THE RESPONSE OF THE LYMPHATIC TISSUE TO BACTERIAL ANTIGEN

STUDIES IN GERMFREE MICE

RICHARD E. HOROWITZ, M.D.*; HEINZ BAUER, M.D.;
FIORENZO PARONETTO, M.D.; GERALD D. ABRAMS, M.D.†;
KENNETH C. WATKINS, D.V.M., AND HANS POPPER, M.D.

From the Department of Germfree Research, Division of Basic Surgical Research, the Department of Experimental Pathology, Walter Reed Army Institute of Research, Washington, D.C., and the Departments of Pathology, The Mount Sinai Hospital and College of Physicians and Surgeons, Columbia University, New York, N.Y.

A previous study [1] indicated that the number and distribution of immunologically competent cells in the lymph node and spleen is different in germfree mice as compared to conventional animals while phagocytes and lymphocytes are not affected. These findings stimulated a study of the response of these organs to bacterial antigenic stimulation in both types of animals by histologic, histochemical, autoradiographic, immunocytochemical and serologic techniques to determine the role of the normal microbial flora in the immune response.

The current view of the response to a single local injection of particulate antigen is as follows: Antigen is first taken up by macrophages of the marginal sinuses,[2–5] followed by intracellular degradation of antigen.[6,7] Then protein-forming cells,[8] so-called "immunoblasts" [9] or hemocytoblasts [10] appear in the lymph nodes and probably give rise to immature and mature plasma cells containing antibody.[11–14] An uncontrollable variable in this scheme is the influence of the microbial flora in conventional laboratory animals. Whether experience with bacterial antigens influences the immune reaction cannot be determined in conventional animals. Studies in "unstressed" germfree mice [1] and guinea pigs [15] have shown that immunologic phenomena of any kind are rare in lymphatic tissue of germfree animals. This indicates that in the intact animal the indigenous microflora is a major source of antigenic material and in its

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* United States Public Health Service Postdoctoral Research Fellow (NIAID). Present address: Department of Pathology, University of Southern California School of Medicine, Los Angeles, Calif.

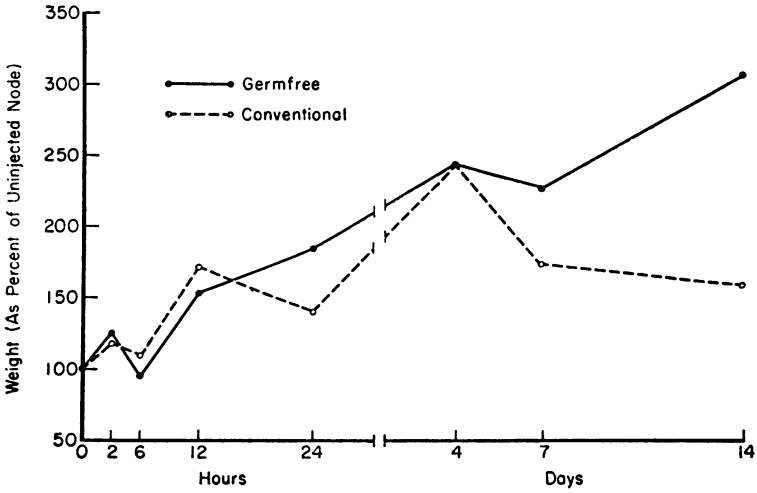
† Present address: Department of Pathology, University of Michigan School of Medicine, Ann Arbor, Mich.

absence immunologic activity is minimal. Thus, in the germfree animal, immune reactions can be studied in a more precise setting.

Material and Methods

Seventy-six germfree (GF) and 76 conventional (CONV) Swiss Webster mice, 3 months of age and 25 to 35 gm. in weight were used. GF and CONV mice were born and reared under identical conditions in plastic isolators as previously described.[1] A sterile suspension (0.05 cc.) of formalin-killed *Escherichia coli* 0128:B12 (Difco Laboratories) containing approximately 700,000 organisms per inoculum was injected subcutaneously into the left forefoot of all mice. The animals were given 40 μc. of sterile tritium-labeled thymidine intravenously 1 hour prior to sacrifice; autoradiographs were prepared as described elsewhere.[16] Groups of animals were anesthetized with ether and exsanguinated by cardiac puncture 2, 6, 12, 24 hours and 4, 7 and 14 days after antigen administration. Bilateral axillary and brachial lymph nodes and the spleen were weighed and prepared for histologic and immunocytochemical study for gamma globulin with fluoresceinated rabbit anti-mouse gamma globulin serum.[1] Acetone-fixed cryostat sections were also treated with fluoresceinated anti-*Escherichia coli* 0128:B12 serum (Difco Laboratories) for antigen localization. The antiserum was absorbed with pork liver powder before use.

