THE PRIMARY IMMUNE RESPONSE IN MICE

II. Cellular Responses of Lymphoid Tissue Accompanying the Enhancement or Complete Suppression of Antibody Formation by a Bacterial Endotoxin*

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The diversity of cell types multiplying rapidly within lymphoid tissues during antibody formation presents a formidable obstacle to the identification of those cells which are actively engaged in the process.

A possible way around this obstacle was suggested by results of a study from this laboratory (1) in which it was shown that single intraperitoneal injections of a bacterial endotoxin into mice led, in less than 24 hr, to a great decrease in the weight of their spleens, lymph nodes, and thymus glands. As will be discussed below, the organs suffered a profound depletion of lymphoid elements followed by slow regeneration. A similar train of events occurred when endotoxin was injected 2 days before giving a single intraperitoneal injection of sheep red blood cells as antigen. Under these conditions, the formation of hemolytic antibody was completely suppressed (1). By sharp contrast, mice injected simultaneously with both endotoxin and antigen, and suffering the same early and severe depletion of lymphoid cells, showed an exceptionally rapid regeneration of these elements and produced extraordinary amounts of antibody (1).

It seemed likely, therefore, that new light might be thrown upon the cellular changes responsible for primary antibody formation by comparing the types of cells appearing first in the cell-impoverished spleens and lymph nodes of mice forming much antibody with those to be seen in the same organs of mice in which antibody formation was suppressed. Under these conditions, the dense cell population normally present in lymphoid tissues would be eliminated during the early phases of antibody response, on the one hand, or during its failure to appear, on the other. Accordingly, in the following pages, the histological changes occurring in the spleens and mesenteric lymph nodes of mice treated with endotoxin alone, hereafter to be designated as group E mice, will be

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contrasted with those of mice which formed no hemolytic antibody when injected with endotoxin 2 days before they got antigen (group E_2A), and in turn, with those from animals with augmented antibody production after receiving antigen and endotoxin simultaneously (group AE). Before these cellular responses to antigen could be profitably compared, it also became necessary to study the histological changes in the spleens and mesenteric lymph nodes of similar mice given single injections of antigen alone, here termed group A mice.

Materials and Methods

Female mice of 20–22 g body weight of the Rockefeller Swiss Nelson-Collins "pathogen-free" strain (1), which are highly resistant to the endotoxin of Gram-negative bacteria, were used exclusively. The doses of endotoxin and of sheep red blood cells given to the animals and the preparation of these materials have been previously outlined (1). Suffice it to add that this paper will detail only the cellular changes taking place in the spleens and mesenteric lymph nodes of those mice that received intraperitoneal injections of either endotoxin (50 μg), or the standard dose of sheep red blood cells (0.1 ml of a 1% suspension), or both in the various ways mentioned above. The curves of the hemolytic antibody content of the blood of the different groups of mice, A, E_2A, and AE, as already defined, have been presented as Fig. 1 in the accompanying paper (1) and demonstrate the complete suppression of antibody production in the mice that received endotoxin 2 days before antigen (group E_2A) and the enhanced formation of hemolysin in the mice given both endotoxin and antigen simultaneously (group AE). The same figure also shows curves indicating the blood antibody of the mice given the antigen only (group A). The mice given endotoxin but no antigen (group E) formed no hemolytic antibody.

In all four groups of mice, batches of three to four animals were sacrificed at 4, 8, and 12 hr, at daily intervals from 1 to 12 days, and at 2 day intervals from 12 to 24 days after the injection of sheep erythrocytes, or after giving endotoxin to the group E mice to which no antigen was given. At these times, the spleens and the mesenteric lymph nodes were taken for histological study. After fixation for 24 hr in neutral formalin, they were washed for an additional day in running water, and then embedded in Tissuemat (Fisher Scientific Co., Pittsburgh, Pa.). Tissue sections 5 μ thick were cut and stained with methyl green and pyronin (2) and, in many instances, with hematoxylin and eosin. Tissues prepared in the same manner from uninjected mice served as controls.

