THE RESPONSE OF THE LYMPHATIC TISSUE TO THE MICROBIAL FLORA. STUDIES ON GERMFREE MICE

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The qualitative and quantitative morphologic pattern of lymph nodes and spleen in germfree and conventional mice has been investigated by histologic, histochemical and immunocytochemical techniques. Particular attention has been given to those elements which differ in germfree and conventional mice and, therefore, reflect the influence of the microbial flora. The definition of these morphologic manifestations of physiologic reactions in relation to the environment has been the specific objective of this study.

In the earliest studies of the lymph node tissue and spleen in the germfree state, Glimstedt[1] observed these to be underdeveloped in guinea pigs. The lack of development was most conspicuous in organs closely associated with the microbial flora. Miyakawa, Iijima, Kobayashi and Tajima[2] found no reaction centers, a decrease in the number of lymphocytes, reticulum cells and "pyronine-positive" cells in the medullary cords, and the presence of only a few scattered plasma cells. Sprinz and associates[3] confirmed these observations. Abrams and Bishop,[4] on the other hand, encountered distinct reaction centers in lymph nodes of germfree guinea pigs and suggested that the development of the centers was not necessarily related to the microbial flora but might reflect nutritional factors. Gustafsson[5] failed to find reaction centers in germfree rats; these animals, as a rule, had less lymphatic tissue than guinea pigs. Thorbecke[6] and Gordon,[7] however, described reaction centers and small numbers of plasma cells in the lymph nodes and spleens of germfree rats and chickens.

The studies reported here extend the above observations to the germfree mouse and serve to examine the two major elements in the lymph node system, the phagocytic and immunologically competent cells. Par-

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ticular regard has been given to the morphologic alterations attributable to the presence of the microbial flora.

Material and Methods

Forty-five germfree and 52 conventional Swiss Webster mice, 3 to 6 months of age and 26 to 48 gm. in weight, were used. The mice were of 2 strains, both originating at the University of Notre Dame, South Bend, Indiana. Germfree and conventional mice were born and reared under identical conditions in Trexler type plastic isolators.[8,9] Technical details of germfree tank operation and microbiologic assay have been described previously.[10,11]

Preliminary investigation had shown that contralateral lymph nodes were histologically similar. Submaxillary and mesenteric lymph nodes were representative of all nodes draining the gastrointestinal and respiratory tracts, and axillary and popliteal nodes were representative of nodes draining other areas, particularly the extremities. Therefore, the following representative nodes were selected for study: both submaxillary, axillary and popliteal nodes and the largest mesenteric node.

The animals were killed with ether, and one submaxillary, axillary and popliteal node, a mesenteric node from alternate animals and half of the spleen were fixed in Helly's fluid. The remaining nodes and portions of the spleen were prepared for histo- or immunocytochemistry. Paraffin sections of each lymph node were checked microscopically until the plane of sectioning was found to show hilum, medulla and an adequate amount of cortex. Serial strips of 5 μ sections were then stained with hematoxylin and eosin, Giemsa, periodic acid-Schiff (PAS) with and without diastase digestion, Gomori's stain for iron, and Sudan black. Acid phosphatase activity was demonstrated in formol-calcium-fixed frozen sections using naphthol AS-TR phosphate as substrate and hexazonium pararosanilin as coupler at pH 5.[12]

Mouse serum for immunocytochemistry was obtained by exsanguination of Swiss Webster pathogen-free mice (obtained through the courtesy of the Division of Veterinary Medicine, Walter Reed Army Institute of Research, Washington, D.C.). The gamma globulin fraction was isolated by electrophoresis and injected into rabbits with Freund's adjuvant until the semi-micro-precipitin titer reached at least 1:512.[13,14] The specificity of the antiserum was determined by double agar diffusion techniques and immunoelectrophoresis.[15] The gamma globulin fraction of the antiserum was isolated, conjugated with fluorescein isothiocyanate and purified by dialysis and elution in a cellulose column.[16,17] Lymph nodes and spleens, embedded in gelatin, were frozen in dry ice-isopentane.[18] Acetone-fixed cryostat sections were treated with fluoresceinated rabbit anti-mouse gamma globulin serum.[19] Immunologic specificity was controlled by treating serial sections with nonfluoresceinated rabbit anti-mouse gamma globulin serum, followed by treatment with fluoresceinated rabbit anti-mouse gamma globulin serum, and by treating random sections with fluoresceinated rabbit anti-human gamma globulin serum.

