THE ENHANCING EFFECT OF THE MICROBIAL FLORA ON MACROPHAGE FUNCTION AND THE IMMUNE RESPONSE

A Study in Germfree Mice*

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Plates 98 and 99

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The lymphatic tissue of germfree animals is immunologically dormant (1) but responds to antigenic stimulation by bacteria (2–5), viruses (6, 7), chemicals (8), soluble proteins (9, 10), and tissue antigens (1, 11, 12). Previous investigations using killed *Escherichia coli* as antigen indicated that the microbial flora of conventional animals conditions macrophages towards an accelerated degradation of antigen (2). The present experiments aim to elucidate this further. Since the possible influence of preexisting antibody to *E. coli* of other serotypes could not be ruled out in the conventional mice of the previous study, the present experiments were designed to eliminate this variable and to compare the response to bacterial and soluble antigen. *Serratia marcescens* was selected as the test organism since the conventional mice used in the present study neither harbored the organism nor had antibody against it. To determine whether enhanced digestion in macrophages applied only to bacterial antigens, horse ferritin, a soluble protein was also injected. The evolution of the immune response was followed serially by semiquantitative histology, by immunocytochemical techniques to observe the fate of the antigens and the production of antibody, by autoradiography after tritiated thymidine administration to identify the proliferating cell populations, and by serum titration to detect antibody.

Materials and Methods

Sixty germfree (GF) and 59 conventionalized (CONV) ICR strain mice, 3 months old and weighing from 25 to 35 g were used. All animals were born and reared GF until 8 wk of age when 59 of them were conventionalized by exposure to cecal contents of open-room CONV

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mice. Within their respective germfree type plastic isolators, all GF and CONV mice were housed, fed, and bacteriologically monitored, as previously described (1).

Serratia marcescens antigen was prepared as follows:[1]

The organisms were grown on Bacto-Veal infusion agar, washed three times in isotonic saline, and resuspended in saline containing 1:5000 merthiolate. The concentration of organisms was adjusted to a density of McFarland's barium standard 10. The suspension was stored in sterile vials and tested for sterility prior to use. No growth was obtained from these vials at any time.

Twice crystallized horse ferritin (Pentex, Inc., Kankakee, Illinois) was recrystallized three more times by precipitation from ammonium sulfate solution with 5% cadmium sulfate (13), then sterilized by passage through a Seitz filter, followed by centrifugation at 100,000 RPM in a Spinco ultracentrifuge for 2 hr, resterilized by passage through a Millipore filter into ampoules, and stored at −20°C. Sterility tests revealed no growth at any time.

The following antisera were prepared in rabbits: (*a*) anti-mouse gamma globulin as described (1); (*b*) antiferritin by injecting 10 mg of purified ferritin with complete Freund's adjuvant 3 times 10 days apart and harvesting the serum 8 days after the last injection; (*c*) anti-*Serratia marcescens* serum following the method of Reitman et al. (14). Specificity of antisera to mouse gamma globulin and ferritin was checked by immunoelectrophoresis (15) and agar double diffusion technique (16).

Serum titers of antibody to ferritin were determined in rabbits and mice with formalinized and tannic acid–treated sheep red cells coated with ferritin according to the method of Stavitsky (17) as modified by Wide (18). Antibody titers to *Serratia* were done using the agglutination method (14) except that agglutination was observed microscopically with a dark field condenser. The reciprocal of the highest dilution of serum giving agglutination was considered as the titer and converted to $\log_{20}$ for tabulation.

Fifty-two GF and 52 CONV mice received 0.05 ml of *Serratia* suspension containing approximately 700,000 organisms per inoculum into the left front foot-pad, followed immediately by 0.05 ml of ferritin solution containing 0.5 mg of ferritin into the opposite foot-pad. The remaining 8 GF and 7 CONV animals received 0.05 ml of sterile saline into these sites and served as controls. Groups of 6 to 7 GF and CONV mice were anesthetized with carbon dioxide (dry ice) in a closed jar and exsanguinated by cardiac puncture 2, 6, 12, and 24 hr, and 2, 4, 7, and 14 days after inoculation. 1 hr prior to sacrifice each animal received 40 μc of tritiated thymidine intravenously.