All of the black and white photomicrographs shown (Figs. 1 *a*–9 *b*) were reproduced from tissue sections stained with methyl green and pyronin using either orthochromatic or panchromatic film. The orthochromatic film renders red-staining, pyronin-positive portions of the cell black, while the emulsion of the panchromatic film depicts blue-staining areas darker than those stained red.

Nomenclature previously employed in the literature is used to distinguish between some of the cells with pyronin-positive cytoplasm which appear in lymphoid tissues after antigenic stimulation. Accordingly, "large pyroninophilic cells," a noncommital term used by Gowans (3) and synonymous with hemocytoblasts (4–6), is employed when referring to the large cells seen around the arterioles in the lymphocytic sheaths as well as those scattered and migrating through the follicles. In contradistinction, the immature blast-type cells found massed together in germinal centers, but otherwise indistinguishable from the large pyroninophilic cells, are termed "activated reticulum cells" as suggested earlier by Marshall and White (7). Other types of cells with pyronin-positive cytoplasm which appear in the red pulp are simply called "pyronin-positive cells," unless they are specifically mentioned as plasmablasts, preplasma cells, or mature plasma cells.

FINDINGS

Cellular Changes in Mice Injected with the Standard Dose of Sheep Red Cells Only as Antigen: Group A Mice

The cellular changes taking place in mice given antigen only were so similar to those described by many authors, as occurring in other species of laboratory animals (4–11 to refer to only a few), and so well known that no special description or documentation needs to be given. Suffice it to remark in passing that the primary cellular response, in the spleens and mesenteric lymph nodes of these mice, to the standard injection of sheep red blood cells is characterized by the appearance, within the first 24 hr after the injection of antigen, of large pyroninophilic, blast-like cells in the lymphocytic sheaths of the white pulp of the spleens. These cells increase very rapidly in numbers and appear to migrate outward from the follicles to reach the splenic red pulp. The behavior of these cells in mice used in the present work was so much like that previously described by others (4–6, 8–11) that no description needs to be given here. By and large our findings confirmed those of Congdon and Makinodan (12).

It should be stressed that the present work also included a careful study of the changes in the mesenteric lymph nodes as well as in the spleen. It revealed the presence of numerous large pyroninophilic cells appearing within the first 24 hr in the cortical regions of the nodes and, by the 4th day, in the medullary cords together with plasmablasts, preplasma cells, and vast numbers of small lymphocytes. Figs. 1 *a* and 1 *b*, taken on orthochromatic film and magnified 400 and 1000 times respectively, show part of a medullary cord in a mesenteric lymph node of a mouse injected intraperitoneally 4 days previously with the standard dose of antigen. In this section, stained with methyl green and pyronin, a diffuse lymphocytic hyperplasia was already present. In the cord, among closely packed blue-stained small lymphocytes which appear gray in the figure, stood numbers of large pyroninophilic cells with pale blue, vesicular nuclei surrounded by intensly red, pyronin-stained cytoplasm appearing like dark black rings. Because of this fact and their large size, these cells are clearly shown in the figures. Typical instances are designated by arrows. In the living animal, all these cell types would, of course, enter the efferent lymph stream and then the blood, to be distributed to various organs where they might take part in antibody formation.

These findings implicate the medullary regions of lymph nodes in the production of antibody during the primary response, and are in general agreement with the findings of White (13) in his study of the lymph nodes of rabbits, after a first injection of antigen, and in the recent work of Nossal and his coworkers (14, 15) who reported plasmablasts in the medullary sinuses of the popliteal lymph nodes of rats 1 day before the appearance of antibody.

Mature plasma cells did not appear in either the nodes or spleens until later, about the 6th or 7th day, that is to say, until antibody had been present in the blood for several days. They remained in evidence for many more days, during the period of decline of circulating antibody (1).

An "activation" of germinal centers in the follicles of both organs, as evidenced by the appearance of closely packed primitive cells with strongly pyronin-positive cytoplasm, took place either 1 day before antibody first appeared in the blood or on the same day. These cells sometimes appeared to leave the centers and stream outward

into the red pulp of the spleen or into the medullary regions and cords of the mesenteric lymph nodes, as also seen by Congdon and Makinodan (12).