Lymph nodes were arbitrarily graded from 0 to 4 for the following features: macrophage population in marginal and medullary sinuses, number of iron-containing macrophages, number of cells in the medullary cords, number of mitotic figures, Russell bodies, eosinophils and immunologically competent cells (plasma cells and precursors).

Results

Basically 3 types of cells were evaluated in spleens and lymph nodes: (a) Macrophages (reticuloendothelial cells, sinus reticulum cells) with bulky, occasionally finely or coarsely vacuolated eosinophilic or amphophilic cytoplasm which contained nonglycogenic PAS-positive granules,

granules with acid phosphatase activity, occasional fat droplets and autofluorescent lipofuscin and iron. (b) Immunologically competent cells (ICC) characterized by the presence of gamma globulin and subdivided morphologically according to the criteria of Vazquez[20]; these exhibited basophilic cytoplasm with small, eccentric, cartwheel nuclei and perinuclear halos. In tallying ICC by conventional microscopy, only the Marschalkó plasma cells were counted. (c) Lymphoid cells (lymphocytes—small, medium, large), characterized by scanty cytoplasm containing none of the histochemical or immunologic features noted above.

The shape, color and consistency of lymph nodes were similar in germfree and conventional mice, although in the former the nodes were slightly smaller. Microscopically all lymph nodes were similar in basic architecture and showed a division into cortex and medulla (Figs. 1 and 2). The submaxillary and mesenteric nodes exhibited an extensive medullary sinus system, but the axillary and popliteal did not (Figs. 3 and 4). With the exception of the popliteal nodes, in which the cortex was uniform, the lymph node cortex could be divided into a narrow peripheral and a wider intermediate zone.[21] The peripheral portion in nodes from both germfree and conventional animals contained nodules of densely packed small lymphocytes in which reaction centers and mitotic figures were seen.[21,22] The intermediate zone formed the major portion of the cortical tissue in all nodes but particularly in those of germfree mice; it was generally less cellular, and the small lymphocyte was the predominant cell type. In the mesenteric nodes of both germfree and conventional mice, the intermediate zone was frequently the site of conspicuous leukocyte destruction, particularly of eosinophils and neutrophils, with attendant macrophage activity. The submaxillary and axillary nodes showed only a few of such degenerating cells and rare macrophages in the intermediate zone.

All nodes had perivascular medullary cords filled with cells which varied in type and number with the anatomic location of the node and the microbial status. The nodes in conventional animals showed larger and more cellular medullary cords comprised of lymphocytes of varying sizes, eosinophils, macrophages and plasma cells. The medullary cords in germfree animals were thin and composed mainly of small and medium-sized lymphocytes (Fig. 5). Mitotic figures were most often seen in medullary cords of mesenteric nodes in conventional animals and were rare in the comparable germfree nodes.

Reaction centers, as defined by Ringertz and Adamson[23] and by Conway,[24] were seen in the conventional state, most commonly in mesenteric nodes (Fig. 10). They were rare in all germfree mice and virtually absent from axillary and popliteal nodes.

The differences in the gross and microscopic characteristics of the

spleen in germfree and conventional mice were not suitable for detailed quantitation.

Macrophages

The findings are summarized in Table I. The incidence and activity varied only with the anatomic location of lymph nodes, not with microbial status. The submaxillary and mesenteric nodes which drain the oropharynx and intestine had the highest number of macrophages (Fig. 9), while the axillary and popliteal nodes showed fewer of these cells. The histologic and histochemical characteristics of macrophages in germfree and conventional subjects were indistinguishable (Fig. 8).

TABLE I

MACROPHAGES IN LYMPH NODES

Lymph nodes	No. of mice		No. of medullary sinus macrophages *		No. of marginal sinus macrophages *	
	Germfree	Conv.†	Germfree	Conv.	Germfree	Conv.
Mesenteric	18	20	2.0 ± 0.2	1.9 ± 0.2	2.4 ± 0.1	2.4 ± 0.1
			$p = 0.5$‡		$p > 0.99$	
Submaxillary	18	20	1.8 ± 0.1	1.8 ± 0.2	2.4 ± 0.2	2.8 ± 0.1
			$p = 0.99$		$p = 0.2$	
Axillary	18	19	1.6 ± 0.1	1.6 ± 0.2	2.3 ± 0.1	2.4 ± 0.2
			$p > 0.99$		$p = 0.5$	
Popliteal	15	14	1.1 ± 0.1	1.7 ± 0.2	1.5 ± 0.2	1.7 ± 0.1
			$p = 0.1$		$p = 0.3$	

* Figure represents mean frequency, graded on a scale of 0 to 4, and the standard deviation of the mean.