Bilateral axillary and brachial lymph nodes and the spleens were weighed on an analytical balance, fixed in formalin for histological and autoradiographic preparations, or were quick frozen in isopentane–dry ice mixture for immunocytochemical studies. Spleens were bisected longitudinally for formalin fixation or freezing. Formalin-fixed sections were stained with hematoxylin and eosin, Giemsa, methyl green–pyronin, and by the Gomori method for iron. Previously described methods (19) were used for the preparation, cutting, fixation, staining, and observation of tissues as well as for the fluoresceination and absorption of antisera and evaluation of the immunologic specificity of staining. Attempts were made to detect antibody to *Serratia* and ferritin in plasma cells by incubation with antigen followed by staining with fluoresceinated antiserum. Sections of formalin-fixed and paraffin-embedded left axillary lymph nodes were also stained with fluoresceinated anti-*Serratia* antiserum.

Lymph nodes were arbitrarily graded from 0 to 4 for: macrophage size, number, and location; diffuse and particulate fluorescence of antigens after treatment with specific antiserum; reaction centers; number and location of hemocytoblasts and of tritium-labeled cells;

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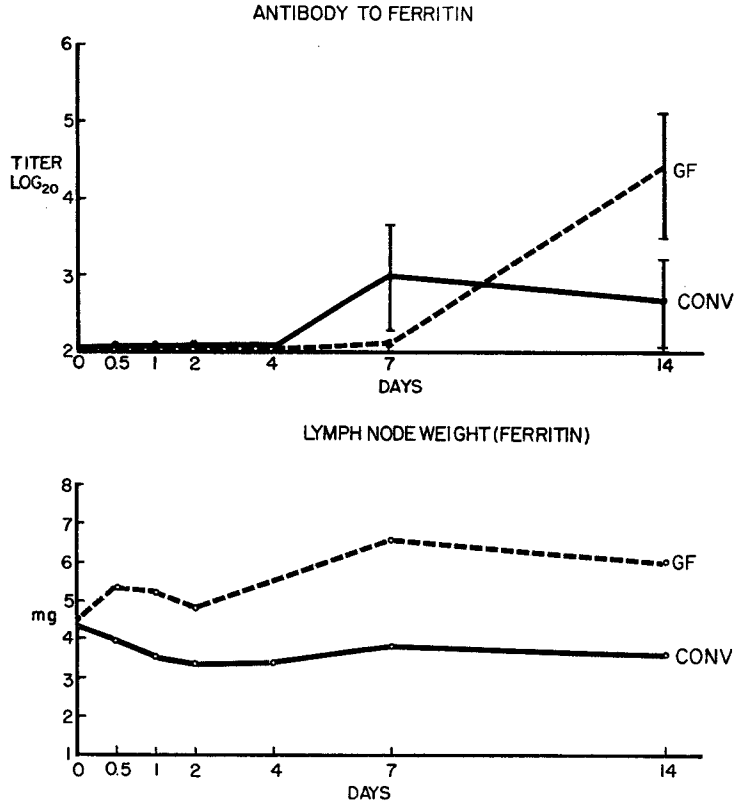
[1] *Serratia marcescens* antigen was prepared by Dr. A. E. Bunner of Difco Laboratories, Detroit. We gratefully acknowledge his cooperation.

number and morphologic appearance of plasma cells by light microscopy on hematoxylin eosin and methyl green–pyronin preparations, and by fluorescence microscopy when stained with anti-mouse gamma globulin serum. In histologic and autoradiographic preparations, reaction centers were counted, compared, and evaluated as to the percentage of tritium-labeled cells within them. Spleens were graded like lymph nodes.

Periodic cultures of food, feces, and bedding never revealed any growth in the GF mice and the mixed flora of the CONV animals never contained *Serratia marcescens*.