Serum antibody titers were determined as follows: Drops of the antigen suspension were placed on ruled microscope slides and allowed to air-dry. They were then fixed in dry acetone for 10 minutes and washed in pH 7.2 phosphate-buffered saline for 10 minutes. Serial dilutions of serum from individual mice were placed on the slides for 30 minutes at room temperature and the slides were again washed in buffered saline for 10 minutes. This was followed by fluorescein-labeled rabbit anti-mouse gamma globulin serum for 30 minutes; the slides were washed, covered, and examined microscopically for fluorescence of serum antibody attached to particles of antigen. Other sero-types of *E. coli* and routine blocking of specific fluorescence by non-fluoresceinated antiserum served as controls. The reciprocal of the highest dilution of serum giving a definite fluorescence was considered as the titer and then converted to $\log_2$ for evaluation. The sensitivity of the method was assayed by comparison with



Text-fig. 1. Mean weight of draining lymph nodes in germfree (GF) and conventional (CONV) mice following foot pad injection of *E. coli* antigen, expressed as per cent of mean weight of nodes from uninjected side.

slide agglutination and precipitin techniques and was found to be 2 to 3 times more sensitive.

Lymph nodes were arbitrarily graded from 0 to 4+ for: macrophage size and number in marginal and medullary sinuses; antigen content of macrophages; number of cells in medullary cords; reaction centers; number of "immunoblasts" and tritium-labeled cells in peripheral and intermediate zones of the cortex and at the corticomedullary junction; and the number of plasma cells and gamma globulin containing cells. In each instance the GF and CONV nodes on the injected side were compared with the paired node from the uninjected side and with nodes from other animals.

Repeated microbiologic tests [17] of GF animals were always negative; tests of the CONV mice showed a mixed flora, including *Escherichia coli* which did not agglutinate with the O128:B12 antiserum.

Results

Lymph Node Weights

At 2 hours both GF and CONV lymph nodes were edematous and showed a slight weight increase (Text-fig. 1). The edema subsided by 6 hours, but from then until 4 days the nodes became progressively heavier, finally weighing almost 2½ times as much as the nodes from the uninjected side. The CONV nodes returned toward baseline weight at 7 days while the GF nodes reached maximum weight at 14 days.

Acute Inflammatory Cells

Two hours after injection, the marginal sinuses of both GF and CONV nodes contained segmented neutrophils and eosinophils. At 6 hours such cells were more numerous and extended into the medullary sinuses; after 12 hours the inflammation subsided and disappeared after 24 hours. The nodes from the uninjected side showed no inflammation at any time.

Macrophages

The number and distribution of macrophages in marginal and medullary sinuses differed neither between injected and uninjected side, nor between GF and CONV animals. In both GF and CONV mice, however, the macrophages on the injected side showed a progressive enlargement, beginning at 6 hours, with fine cytoplasmic vacuolation and dispersion of PAS-positive granules (Fig. 1). These changes reached a peak at 12 hours in the CONV and at 24 hours in the GF animals (Fig. 2) and were no longer apparent at 4 days in either.

Antigen Localization

Fluoresceinated anti-*E. coli* serum combined with macrophages in 2 forms—a particulate form with the characteristics of whole bacteria, and a diffuse form, not recognizable as bacteria. No extracellular material bound the antiserum. Some cells contained only particulate antigen;

other cells contained only diffuse antigen, while yet others contained antigen in both forms. At 2 hours, antigen was present in macrophages of all lymph nodes on the injected side. It was mainly particulate and limited to the marginal sinuses (Fig. 3). At 6 hours, particulate antigen was equally present in GF and CONV macrophages but diffusely staining antigen was more common in the CONV nodes. This difference continued at 12 hours and was even more marked at 24 hours when virtually all CONV macrophages contained diffuse antigen (Fig. 4) while most antigen in GF macrophages was still particulate. By 4 days, particulate antigen had disappeared from both GF and CONV nodes. Diffuse antigen had reached equal levels and was demonstrated in occasional macrophages for the remainder of the experiment. No antigen was ever demonstrated in lymph nodes from the uninjected side or in the spleens.

"Immunoblasts" (Hemocytoblasts)

A few of these large basophilic and pyroninophilic cells were present in the cortex of all lymph nodes, and no changes were observed on the uninjected side at any time. The nodes draining the site of inoculation showed a progressive increase in "immunoblasts," beginning at 24 hours. The first area of the node to show increased numbers of these cells was the intermediate zone at 24 hours, and large numbers were present at 4 and 7 days. Accumulation of "immunoblasts" at the corticomedullary junction was first noted at 4 days, reached a peak at 7 days, and declined to baseline levels in the 14-day CONV nodes, while at 14 days the corticomedullary junction of GF nodes still showed a heavy concentration of these cells. In the subcapsular cortical nodes (primary follicles, solid nodules) "immunoblasts" did not appear until the seventh day, and by the 14th day these peripheral concentrations had formed into reaction centers in 4/5 of all GF and CONV animals (Fig. 11).