† Conv. = conventional.

‡ Probability according to chi ^{2} distribution.

The lumens of the marginal and adjacent medullary sinuses contained macrophages in approximately equal numbers in both groups of mice. Lymphocytes in this location were more numerous in germfree animals in all anatomic locations. The marginal and adjacent medullary sinuses of all nodes had varying numbers of mast cells. They were rare in mesenteric lymph nodes, more frequent in the axillary and submaxillary region, and most common in the popliteal nodes. The mast cells were well seen after staining with fluoresceinated anti-mouse or anti-human gamma globulin since their granules bound either serum nonspecifically. The microbial state of the host did not influence the number or appearance of these cells.

In the spleen were many iron-containing macrophages and extracellular aggregates of hemosiderin in the red pulp. There was no difference in the number or histochemical characteristics of the splenic macrophages in germfree and conventional mice.

TABLE II

IMMUNOLOGIC REACTIONS IN LYMPH NODES

Lymph nodes	No. of mice		No. of ICC *		No. of reaction centers †		No. of mitotic figures †		Cellularity of medullary cords †	
	Germfree	Conv.‡	Germfree	Conv.	Germfree	Conv.	Germfree	Conv.	Germfree	Conv.
Mesenteric	19	21	0.8 ± 0.2	2.9 ± 0.2	0.5 ± 0.2	2.9 ± 0.3	1.0 ± 0.1	2.9 ± 0.2	2.4 ± 0.2	3.0 ± 0.1
			$p < 0.01$§		$p < 0.01$		$p < 0.01$		$p = 0.05$	
Submaxillary	18	19	1.0 ± 0.2	3.2 ± 0.2	0.5 ± 0.1	1.4 ± 0.3	0.7 ± 0.2	1.1 ± 0.2	1.7 ± 0.1	2.5 ± 0.1
			$p < 0.01$		$p < 0.01$		$p = 0.05$		$p = 0.01$	
Axillary	18	19	0.8 ± 0.2	1.7 ± 0.2	0.4 ± 0.1	0.6 ± 0.2	0.8 ± 0.2	1.5 ± 0.2	1.5 ± 0.1	1.8 ± 0.2
			$p = 0.5$		$p = 0.7$		$p = 0.1$		$p = 0.2$	
Popliteal	16	14	0.6 ± 0.2	0.7 ± 0.2	0.2 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.6 ± 0.2	1.5 ± 0.1	1.7 ± 0.2
			$p = 0.5$		$p = 0.2$		$p = 0.8$		$p = 0.3$	

* Figure represents mean frequency of gamma globulin-containing cells (fluorescence) or Marschalkó plasma cells (by conventional microscopy), graded on a scale of 0 to 4, and the standard deviation of the mean.

† Figure represents mean frequency, graded on a scale of 0 to 4, and the standard deviation of the mean.

‡ Conv. = conventional.

§ Probability according to chi ^{2} distribution.

Immunologically Competent Cells (ICC)

The findings are summarized in Table II. ICC were more common in lymph nodes in conventional animals than in the germfree group. The difference was most striking in comparisons of nodes draining the oropharynx and intestine; the submaxillary and mesenteric nodes in conventional subjects were rich in ICC which were found at the border of the cortex and medulla, in the medullary cords, in the hilum and occasionally in germinal centers. Comparable nodes in the germfree mice showed only rare ICC (Figs. 6 and 7). Most of the gamma globulin-containing cells were large with large central nuclei and a scant to moderate rim of specifically fluorescing cytoplasm, corresponding to types A and B of Vazquez.[20] Only a minority of the fluorescing cells had the extensive cytoplasm, small eccentric nuclei and perinuclear halos characteristic of Vazquez's type C; these corresponded to plasma cells of the Marschalkó type. With conventional staining, however, the Marschalkó cells seemed to predominate among the basophilic cells, indicating that only some of these cells contained demonstrable gamma globulin. PAS-positive plasma cells and Russell bodies similar to those recently described by Welsh[25] in man, were observed in the lymph nodes of both germfree and conventional mice and did not vary significantly with the location or microbial status.