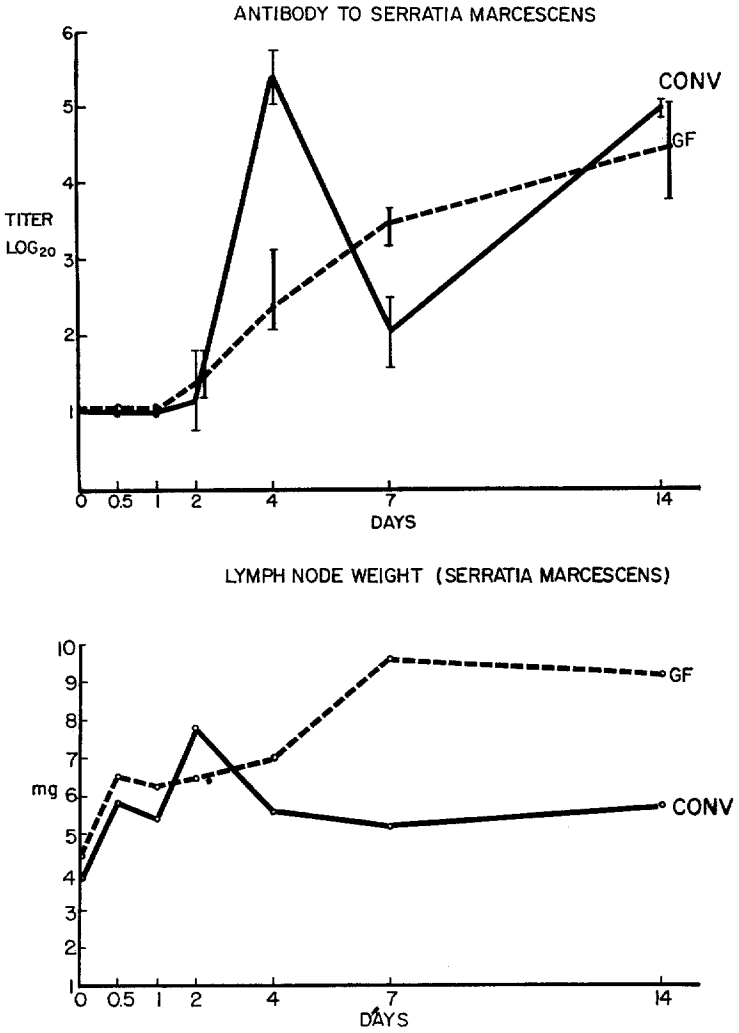


TEXT-FIG. 1. Mean and range of serum antibody titers to ferritin and mean weights of right axillary lymph nodes in germfree (GF) and conventionalized (CONV) mice following right front foot-pad injection of sterile horse ferritin.

RESULTS

Lymph Node and Spleen Weights.—GF lymph nodes generally showed a greater and more sustained weight gain than those of CONV mice. This difference was particularly striking in lymph nodes from the ferritin-injected side (Text-fig. 1). Lymph nodes of CONV mice showed little change while the GF

nodes gained weight rapidly, were 50% heavier than the controls by the 7th day after inoculation, and remained at that level. In lymph nodes receiving *Serratia* (Text-fig. 2), weight increased rapidly both in GF and CONV mice. By the 2nd day, CONV nodes had doubled their size but 2 days later the weights had begun to decrease. In GF mice, lymph node weights rose more slowly and showed a 50% gain at 4 days and a peak increase of 100% at 7 days which persisted thereafter.



TEXT-FIG. 2. Mean and range of serum antibody titers to *Serratia marcescens* and mean weights of left axillary lymph nodes in germfree (GF) and conventionalized (CONV) mice following left front foot-pad injection of killed *Serratia marcescens* organisms.

The weights of the spleens rose rapidly in all mice. By 4 days, both GF and CONV animals showed a 50% increase which declined after 1 wk and had returned to control levels by the end of the experiment.

Inflammatory Cells.—Beginning at 2 hr after inoculation, the marginal sinus of most lymph nodes was infiltrated by polymorphonuclear leukocytes. In the nodes draining the site of *Serratia* injection (left), leukocytosis was most conspicuous at 6 hr and disappeared after the 1st day. The axillary lymph nodes receiving ferritin showed appreciable numbers of polymorphonuclear leukocytes at 2 and 6 hr after inoculation. Eosinophiles began to appear near the corticomedullary junction and in the medullary cords of most lymph nodes at 2 hr and reached a numerical peak at 12 to 24 hr after antigen injection. The timing and intensity of leukocytosis and eosinophilia did not differ between the nodes of GF and CONV mice. The spleens showed no inflammatory changes at any time.