Tritium-labeled Cells

Lymph nodes from the uninjected side of all mice showed no changes in the number and location of labeled cells (Fig. 5). In the intermediate zone the incidence and time of appearance of labeled cells paralleled the incidence and time of appearance of "immunoblasts." At 24 hours, labeled cells were numerous in the intermediate zone (Fig. 6). Only about 20 per cent of the labeled cells were "immunoblasts," however; the others were large and medium lymphocytes. At the corticomedullary junction labeled cells were at their peak by 4 days and thus were seen earlier than the "immunoblasts" identified in conventional sections (Fig. 7). Most of the labeled cells at the corticomedullary junction

were large and medium lymphocytes. In the cortical nodules the maximum number of labeled cells occurred at 7 days, having the appearance and incidence of the "immunoblasts" demonstrated histologically. At 14 days the majority of hemocytoblasts and large lymphocytes in the reaction centers contained tritium (Fig. 8). Such reaction centers were only rarely seen in uninjected nodes and, when present, contained less label.

Gamma Globulin-containing Cells

Until 24 hours after injection their number was similar in the nodes from the injected and uninjected sides, but as in previous studies,[1] they were fewer in the GF. On the injected side, increasing numbers of gamma globulin-containing cells began to appear in both GF and CONV animals at 4 days and reached a peak at 7 days (Figs. 10 and 12). At these times most of the cells were the more mature B and C types of Vazquez,[18] and were located at the corticomedullary junction and in the medullary cords. At 14 days, gamma globulin-containing cells in the GF had returned toward baseline levels while they remained high in the CONV. The incidence and distribution of gamma globulin-containing cells in nodes of the uninjected side and in the spleens did not change throughout the experiment.

Plasma Cells

The incidence and order of appearance of plasma cells identified by light microscopy paralleled that of gamma globulin-containing cells. The early injected and uninjected CONV nodes contained larger numbers of such cells than the GF, but did not exceed their respective baseline.[1] On the injected side plasma cells increased both in GF and CONV nodes beginning at 24 hours, reaching a peak at 7 days with many plasma cells still present 14 days after inoculation (Fig. 9).

Spleen

The spleens in all mice increased slightly in weight from 24 hours to 7 days after injection of antigen but revealed no histologic, autoradiographic or immunocytochemical changes.

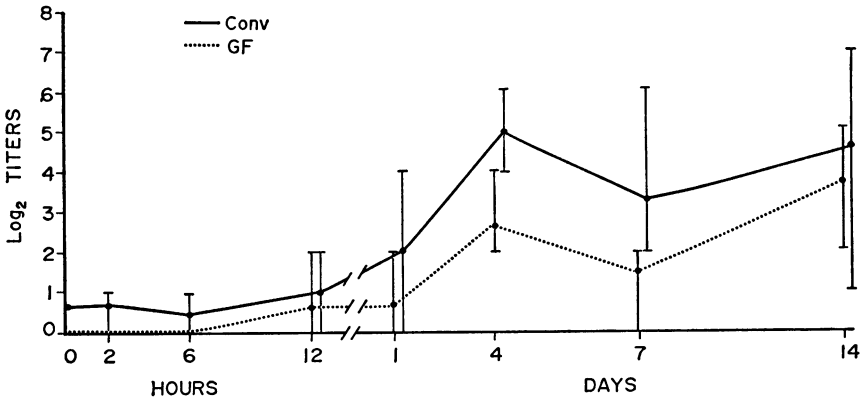
Antibody Titers

Serum antibody against *E. coli* 0128:B12 (Text-fig. 2) was not detectable in the GF mice at 2 and 6 hours after inoculation. At 12 and 24 hours an occasional GF animal had a low titer. Starting at 4 days most GF mice showed antibody titers which were highest at 4 and 14 days. Most 2 and 6 hour CONV mice and some uninjected CONV controls showed low antibody titers. Beginning at 1 day after inoculation,

titers increased progressively, reaching a peak at 4 days and remaining elevated thereafter.

Discussion

These experiments demonstrated no fundamental differences between the response of GF and CONV animals to local injections of killed bac-



Text-fig. 2. Titers of anti-*E. coli* antibody in germfree (GF) and conventional (CONV) mice following foot pad injection of *E. coli* antigen, expressed as $\log_2$ (mean and range) of the reciprocal of the highest dilution giving definite fluorescence.

teria, but a more rapid reaction of CONV lymphatic tissue. The inaccuracies inherent in the quantitative evaluation of a dynamic process by histologic techniques were partly eliminated through the application of multiple independent parameters, i.e., autoradiographic, immunocytochemical and serologic techniques.