Cellular Changes in Mice Injected With 50 μg of Endotoxin: Group E Mice

On the 1st day after injection: Within 24 hr after injecting 50 μg of endotoxin intraperitoneally, the spleens became much lighter than normal. Fig. 3 *a* presents a transverse section of one of these spleens stained with methyl green and pyronin and photographed on panchromatic film at a low magnification (× 30). This photograph is to be compared with Fig. 2, which is a photograph of a typical, normal spleen also in transverse section at the same magnification. In the spleen shown in Fig. 3 *a*, the red pulp by comparison had become almost devoid of cells and contained few lymphocytes. The arrow in Fig. 3 *a* indicates the area of the section shown at higher magnification, × 200 and × 400, respectively, in Figs. 3 *b* and 3 *c*. Polymorphonuclear cells had infiltrated both the red pulp and the follicles. The two arrows in Fig. 3 *b* indicate two of these collections of cells, and the left hand arrow of Fig. 3 *b* points to the same group as the one in Fig. 3 *c*. These cells are not easily identifiable in the pictures since they are stained poorly with methyl green and pyronin. The follicles were composed mostly of closely crowded cells with large blue nuclei and pyronin-positive cytoplasm, scattered among which were very few large pyroninophilic blast cells, not nearly as many as in mice given only sheep red blood cells as antigen. These cells do not show in the picture since panchromatic film does not bring out clearly their pyronin-positive cytoplasm. There were no germinal centers present.

The mesenteric nodes, less cellular than those of normal animals, were nevertheless more cellular than the spleens, as evidenced by medullary cords filled with blue lymphocytes apparently ready to enter the efferent lymph. Germinal centers were absent.

On the 2nd day: 48 hr after injecting the endotoxin, the cellularity of the red pulp of the spleens had increased only slightly. Fig. 4 shows a transverse section from such a spleen stained and photographed like that shown in Fig. 3 *a*. The lymphocyte-poor follicles, although fairly well defined, contained no germinal centers. In the spleen, but not shown at this low magnification, there were some large pyroninophilic cells in which the nuclei stained poorly and the cytoplasm seemed smudged. By contrast, in the mice given sheep cell antigen the large pyroninophilic cells were very numerous 2 days later. In the section under consideration, some polymorphonuclear cells persisted, but their numbers were much smaller than those in the sections taken 24 hr after the endotoxin had been given.

Again, on this day, the nodes were more cellular than the spleens, although no lymphatic hyperplasia had developed as yet. There were some poorly developed follicular centers and only very few large pyroninophilic cells, all of which were in the mid-regions of the nodes and none at all in the medullary cords. This stood in sharp contrast to the great number of these cells seen all through the nodes, including the medullary cords, of the group A mice 2 days after these animals were injected with sheep red blood cells, as described above.

On the 3rd day: During the next 24 hr, the spleens began to increase in cellularity and to look more like normal spleens. Fig. 5 shows a photomicrograph at a low magnification, × 30, of one of these spleens stained, as usual, with methyl green and pyronin and photographed on panchromatic film. It should be compared with Fig. 2 show-

ing the normal histological picture. The organ shown in Fig. 5 of this paper contained enlarged follicles, nearly half of which held the possible beginnings of germinal centers, that is to say small clusters of cells with pyronin-positive cytoplasm, not shown at this low magnification. Scattered through the follicles were some large pyroninophilic cells, also not shown. Since they were no more numerous than on the 2nd day and fewer in number than those found in the group A mice, 3 days after an injection of antigen, they may simply have been cells present in these areas at the time of injection and retained there without multiplying.