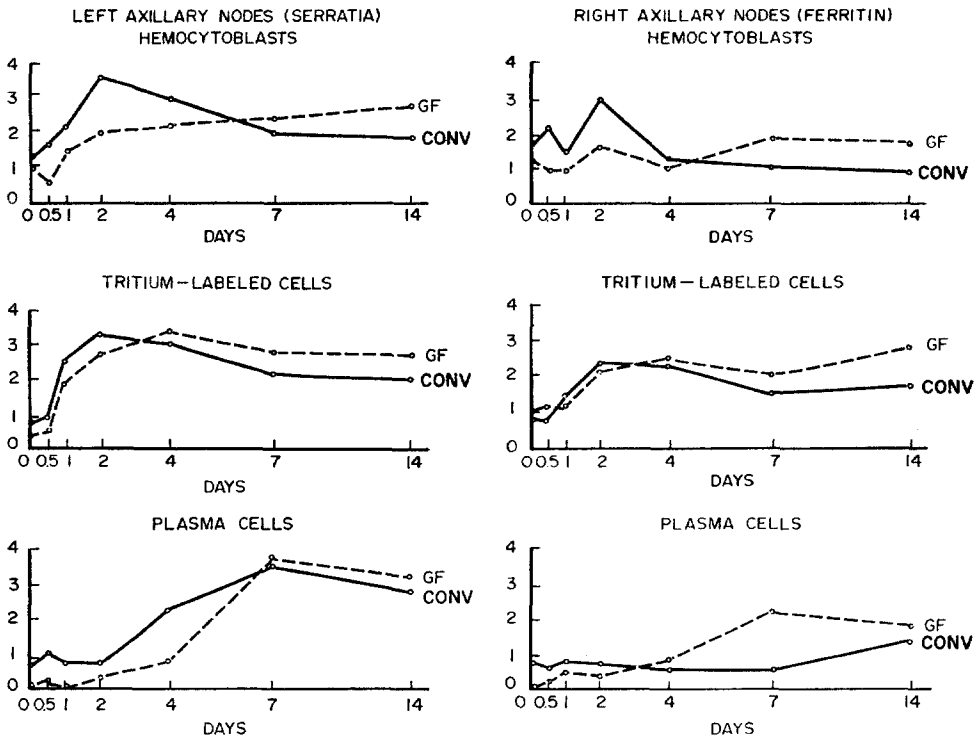
Macrophages.—The number of macrophages in the sinuses was slightly above control levels for the first 4 days in all antigen-injected mice. Thereafter, the population size resembled that of the saline-treated animals. For 6 to 48 hr after antigen injection, the macrophages in the sinuses of most lymph nodes showed moderate enlargement when the peripheral inoculum consisted of ferritin, and a more marked increase in size where the inoculum was *Serratia.* In addition to enlargement, the macrophages of the left axillary nodes (*Serratia*) also showed varying degrees of cytoplasmic vacuolization. The distribution, incidence, and cytoplasmic changes of macrophages were the same when homolateral lymph nodes were compared in GF and CONV mice. After 2 days, the size of macrophages in all lymph nodes was within normal limits. No changes could be detected in the macrophages of the spleens.

Antigens.—After staining with antiserum, the antigens were always found within macrophages and fluoresced either in a granular or diffuse pattern or showed both types in varying proportions. The distinction between particulate and diffuse fluorescence was easier to make with *Serratia* than with ferritin but both antigens showed these characteristics. *Serratia* antigen could be identified immunocytochemically in sections of left axillary lymph nodes whether they were formalin-fixed and paraffin-embedded or frozen and cut in a cryostat. Neither *Serratia* nor ferritin were ever demonstrated in lymph nodes contralateral to their respective site of injection, nor in the spleens.

All GF and CONV lymph nodes contained antigen at 2 hr after injection and particulate fluorescence predominated in all. (Figs. 1 to 3). By 6 and 12 hr, CONV macrophages with *Serratia*, and to a lesser extent with ferritin, began to show diffuse as well as particulate fluorescence. At 1 and 2 days, many *Serratia*-containing macrophages stained only diffusely while both types of fluorescence were seen on the ferritin-injected side. In the CONV animals ferritin could no longer be demonstrated with fluoresceinated antiserum by 4 days and at 7 days *Serratia* had also disappeared from CONV lymph nodes. In the GF animals, granular staining always predominated but diffuse fluorescence began to increase

at 2 and 4 days after antigen administration. Both antigens remained immunocytochemically demonstrable throughout the entire study in all lymph nodes (Fig. 4).

Iron-positive granules probably representing ferritin were numerous in macrophages of the right axillary lymph nodes of GF and CONV animals at all



TEXT-FIG. 3. Mean frequency, graded on a scale of 0 to 4, of hemocytoblasts, tritium-labeled cells, and plasma cells in bilateral axillary lymph nodes of germfree (GF) and conventionalized (CONV) mice following injection of killed *Serratia marcescens* organisms into left, and sterile horse ferritin into right front foot-pad.

times. The number of iron-containing cells decreased, however, in CONV mice after the 4th day, while it remained high in the GF animals. No iron could be demonstrated in macrophages of lymph nodes from the *Serratia*-injected side at any time.