In the spleen the ICC were of the same type as in lymph nodes and were abundant in conventional mice, in which they formed clusters throughout the red pulp, particularly along penicilliary arteries, occasionally within and at the edge of the white pulp and sometimes near the capsule. The spleens in germfree mice exhibited gamma globulin-containing cells in the same location but in lesser numbers. With conventional staining, Marschalkó plasma cells were rare in all spleens, and their immature precursors could not be distinguished from hematopoietic blast cells, which abounded in the red pulp. PAS-positive plasma cells and Russell bodies were present in the majority of spleens.

All the above criteria were applied separately in the analysis of the lymph nodes and spleens in both strains of mice. The results showed no strain differences and were, therefore, evaluated and tabulated together. Age and sex of the animals also failed to influence the characteristics of the lymphatic tissue.

Discussion

The major morphologic differences between lymph nodes and spleens in the germfree and conventional state were related to structural and cytologic phenomena reflecting immunologic reactions. They involved

the reaction centers where response to antigenic stimulation probably begins, the medullary cords where ICC are known to congregate, and also the total number of ICC, whether defined as cells containing gamma globulin or as Marschalkó type plasma cells.[26,27] In all 3 respects, germfree animals were deficient. Reaction centers were fewer and less active, medullary cords were less cellular because of fewer plasma cells, and ICC were rare. Strain differences played no role in these phenomena.

In conventional mice, the morphologic evidence of response to antigenic stimulation was most conspicuous in the spleen and those lymph nodes which drained the areas of heaviest microbial contamination, namely, the upper and lower digestive tract. Conversely, in germfree animals in which these areas were not exposed to the effect of microorganisms, ICC response was minimal. This suggested that the products of physiologic tissue breakdown did not evoke a substantial antibody response as Springer had suggested.[28] That the altered tissue products derived from pathologic processes, however, might induce a conspicuous reaction in germfree animals was indicated by a recent observation in a small group of germfree mice with sterile, self-inflicted, chronic inflammatory lesions. The regional nodes in these animals had morphologic evidence of antibody formation, while the other lymph nodes and the spleens retained their characteristic "germfree" appearance.[29]

The paucity of immunologic phenomena, recognizable by conventional and fluorescence microscopy, in the lymph nodes and spleens of germfree mice supported the histologic findings in germfree chickens, rats and guinea pigs.[7] The low incidence of ICC in lymph nodes correlated with the hypogammaglobulinemia in germfree animals.[30–33]

In contrast to the differences in the immunologic response of germfree and conventional mice, the incidence, distribution and activity of macrophages throughout the lymph nodes and spleens did not vary with the microbial status. All of these reticuloendothelial elements stained strongly for acid phosphatase and showed an equal ability to store iron and lipofuscin. The intensity of the acid phosphatase reaction has been shown to parallel the phagocytic activity.[34,35] This indicated that macrophages were functionally as well as morphologically similar in conventional and germfree mice, and confirmed studies *in vivo* showing an equal ability of germfree and conventional animals to clear materials from the blood.[36,37]

The magnitude of the sinus system and its population of cells was apparently not dependent on the presence of micro-organisms but only on the anatomic site of the node. Meneghelli[38] also found a prominent system of sinuses in human mesenteric lymph nodes and fewer sinuses in axillary nodes.

The lymph nodes and spleens in germfree mice, thus, appeared immunologically dormant, while engaged in conspicuous phagocytic activity. Phagocytosis, therefore, does not necessarily render materials antigenic. In the mouse not subjected to injury or other antigenic challenge, the microbial flora was the main stimulus for reaction center formation, plasma cell maturation and gamma globulin production.

Summary

Lymph nodes and spleens from 45 germfree and 52 conventional Swiss-Webster mice of two strains were examined by histologic, histochemical and immunocytochemical techniques.

The number, distribution, histologic and histochemical characteristics of macrophages in lymph nodes and spleens in germfree and conventional animals did not differ; in lymph nodes the macrophages were found to be related only to the area of drainage, being more prominent in nodes related to the oropharynx and intestine than in those related to the extremities.

Reaction centers and immunologically competent cells, however, whether identified as Marschalkó plasma cells by conventional microscopy or as gamma globulin-containing cells by immunocytochemistry, were rare in lymph nodes and spleens from germfree animals and abundant in comparable tissues from conventional mice. This indicated that the presence of a microbial flora affected the immunologic but not the phagocytic function of lymph nodes and spleens.