The nodes, too, had become slightly more cellular. They began to show a mild lymphocytic hyperplasia, but still only very few large pyroninophilic cells. The medullary cords had filled with small, blue-stained lymphocytes, but among the latter there were no large pyroninophilic cells. Indeed only a few had appeared elsewhere in the nodes. Again there was a sharp contrast between these findings, on the 3rd day after the mice received endotoxin, and those obtained 3 days after giving mice sheep red blood cells, in group A, in which the large pyroninophilic cells were very numerous.

On the 4th day: The red pulp of the spleens had become still more cellular. The follicles had enlarged, some had become confluent, and many contained a few "activated reticulocytes" with pyronin-positive cytoplasm (7). These appeared as clusters large enough to be considered as early forming germinal centers, not yet fully developed. Extending out from the follicular arterioles to the red pulp, in a few areas, were some large pyroninophilic cells, still not nearly as numerous as in the mice given sheep cell antigen (group A).

In the nodes on the 4th day, the centers in the cortex were not as well developed as on the 3rd day, and the lymphocytic hyperplasia was no greater. The medullary cords did not contain as many lymphocytes as on the day before, and there were almost no large pyroninophilic blasts to be seen. In other words, the characteristic cellular changes accompanying antibody formation as seen in the group A mice, after injection of antigen, were largely lacking.

On the 5th day: There was no marked change in the nodes, the centers remained small, poorly developed, and there were still no large pyroninophilic cells to be seen.

On this day, the red pulp of the spleens appeared no more cellular than on the day before, although about half of the follicles of the white pulp now showed a delayed formation of some germinal centers with pyronin-positive activated reticulum cells, but even on this 5th day after receiving endotoxin there were only a few large pyroninophilic cells.

On the 6th and 7th days: Even 6 and 7 days after injecting endotoxin, there were still only a few, poorly-stained, large pyroninophilic cells. These had failed either to multiply or to migrate out into the red pulp as they did in the group A mice that received the sheep cell antigen. Indeed, in that group of mice the explosive increase in the number of these cells that usually follows injections of antigen had not only taken place, but, by the 6th or 7th day, these cells had either disappeared or had migrated into the red pulp which then became filled with various kinds of pyronin-positive cells.

There were more germinal centers in the splenic follicles than had been present on the 5th day, but they had appeared much later than in the mice given sheep blood cells as antigen, mice which by the 6th or 7th days after challenge, had already reached or passed the peak of their antibody formation.

By this time, the nodes had become almost as cellular as those of normal mice. The

germinal centers appeared redder since they contained more pyronin-staining, activated, center cells, but these structures were not as well formed as those in the spleens. There were still only a few of the large pyroninophilic cells, but as yet none had entered the medullary cords which were filled instead with blue-stained lymphocytes only.

During the next few days: The cellularity of the nodes and the spleens began to look more like that of normal mice in contrast to the antibody-forming reaction seen in those that received sheep red blood cells (group A). The nodes never developed a strong hyperplastic lymphocytosis as those in the latter had. By the 10th day, the nodes also contained some mature germinal centers like those frequently seen in the spleens of normal mice. Some large pyroninophilic cells had appeared in their midregions, but not in the cords, a phenomenon which can also be seen in normal mice.

By this time, the splenic germinal centers became well developed and ringed with mantles which, although they were clearly visible, were not as broad and well defined as those described and pictured by Ward, Johnson, and Abell (16) in rabbits given not only endotoxin but antigen as well. In the red pulp, many pyronin-positive cells, perhaps preplasma cells, were present along with other unidentified units having pyronin-positive cytoplasm like those seen in normal spleens.

In summary, the mice injected with endotoxin but no antigen failed to show the characteristic cellular changes in the spleens and mesenteric lymph nodes that appeared in the animals given sheep red blood cells. In particular, the great overgrowth of large pyroninophilic cells and the subsequent appearance of members of the plasma cell family were lacking, and the follicular development of germinal centers was poor and late.

The Effects of Endotoxin Given 2 Days Before Antigen: Group E_2A Mice

The histological appearance of the lymphoid organs of this group of mice (E_2A), during the first 3 days after giving 50 μg of endotoxin and before injecting the antigen was, of course, just like that already described for the group E mice (given endotoxin only) during the same time interval.