Hemocytoblasts.—Bilateral axillary lymph nodes of CONV mice showed earlier, more intense, but less sustained hemocytoblast formation than those of the GF animals (Text-fig. 3). In GF mice peak levels occurred at 7 and 14 days after inoculation while CONV mice showed the greatest response at 2 days

(Figs. 5 and 6), followed by a decline towards baseline levels. When compared with the axillary nodes of saline-injected controls, hemocytoblasts increased up to 100% of baseline values. Hemocytoblasts appeared first in the intermediate zone of the cortex, then at the corticomedullary junction and finally (7 to 14 days) in the subcapsular cortical nodules.

In the spleens of GF and CONV mice, hemocytoblasts were noted first around the central arteriole of the follicles (24 to 48 hr after inoculation), then throughout the white pulp (2 to 4 days), and later in the red pulp where they formed clusters with plasma cells.

Reaction Center.—The reaction centers in lymph nodes and spleens were composed of strongly pyroninophilic hemocytoblasts, large lymphocytes, and cellular debris. The dense fields of small and medium lymphocytes around the reaction centers usually contained scattered hemocytoblasts. In the lymph nodes and spleens of most GF and CONV mice, increasing numbers of reaction centers appeared at 7 and 14 days after inoculation. Their proliferation was more prominent in left axillary nodes and in the spleens than in lymph nodes from the ferritin-injected side.

Tritium-Labeled Cells.—Both GF and CONV control mice showed little proliferative activity. After antigen injection, the times of appearance, location, and relative incidence of tritium-labeled cells resembled those of the hemocytoblasts (Text-fig. 3). The number of labeled cells rose sharply and reached its peak at 2 days in the CONV nodes (Fig. 7) while GF lymphatic tissue showed less uptake (Fig. 8) and attained maximum labeling 2 days later than in the CONV mice. Large and medium lymphocytes and hemocytoblasts accounted for most labeled cells. Small lymphocytes, plasma cells, and macrophages were never labeled. Reaction centers contained more tritiated cells at 7 and 14 days after inoculation than in earlier groups. Left axillary nodes which drained the site of *Serratia* inoculation generally showed more tritium uptake than contralateral nodes receiving ferritin.

In the spleens, labeled cells were also mainly large and medium lymphocytes and hemocytoblasts. As in the lymph nodes, reaction centers in spleens of mice sacrificed 7 and 14 days after inoculation contained more labeled cells than those of animals sacrificed earlier.

Plasma Cells.—Germfree saline-treated animals showed virtually no plasma cells and their incidence in CONV control mice was low (Text-fig. 3). In left axillary lymph nodes, beginning at 4 days after *Serratia* inoculation, plasma cells increased slightly in GF and more markedly in CONV mice. Both types of animal showed the highest incidence at 7 days, followed by a slight decline but values remained well above baseline levels. In lymph nodes receiving ferritin, GF animals showed an increased number of plasma cells at 4 and 7 days, but in CONV nodes a rise occurred only at the end of the experiment. Plasma cells recognizable histologically or immunocytochemically by their gamma globulin

content were first located at the corticomedullary junction and appeared immature. Later, the cells became mature and were most numerous in the medullary cords. Specific antibody to ferritin could be demonstrated in some plasma cells of lymph nodes draining the site of ferritin injection. Attempts to identify intracellular antibody to *Serratia* in plasma cells by treatment of sections with *Serratia* followed by fluoresceinated anti-*Serratia* serum, were unsuccessful because of the highly particulate nature of the antigen even after sonification.

In the spleen, plasma cells were located mainly in the red pulp, began to increase in number at 4 days and then occurred in clumps together with hemocytoblasts along follicles and trabecular vessels. The spleens of GF and CONV mice did not differ in any of these developments.

Serum Antibody.—While serum antibody titers to *Serratia* rose more slowly in the GF mice and reached their peak at 4 days, antibodies in the CONV animals exhibited their highest levels at 4, and again at 14 days, at which time the titers resembled those observed in the GF mice (Text-fig. 2). Antibody to ferritin appeared at 7 days in the CONV and at 14 days in the GF mice which showed higher titers than the CONV animals (Text-fig. 1).