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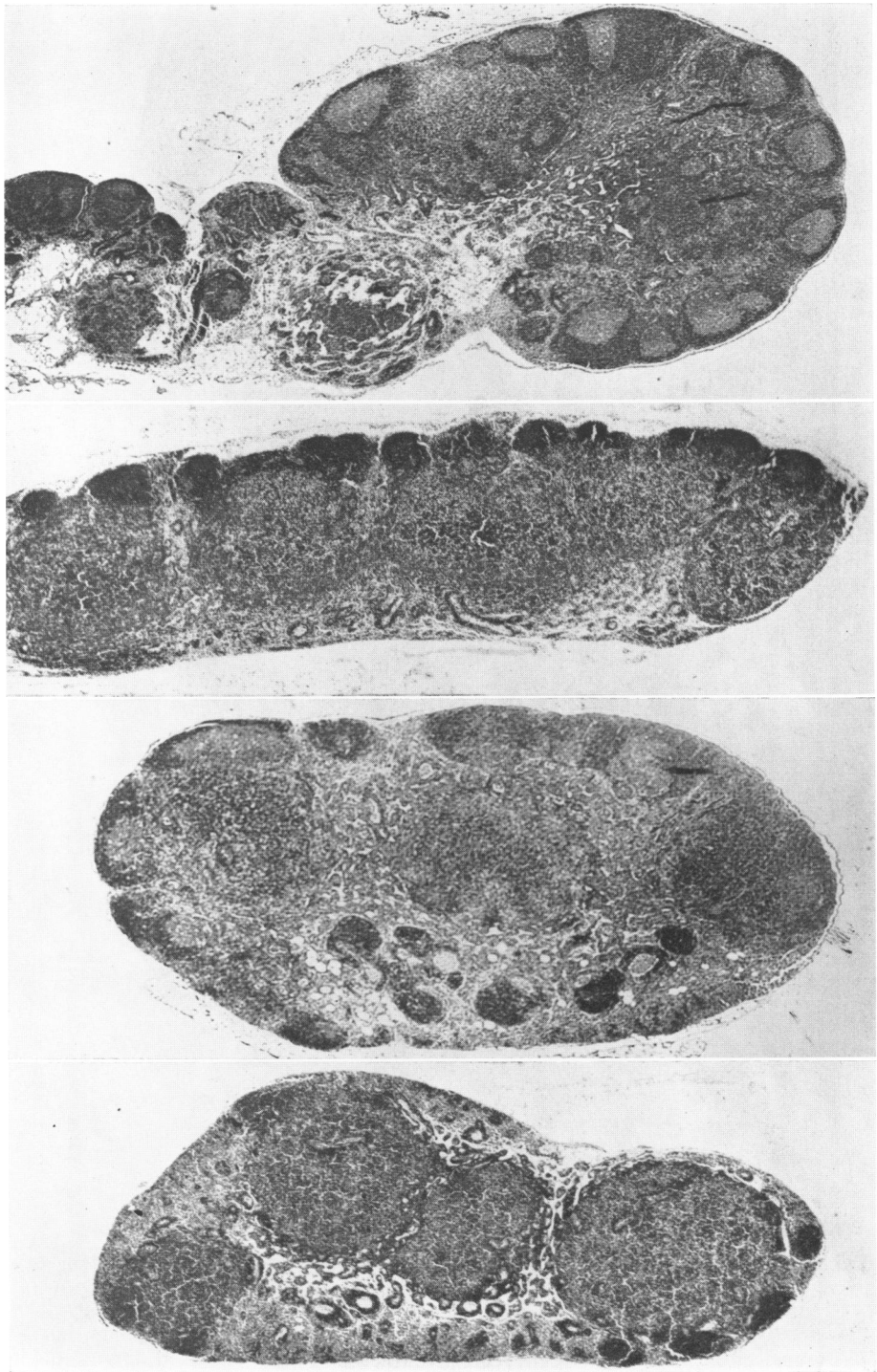
LEGENDS FOR FIGURES

FIG. 1. Mesenteric lymph node in a conventional mouse. Numerous cortical reaction centers are manifest. Hematoxylin and eosin stain. × 35.

FIG. 2. Mesenteric lymph node in a germfree mouse. The numerous cortical follicles contain no reaction centers. Hematoxylin and eosin stain. × 35.