Even during an initial period of mild acute lymphadenitis, sinus macrophages of all nodes contained ingested bacteria. Therefore, the flow of materials from peripheral tissues to regional lymph nodes through lymphatic vessels was unimpaired in GF animals, and GF macrophages were fully capable of phagocytosis. This correlated well with our previous morphologic studies [1] and the clearance experiments of Thorbecke [19] and Doll.[20]

The next phase in the immune reaction, the intracellular digestion or degradation of antigen within phagocytic cells, has been extensively studied,[3,6,21,22] with conflicting indications as to enhancement of the rate of intracellular breakdown of antigen following prior experience with the antigen. In the observations presented here the transition from particulate to diffuse antigen occurred sooner in the macrophages of the CONV mice. It could not be determined whether this accelerated intracellular digestion was nonspecific and related to the presence of

a microflora *per se,* or whether it was related to prior experience with antigenically similar organisms.

After the intracellular degradation of particulate antigen, immunologically active cells (hemocytoblasts, "immunoblasts") proliferated, as shown by their avid uptake of tritiated thymidine injected only one hour prior to sacrifice. These cells first appeared in the intermediate zone of the cortex, then in large numbers at the junction of cortex and medulla, and, still later, in the medullary cords. The formation of the "immunoblasts" preceded the appearance of gamma globulin-containing cells which were histologically recognizable as young and mature plasma cells. Gamma globulin first appeared in immature cells at the corticomedullary junction and in the proximal medullary cords, and only later were the more mature cells seen in the medullary cords. Plasma cells similarly were at first immature and located in sheets at the corticomedullary junction before they appeared in more mature form and constituted the majority of cells in the medullary cords. This sequence of events was identical in GF and CONV mice and confirmed the kinetic studies of Nossal with teased preparations of lymph nodes from immunized CONV rats.[23]

When gamma globulin production was well under way and when the number of tritium-labeled cells and "immunoblasts" in the intermediate zone, corticomedullary junction and in the medullary cords decreased, "immunoblasts" suddenly proliferated in the peripheral cortical nodules and formed classic reaction centers two weeks after antigen inoculation. The individual "immunoblast" in the cortical nodules as well as those congregated with the reaction centers were strongly labeled with tritium, attesting to their sudden formation. These late phenomena were alike in GF and CONV nodes. The cells in the germinal centers at no time contained gamma globulin; this confirmed White's observation [13] in conventional rabbits that no antibody was formed in germinal centers after a single injection of antigen.

The method used in this experiment for determining antibody titers to *E. coli* organisms has not been described before and depends on the fact that antibodies from the infected mouse bind specifically to antigen on a slide. The antibody is then detected with fluorescein-labeled anti-mouse gamma globulin serum. This is in essence the indirect Coons technique,[7] utilizing specific antigen previously placed on a slide. The method is more sensitive than standard agglutinin or precipitin reactions and specificity is maintained since no cross-reactions with other serotypes of *E. coli* occurred.

The development of circulating antibody correlated well with the morphologic changes but was quantitatively less in the GF. The quantita-

tive difference probably reflected a slower rate of production rather than an impaired ability to form antibody. One possible reason for this lag could be the slower degradation of antigen within GF macrophages. Another possibility was the superimposition in CONV mice of a minor anamnestic response as a result of prior contact with *E. coli* of similar antigenicity. Although the response of GF and CONV animals to a single injection of particulate bacterial antigen differed quantitatively, the essential sequence of events was the same. Thus, the normal microflora did not influence the immune response, and the virginal immunologic system of the GF mouse responded to antigen challenge in a manner similar to that of the immunologically experienced CONV animal.

Summary

Lymph nodes from 76 germfree and 76 conventional mice were examined by histologic, histochemical, autoradiographic and immunocytochemical techniques, at intervals of 2 hours to 14 days after foot pad injection with killed *E. coli* organisms. All nodes draining the injection site showed transient initial acute lymphadenitis and persistent weight gain which was maximal 4 days after injection in the conventional and at 14 days in germfree mice. Particulate antigen appeared in sinus macrophages 2 hours after injection in both germfree and conventional nodes but disintegrated more rapidly in the latter. "Immunoblasts" (hemocytoblasts) proliferated first in the intermediate zone of the cortex and later at the corticomedullary junction. This was followed by the appearance of plasma cells, and gamma globulin-containing cells at the corticomedullary junction and in the medullary cords in both germfree and conventional lymph nodes. Circulating antibody developed in all mice after 4 days, with higher titers in the conventional animals, and reaction centers appeared at 7 and 14 days in the lymph nodes of both germfree and conventional mice.