DISCUSSION

In GF and CONV mice, the response of lymphatic tissue to two dissimilar antigens evolves in a similar manner and generally conforms to observations of lymph nodes, (8, 20–25) and spleens (26–31) in CONV animals after primary antigenic stimulation. Since the antigens were injected locally, the major changes occurred in the regional lymph nodes but the spleens also reacted.

The earliest cellular reaction in the lymph nodes was an infiltration of polymorphonuclear leukocytes and eosinophiles which subsided after 24 hr. This known response to antigen injection (32) was the same in GF and CONV mice which indicates that the microbial flora does not affect this reaction. All subsequent events were more pronounced in nodes draining the site of *Serratia* inoculation, confirming the observation that particulate antigens evoke a more dramatic response than soluble proteins (33). The lymph nodes rapidly increased in size and cellularity, both of which rose more steeply in the CONV but were maintained longer in the GF animals. All cytologic, immunocytochemical, and serologic parameters confirmed the pattern of a slower but eventually equal, and more sustained response of GF lymphatic tissue.

The reason for this difference in the immune response of GF and CONV mice seems to be the ability of CONV macrophages to digest antigen faster and thereby to hasten the immune response. Morphologically, this manifests itself as a more rapid change from a particulate to a diffusely distributed and, therfore, partially digested antigen and by the earlier disappearance of immunocytochemically demonstrable antigen from CONV macrophages. The slower and more prolonged digestion of antigen in GF macrophages was in turn reflected in

a more slowly developing but more persistent lymphatic tissue response than that observed in the CONV mice.

The antigens appeared at the same time and were phagocytized equally well by the lymph node macrophages of the GF and CONV mice. Lymphatic drainage or unequal phagocytosis can, therefore, not explain the differences in the speed of antigen digestion. The physical characteristics and source of the antigens also seem unimportant since one was bacterial and particulate and the other nonbacterial and soluble. Finally, serum antibody also played no part since none existed in the animals prior to antigen inoculation. The difference in the digestive capacity of GF and CONV macrophages must thus be intrinsic, related to the difference in the microbial state of the animals prior to challenge and, at least in this experiment, unrelated to the type of antigen or the circumstances of its uptake.

The intracellular fate of antigens in macrophages has been studied with isotope-labeled antigen (23), by the residual immunogenicity of antigen fragments (34), and by flourescent antibody technique (35). These and other investigations have shown the importance of macrophages (36) and of the rate at which they dispose of antigen (37) which they have rendered immunogenic (38). Although bacteria and bacterial products are known to have an adjuvant effect on antibody formation (39) and may activate phagocytic cells (40), the influence of the total microflora has received little attention. The experiments reported here suggest that immunologic experience with the microbial environment not only determines the level of overall immunologic activity, but enhances the immunogenic capacity for new antigenic challenges and accelerates the onset of specific antibody formation.

SUMMARY

The immune response to bacteria and to a soluble protein was compared in germfree and conventionalized mice. Sixty germfree and 59 conventionalized mice received a suspension of killed *Serratia marcescens* into one front foot-pad and sterile horse ferritin into the other and were sacrificed in groups from 2 hr to 14 days after inoculation. All mice had no pre-existing antibody to either antigen and the flora of the conventionalized mice never contained *Serratia*. Lymphatic tissue changes and the fate of the antigens were followed in axillary lymph nodes and the spleens by histologic, fluorescent antibody, and autoradiographic techniques after tritiated thymidine injection. Individual serum antibody titers for both antigens were determined at each time period.

The cellular and serologic responses were slightly delayed in the germfree mice but later equaled and sometimes exceeded those of the conventional animals. In all animals, lymph nodes draining the site of *Serratia* injection showed a more vigorous response than those on the ferritin-injected side but the reaction was qualitatively the same for both antigens. All lymph nodes contained the antigens by 2 hr after foot-pad injection. With time, both antigens lost their

particulate nature sooner in conventionalized than in germfree macrophages. In the latter, both antigens persisted throughout the study while no longer demonstrable with fluoresceinated antiserum in conventional macrophages after the first week.

While phagocytosis is equal in germfree and conventional mice, a greater digestive capacity of macrophages for antigens seems to result from the continuous exposure of conventional animals to the immunologic effects of the microbial flora. Conversely, the lack of substantial antigenic stimulation of lymphatic tissue in germfree animals fails to develop these macrophage functions beyond their basic ability to degrade foreign substances. Although the onset of the immune response is delayed in germfree mice, the relatively prolonged antigen digestion and the presumably slower release of immunogenic antigen fragments result in a more sustained and sometimes greater response than in conventional animals. This modifying effect of the microflora on the function of macrophages during the immune response is independent of previous experience with, or the nature of, the antigen.