FIG. 3. Axillary lymph node in a conventional mouse. Several cortical reaction centers and cellular medullary cords are evident. Hematoxylin and eosin stain. × 35.

FIG. 4. Axillary lymph node in a germfree mouse. There are no reaction centers and thin medullary cords, but the intermediate cortical zone is equal in size to that in nodes in conventional animals. Hematoxylin and eosin stain. × 35.



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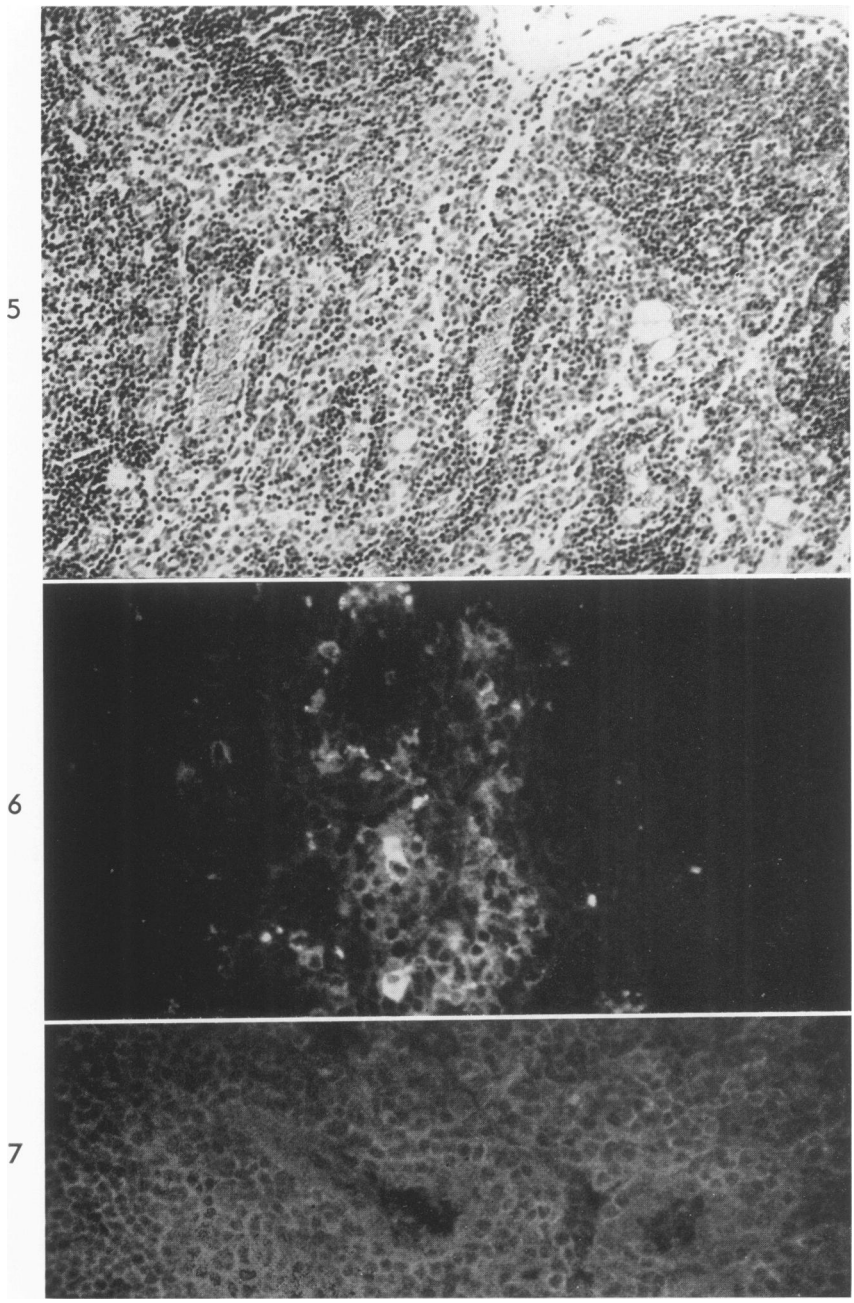

FIG. 5. Submaxillary lymph node in a germfree mouse. The medullary cords are thin. Giemsa stain. × 101.

FIG. 6. Submaxillary lymph node in a conventional mouse. Numerous immunologically competent cells comprise the medullary cords. Rabbit anti-mouse gamma globulin serum. × 400.