These studies indicate that the lymphatic tissue of the germfree animal is capable of responding to antigenic stimulation. Previous experience with a microbial flora confers only minor advantages upon conventional animals, resulting in earlier and greater antibody production. In other respects the dormant lymphatic system of germfree animals reacts like its immunologically experienced conventional counterpart.

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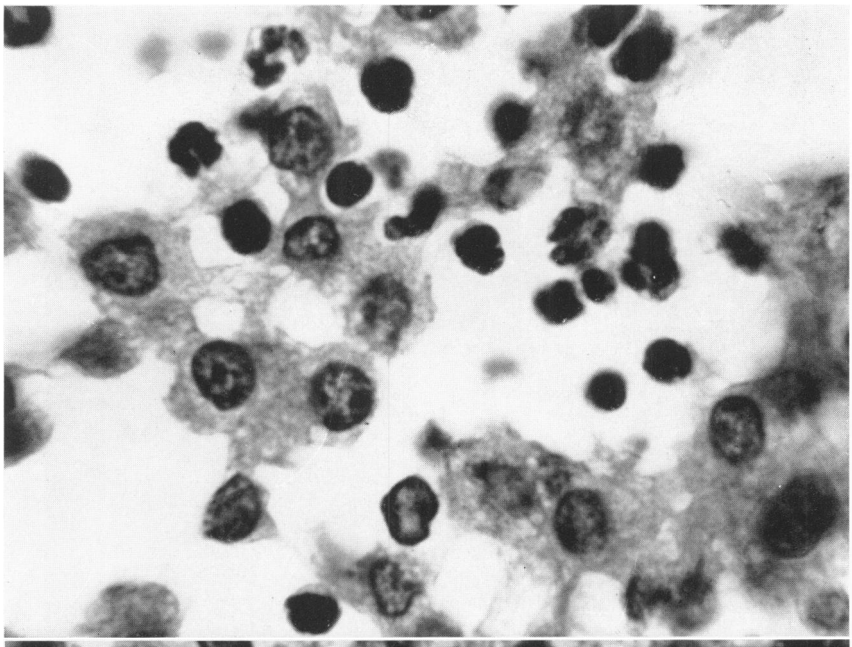
LEGENDS FOR FIGURES

FIG. 1. Marginal sinus of a draining node 6 hours after injection of antigen. Macrophages are moderately enlarged. Hematoxylin and eosin stain. × 1,224.

FIG. 2. Marginal sinus of a draining node 24 hours after injection of antigen. Macrophages are markedly enlarged. Hematoxylin and eosin stain. × 1,224.



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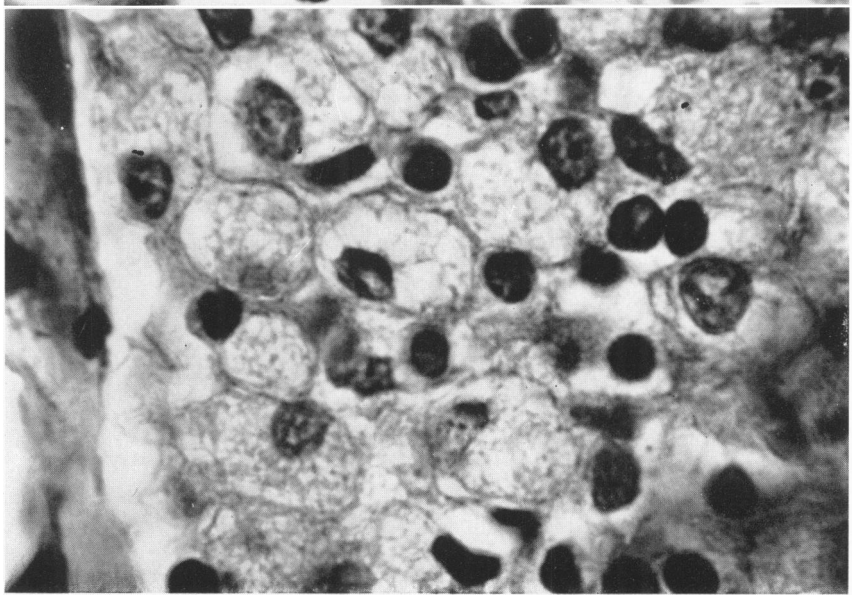