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EXPLANATION OF PLATES

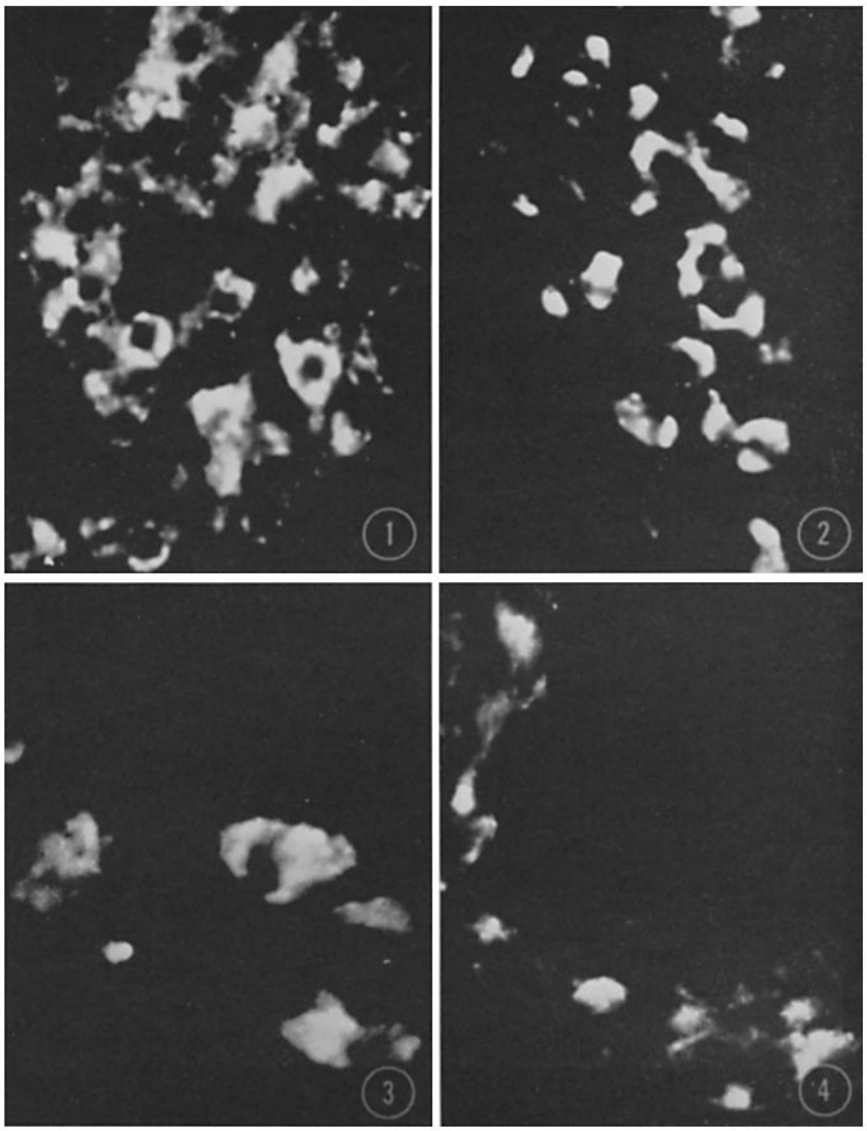
PLATE 98

FIG. 1. Left axillary lymph node of germfree mouse, 2 hr after injection of *Serratia marcescens* into left front foot-pad. Note sinuses with many macrophages containing phagocytozed bacteria. Anti-*Serratia* antiserum labeled with fluorescein. × 250.

FIG. 2. Left axillary lymph node of conventionalized mouse 2 hr after injection of *Serratia marcescens* into left front foot-pad. Note many bacteria in macrophages. Anti-*Serratia* antiserum labeled with fluorescein. × 250.

FIG. 3. Same animal as Fig. 1. Note particulate nature of ingested antigen. Anti-*Serratia* antiserum labeled with fluorescein. × 1000.

FIG. 4. Right axillary lymph node of germfree mouse 7 days after injection of ferritin into right front foot-pad. Note that macrophages still contain antigen. Anti-ferritin antiserum labeled with fluorescein. × 250.