Changes on the Ist day after giving antigen (3 days after endotoxin): 48 hr after injecting the endotoxin, the standard dose of sheep red blood cell antigen was given. On the next day, no visible change attributable to the latter was to be found in either the spleens or the mesenteric lymph nodes of these animals. This is apparent from an inspection of Fig. 6, a photomicrograph of a section taken from one of these mice. The tissue was stained with methyl green and pyronin, and photographed on panchromatic film at a magnification of $\times$ 30. These spleens somewhat resembled those of the mice that got endotoxin only (group E) 3 days previously, as can be seen by comparing Fig. 6 with Fig. 5. No germinal centers existed in the follicles. The latter, observed at higher power, held only a few poorly stained large pyroninophilic cells, far fewer than were found in the spleens of the mice injected 1 day previously with sheep red blood cells. The red pulp held a few pyronin-positive cells, none of which could be identified as members of the plasma cell line.

The nodes of the E_2A mice were still less cellular on this day than those of normal animals, and not even as cellular as those of mice 3 days after an injection of endotoxin.

Only a few large pyroninophilic cells appeared, and germinal centers were rare and poorly developed.

On the 2nd day after antigen (4 days after endotoxin): The red pulp of the spleens had become somewhat more cellular. Fig. 7, a photograph taken of a section of one of these spleens, by the same techniques used to obtain Figs. 5 and 6, illustrates the change. There was no evidence of a beginning immune response as yet. In contrast to the mice that received antigen only, the group A animals, far fewer large pyroninophilic blast cells presented themselves. Since they were visible in approximately the numbers that one would expect to find in normal mice, it seems probable that they were cells which had already been present in the follicles when the endotoxin was given. After the injection of antigen, they had not increased in numbers as they do in mice stimulated by antigen alone.

On this day, the tissues of the nodes had become more densely populated with cells. The germinal centers had increased slightly in number and contained macrophages filled with cellular debris. This "starry sky" appearance was not seen in the mice 4 days after receiving only endotoxin (group E). A lymphocytic hyperplasia in these E_2A mice had become evident, and lymphocytes filled the mid-regions of the nodes and the medullary cords. Again only a few large pyroninophilic cells were to be seen. In brief, the nodes were much like those of the mice given endotoxin only 4 days previously.

On the 3rd day after receiving antigen (the 5th day after endotoxin): On this day, the splenic sections still presented only a few poorly stained large pyroninophilic cells in the follicles and no germinal centers, although some were present in the nodes. The red pulp, although more cellular than on the day before, was not as well filled as in mice 5 days after receiving endotoxin only. Obviously the antigen had elicited no specific cellular reaction in the spleens.

In the nodes, by contrast with the spleens, somewhat poorly formed germinal centers contained many macrophages filled with debris presumably derived from the injected sheep red blood cells.

On the 4th day after getting the antigen (6 days after endotoxin): The splenic follicles became denser, and many contained small clusters of cells which appeared to be in the early stages of germinal center formation. Near the follicular arterioles and scattered throughout the follicle, there were still only a few smudged, large pyroninophilic cells. This appearance formed a marked contrast with that seen in the splenic sections from mice taken 4 days after injecting antigen only. In these, as not mentioned previously, the germinal centers were well formed, and the large pyroninophilic cells had not only reached their numerical peak but many had already migrated to the edges of the follicles.

There was little change in the nodes. They, too, contained far fewer large pyroninophilic cells than normal mice. The medullary cords held only lymphocytes but no large pyroninophilic cells like those seen in the nodes of mice 4 days after receiving the same antigen but no endotoxin.

On the 5th day after antigen (the 7th after endotoxin): The splenic sections contained practically no large pyroninophilic cells. This substantiated the suggestion made above that, since they had not increased in number, these cells had probably been present in the follicles when the endotoxin was given. They had not responded to the

subsequent injection of antigen as they had in the mice that received antigen only. Further, the clusters of activated reticulum cells seen on the 4th day had increased but moderately in size, instead of developing rapidly into germinal centers as in antigen-injected mice. The cellular antibody-forming mechanism did not appear to be activated.