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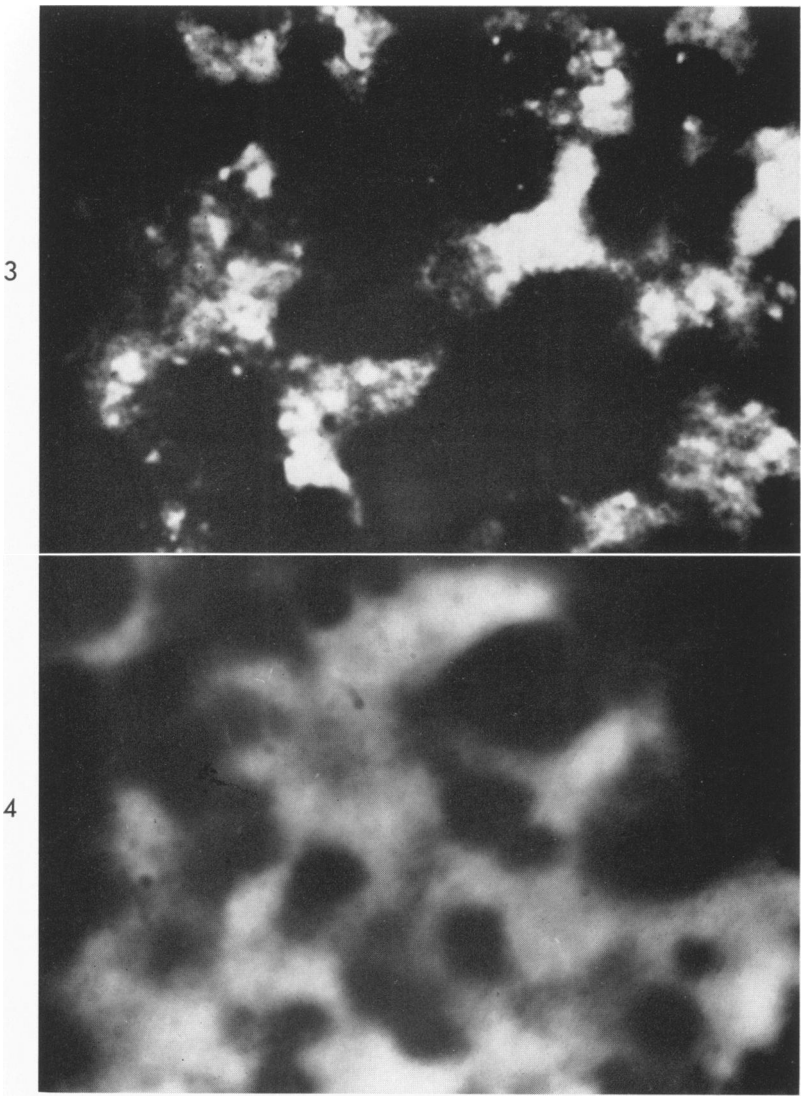
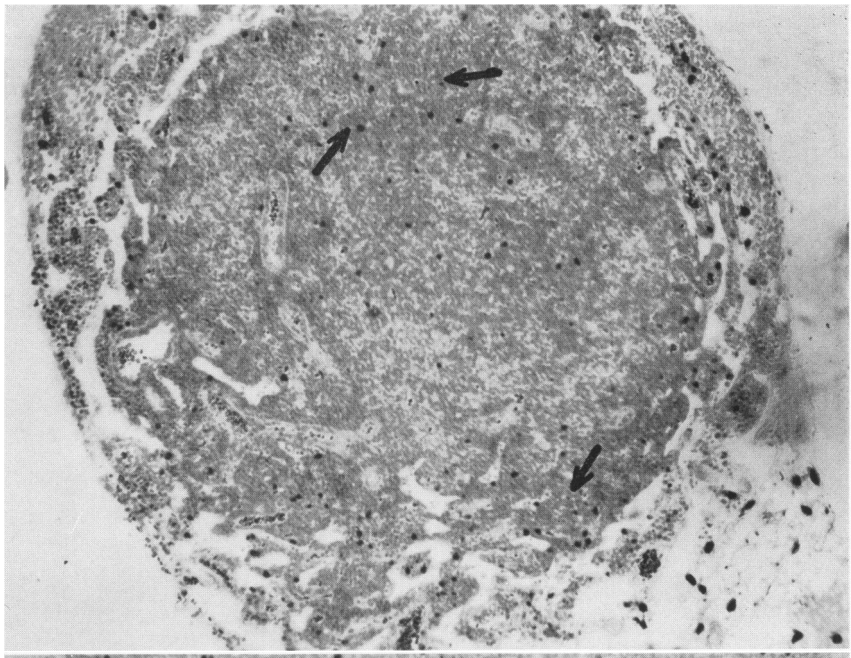


FIG. 3. Marginal sinus of a draining node in a germfree mouse 12 hours after injection of *E. coli*. The particulate nature of antigen is apparent in macrophages. Fluoresceinated anti-*E. coli* serum. × 1,200.