FIG. 7. Submaxillary lymph node in a germfree mouse. The medullary cords are devoid of immunologically competent cells. Rabbit anti-mouse gamma globulin serum. × 400.

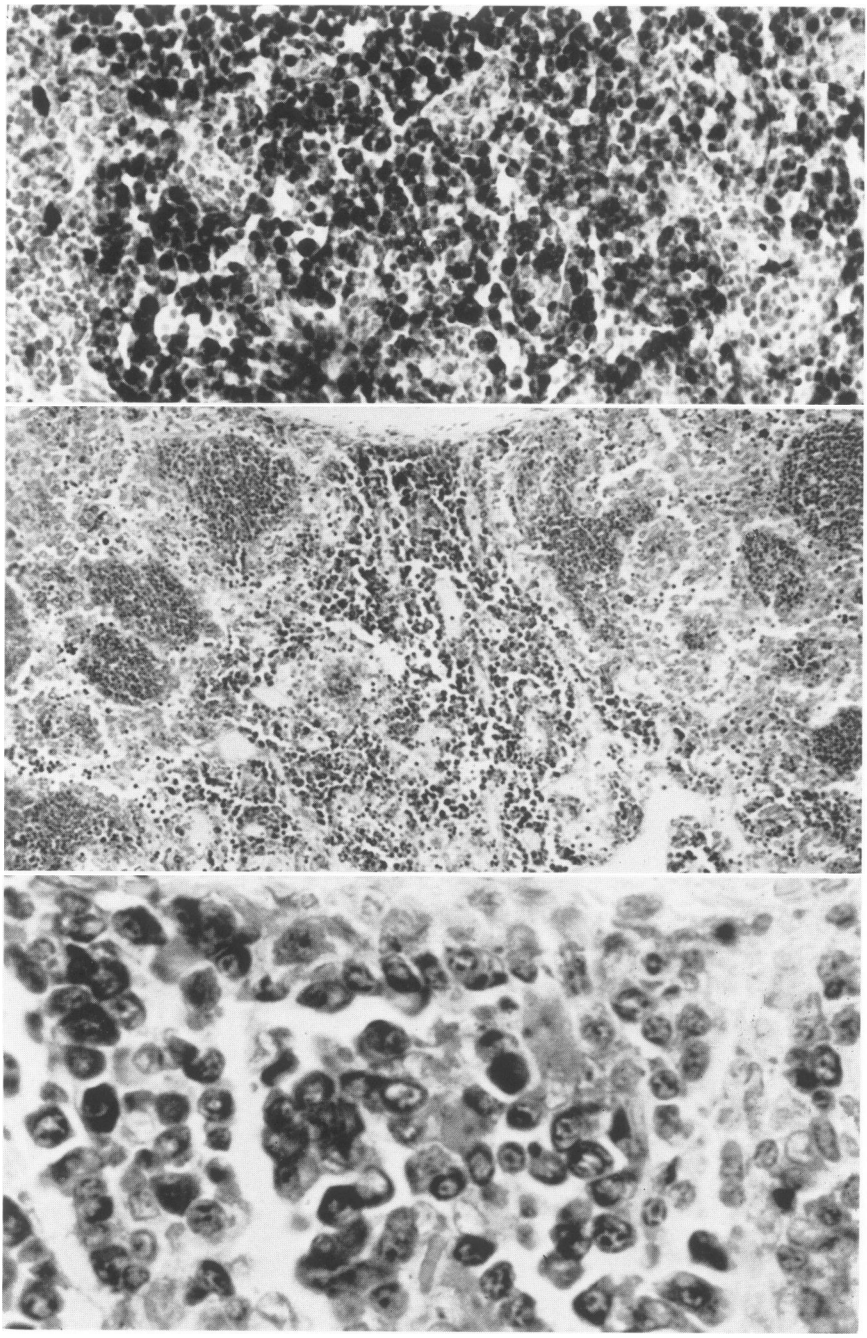

FIG. 8. Mesenteric lymph node in a conventional mouse. Numerous cells in the marginal and medullary sinus show positive reactions for acid phosphatase. Acid phosphatase (Barka [12]). × 270.

FIG. 9. Mesenteric lymph node in a conventional mouse. Hilar sinuses are packed with macrophages and immunologically competent cells. Giemsa stain. × 110.

FIG. 10. Mesenteric lymph node in a conventional mouse. Marschalkó plasma cells appear in the outflow tract. Giemsa stain. × 736.