(Bauer et al.: Macrophage function in germfree mice)

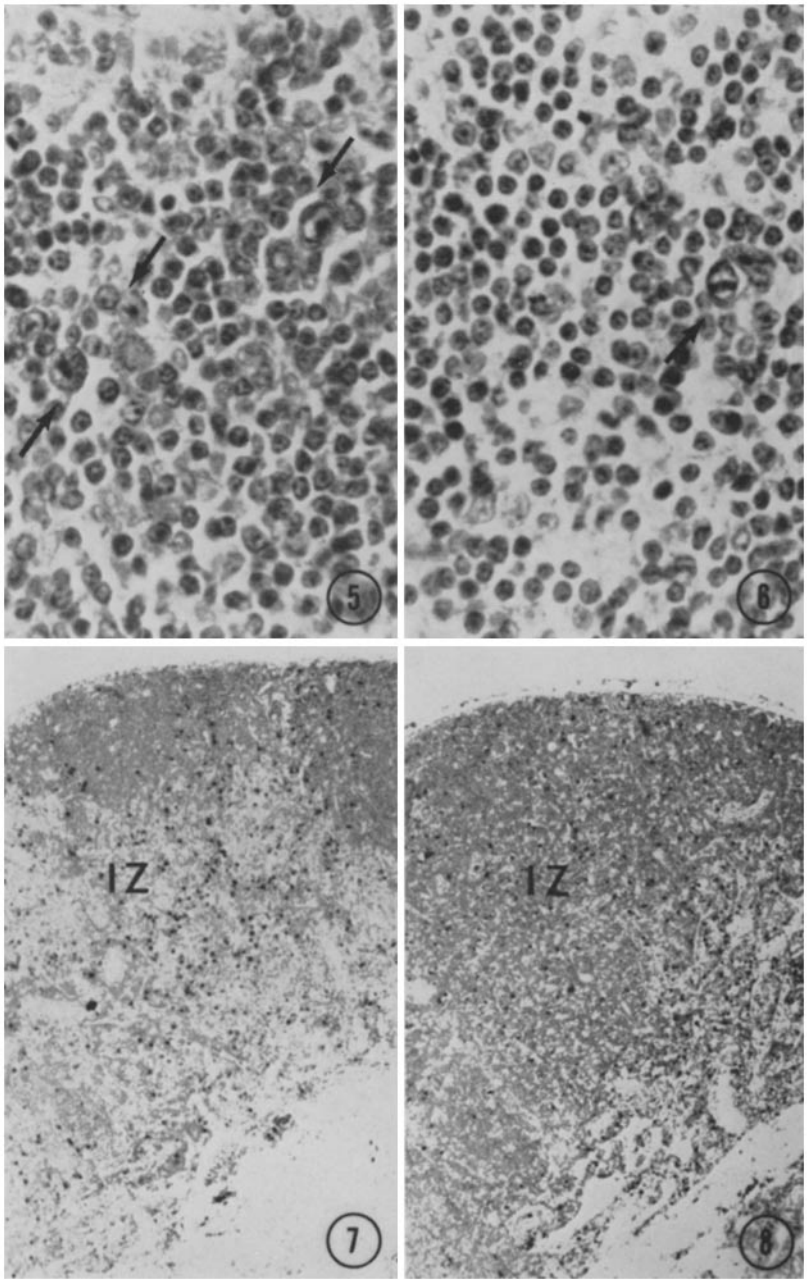
PLATE 99

FIG. 5. Cortex of left axillary lymph node of conventionalized mouse, 2 days after injection of killed *Serratia marcescens* into left front foot-pad. Note many hemocytoblasts (arrows). Methyl green–pyronin stain. × 635.

FIG. 6. Cortex of left axillary lymph node of germfree mouse, 2 days after injection of killed *Serratia marcescens* into left front foot-pad. Note single hemocytoblast (arrow). Methyl green–pyronin stain. × 635.

FIG. 7. Same lymph node as Fig. 5. Note many tritium-labeled cells in intermediate zone (*IZ*) of cortex. Autoradiograph-hematoxylin stain. × 80.

FIG. 8. Same lymph node as Fig. 6. Note relative paucity of tritium-labeled cells in intermediate zone (*IZ*) of cortex. Autoradiograph-hematoxylin stain. × 80.



(Bauer et al.: Macrophage function in germfree mice)