FIG. 4. Marginal sinus of a draining node in a conventional mouse 24 hours after injection of *E. coli*. The antigen in macrophages has a diffuse appearance. Fluoresceinated anti-*E. coli* serum. × 2,200.



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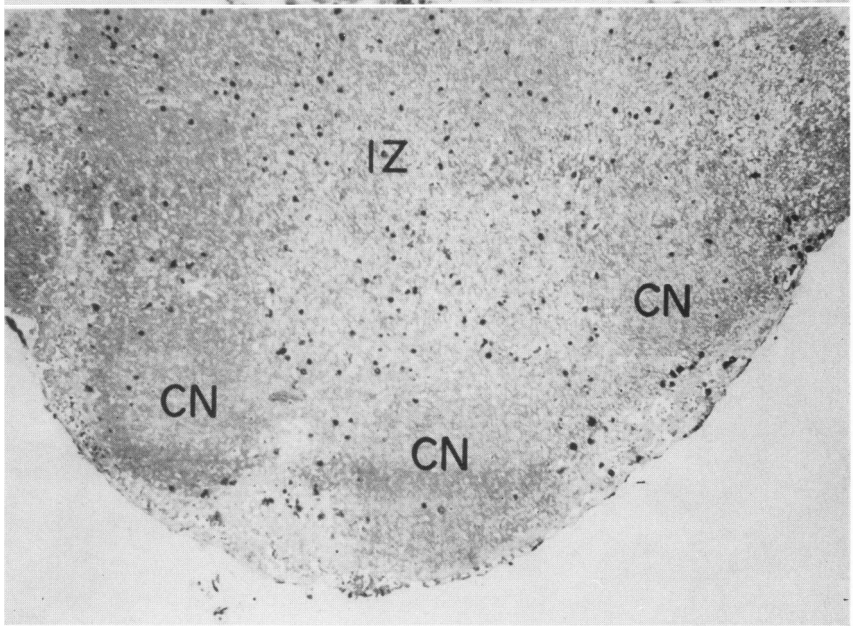

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FIG. 5. Lymph node from the uninjected side of germfree control mouse 1 week after injection with *E. coli*. There is a paucity of labeled cells (arrows). Radioautograph-Giemsa stain. × 86.

FIG. 6. Draining lymph node of a conventional mouse 24 hours after injection. The node is enlarged and contains many labeled cells in the intermediate zone (IZ) and a paucity in the cortical nodules (CN). Radioautograph-Giemsa stain. × 86.

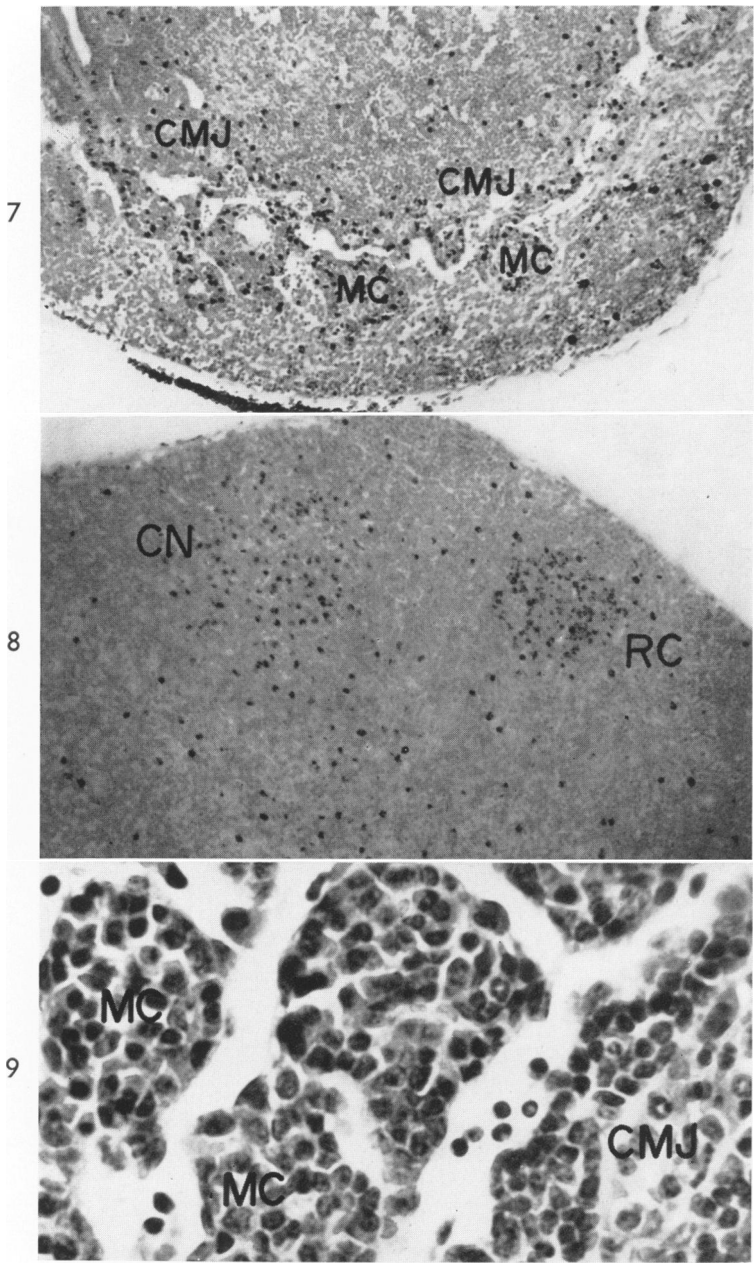
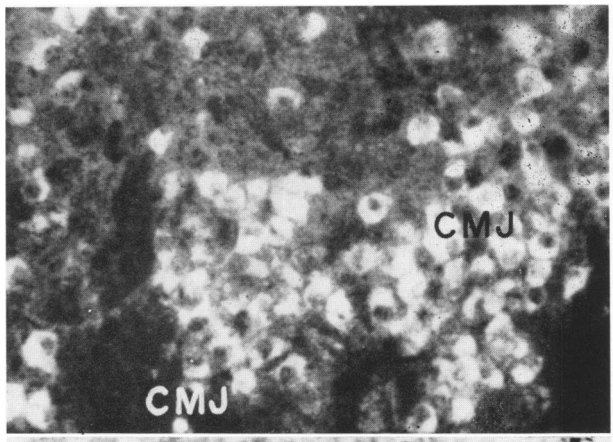