At this time the nodes, although some contained large centers with heavy mantles, lacked the other characteristics of an early immune response, and resembled those of mice given endotoxin only at a similar time interval.

Changes in the spleens and nodes on the 6th and subsequent days: The splenic sections, obtained on and after the 6th day after the injection of antigen (the 8th after endotoxin), presented a very different appearance from those of the earlier periods. "Active" germinal centers with massed pyronin-positive, activated reticulum cells had now fully developed. Although the follicles still contained only a small number of large pyroninophilic cells, they now appeared to be better stained, perhaps were newly formed. The red pulp also contained a few smaller pyronin-positive cells. This awakening or "activation" of the splenic centers increased on the 7th day, while the cellularity of the red pulp remained much like that of normal spleens.

The nodes, like the spleens, showed marked changes on the 6th day after the injections of antigen. Many more germinal centers made their appearance containing great numbers of activated reticulum cells. For the first time, the number of large pyroninophilic blast cells increased slightly. These cells had, at last, begun to multiply, 6 days after the injection of antigen, in contrast to their appearance on the 1st day in the mice that received antigen but no endotoxin.

By the 7th day, the germinal centers of the nodes increased in size and numbers. Many more large pyroninophilic cells lay scattered throughout the follicles, and the cords near the hilus contained moderate numbers of cells with pyronin-positive cytoplasm, probably plasmablasts and preplasma cells. This histological reaction closely resembled the tissue response during early antibody formation, but it was not accompanied, under these experimental conditions, by the appearance of serum hemolysin. Since endotoxin is antigenic, the organs may have been reacting to it.

The "activity" in the spleens was short lived. By the 10th day, the red pulp had come to look like that of normal animals. Nevertheless active germinal centers were present and they had become surrounded by compact mantles of blue-stained cells. These spleens resembled those of mice 10 days after an injection of endotoxin only (group E). By the 14th day, the splenic tissue looked like that of normal mice. There had been no evidence of a preplasma cell or mature plasma cell reaction in the spleens.

By contrast, the nodes appeared to be somewhat active between the 10th and 14th days. Germinal centers remained present, and the large pyroninophilic cells increased slightly in number, some of them appearing among the masses of blue-stained lymphocytes in the cords together with a few cells with dark-stained and even eccentric nuclei and pyronin-positive cytoplasm, probably preplasma cells. Through the 12th day, some lymphocytic hyperplasia persisted. In spite of all this, no hemolysin was found in the blood (1).

Up until the 20th day, the nodes continued to show more active germinal centers than are usually found in normal organs. In the cords, near the hilus, many preplasma cells and a few mature forms remained as if these nodes had been forming antibody to

something, but not to the sheep red cell antigen because no hemolysin was found in the blood of these mice at any time.

Striking Cellular Changes in the Spleens and Mesenteric Nodes After Simultaneous Injection of Antigen and Endotoxin: Group AE Mice

The simultaneous injection of 50 μg of endotoxin and the standard dose of sheep red blood cells into mice gave rise to an enhanced production of antibody (1) as well as to interesting differences in the cellular reactions taking place in the lymphoid tissues when compared with those found in other groups of mice used in this study. Besides these, notable differences also appeared between the cellular reactions of the nodes and those of the spleens of these animals.

Changes on the Ist day: At this early stage, 1 day after a simultaneous injection of endotoxin and antigen, both the red pulp and the follicles were deprived of almost all of their lymphocytes. The red pulp contained great numbers of polymorphonuclear cells lying close to the follicles and in clusters in open spaces within them, more than were seen in any of the other experimental groups of mice at this time after injections of antigen or endotoxin. Only a few pyroninophilic cells, so prominent in the mice given red blood cells only, were present.

