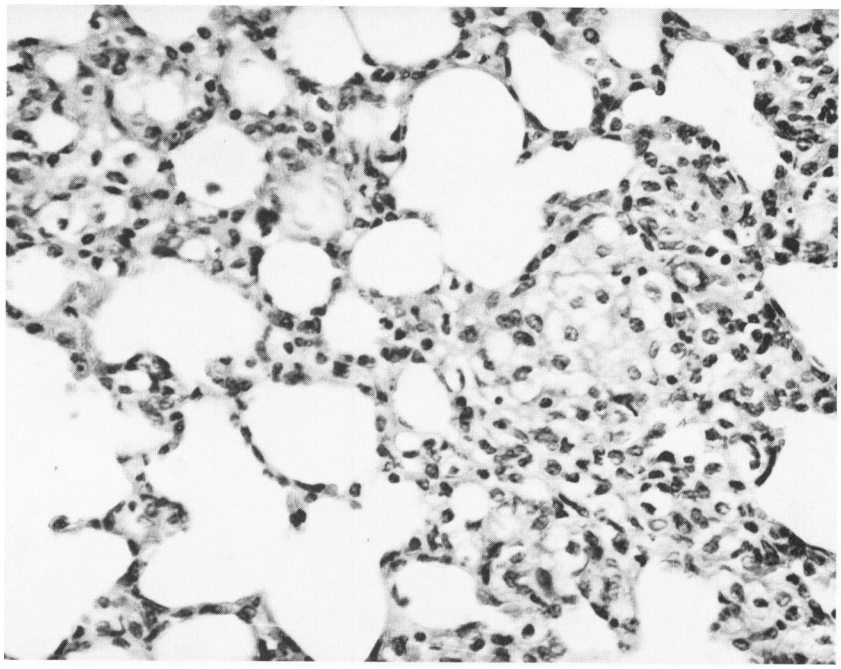


Figures 1A and B—Lung at 7 days after intravenous challenge with BCG in guinea pigs immunized with CFA (Experimental series 3). Complete replacement of lung parenchyma by epithelioid cells is observed, with a few giant cells (**B**) and evidence of organization (**A** and **B**). Lungs from experimental groups in Series 1 and 2 have shown similar histology, although it took longer (2 weeks) to form the well-organized granulomatous lesions. (H&E; **A**, × 500; **B**, × 1000)

Figure 2—Lung at 7 days after intravenous challenge with BCG in guinea pigs immunized with IFA, showing a microgranuloma predominantly composed of histiocytes surrounded by mild interstitial hystiocytic infiltration. (H&E, × 1000)

Figure 3—Lung from an immune guinea pig injected with DNP-BSA-coated beads. At 4 days after the injection, extensive infiltrate around the bead is composed of epithelioid cells and occasional giant cells. A few neutrophils can also be seen around the bead. (H&E, × 1000)



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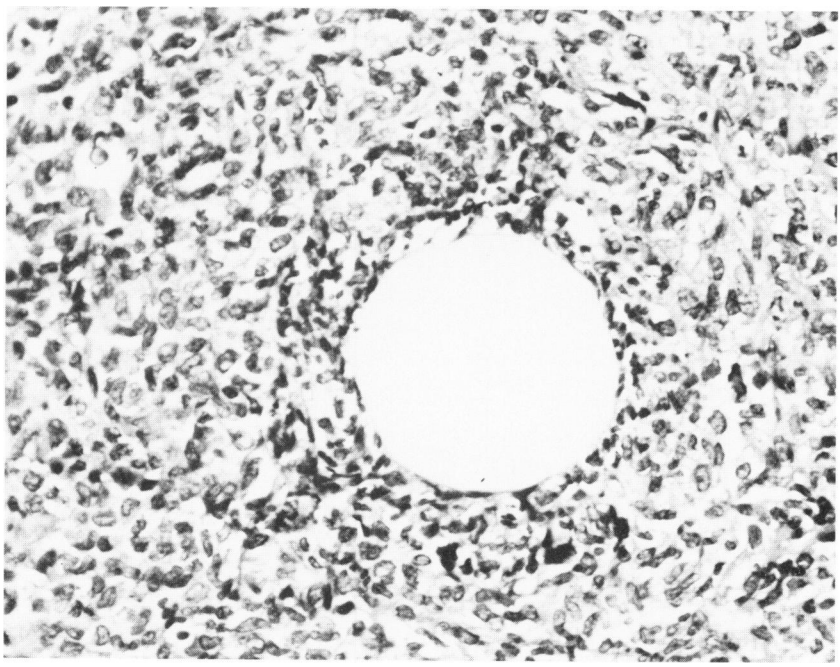

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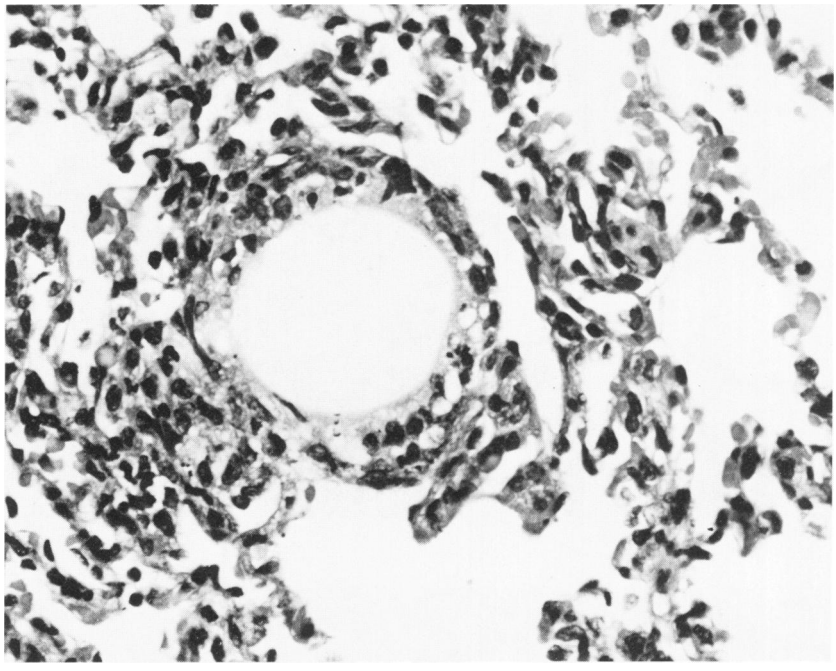

Figure 4—Lung from an immune guinea pig injected with uncoated beads. At 4 days after the injection, a small number of histiocytes are noted around the beads. (H&E, × 1000)