FIG. 7. Draining lymph node in a conventional mouse 4 days after injection. Labeled cells are concentrated at the corticomedullary junction (CM) and in medullary cords (MC). Radioautograph-Giemsa stain. ×86.

FIG. 8. Draining lymph node in a germfree mouse 14 days after injection. Enlargement of node persists and there is concentration of labeled cells in a reaction center (RC) and a cortical nodule (CN). Radioautograph-Giemsa stain. × 86.

FIG. 9. Draining lymph node in a germfree mouse 7 days after injection. Plasma cells are evident at the corticomedullary junction (CM) and in medullary cords (MC). Methyl-green-pyronine stain. × 512.



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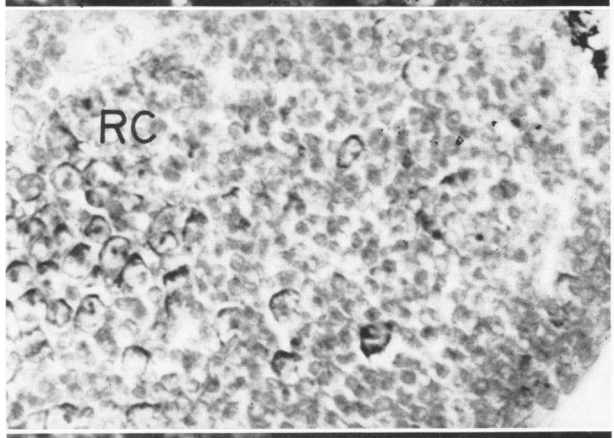

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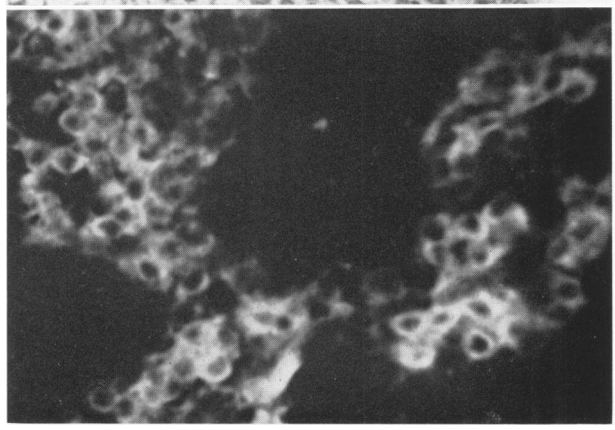

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FIG. 10. Draining lymph node in a germfree mouse 4 days after injection. Gamma globulin-containing cells appear at the corticomedullary junction (CM). Rabbit anti-mouse gamma globulin serum. × 400.

FIG. 11. Draining lymph node in a germfree mouse 14 days after injection. Numerous "immunoblasts" and an early reaction center (RC) appear in a cortical nodule. Methyl-green-pyronine stain. × 420.

FIG. 12. Draining lymph node in a germfree mouse 7 days after injection. Gamma globulin-containing cells are manifest in the medullary cords. Rabbit anti-mouse gamma globulin serum. × 400.