Fig. 8 *a* shows a transverse section of one of these spleens stained with methyl green and pyronin, photographed on panchromatic film, and magnified × 30. The pale, empty red pulp is plainly evident. The atypical appearance of the follicles is not shown at this magnification. The section looked like those obtained from the group E mice 1 day after they had been given endotoxin only, as shown in Fig. 3 *a*. Some of the cellular changes in the section seen in Fig. 8 *a* appear to better advantage in Fig. 8 *b* at a higher magnification, × 150. The figure shows the right tip of the section illustrated in Fig. 8 *a* as it appeared when taken on orthrochromatic film using a yellow K-2 filter. Under these circumstances, the pyronin-stained portions of the cells appear black. This photograph shows not only the empty red pulp but also one large atypical follicle in the central portion of which, and also slightly above and to the right, are collections of polymorphonuclear cells. Three of these collections are indicated by arrows. The loss of cells in this spleen is obvious when the picture is compared with that of the normal spleen shown in Fig. 2. There were no germinal centers.

In Fig. 8 *c*, the areas of Fig. 8 *b*, indicated by the two lower arrows, are shown at a still higher magnification, × 700, as they appeared on orthochromatic film. The arrows point to separate collections of polymorphonuclear cells, which show poorly since they did not stain well with the methyl green and pyronin used in this work. The white pulp contained many primitive cells with pyronin-positive cytoplasm, which appears in each cell of this kind as a black ring.

In contrast to the findings in the spleens, a study of four mesenteric nodes from the same animals disclosed highly cellular organs, more cellular than those of the E mice 1 day after the latter received endotoxin only. There was an active lymphocytic hyperplasia. The cortical regions showed modified germinal centers which had apparently suffered a pronounced loss in cellularity. The medullary cords held about the same number of lymphocytes that are found in normal mesenteric nodes, but many large

pyroninophilic blast cells were present. As already stressed above, these cells were not found in the cords of the nodes of mice examined 1 day after they got antigen when endotoxin had been given 2 days previously, that is to say in the E_2A mice, nor were they seen so early in the cords of the mice that got antigen only, the group A mice.

Changes on the 2nd day: By this time, striking cellular changes, brought about by the simultaneous injection of endotoxin and antigen, appeared in both the spleens and nodes. This is best illustrated by a comparison of three splenic sections, one from a mouse given endotoxin only 2 days previously (Fig. 4), one from a mouse given endotoxin 2 days before the antigen (Fig. 7), and one from a mouse of the group presently under consideration and given endotoxin and antigen simultaneously, 2 days before the picture was taken (group AE, Fig. 9 *a*).

Fig. 9 *a* shows a transverse section of one of the latter magnified × 30, stained with methyl green and pyronin, and photographed on orthochromatic film. Since the follicles appeared red, not blue as usual, because most of their cells were pyronin-positive, the orthochromatic film rendered them darker than in the other pictures. Several of the follicles show pale centers because they contained either fewer cells, or more bluish ones. One of the former follicles, with fewer cells, is indicated by the lower arrow in the figure. Other follicles show relatively open spaces which can be seen, at higher magnifications, to contain collections of bluish-tinted polymorphonuclear cells instead of the usual types of cells. The upper arrow in the figure points to one of these spaces, which is also photographed at a higher magnification, × 600, in Fig. 9 *b*. This photomicrograph, also taken on orthochromatic film, renders the pyronin-stained follicle cells darker than the more bluish cells of the red pulp, as shown by the lower arrow in the picture. A collection of leukocytes within an open space in a follicle is indicated by the upper arrow. These cells do not show up clearly because they stain poorly with methyl green and pyronin.

Fig. 9 *b* illustrates the huge increase in the cell density which had taken place by the 2nd day not only in the follicles, but, as indicated by the lower arrow, in the red pulp as well. Among the cells of the follicles, as opposed to the open space containing the leukocytes, there were also greatly increased numbers of the large pyroninophilic cells, far more than in any of the other groups of mice on the corresponding day. These cells do not stand out in Fig. 9 *b* because their pyronin-positive cytoplasm was much the same color as that of the other cells, all being pyronin-positive. All this contrasts sharply, as mentioned above, with the lack of cellular proliferation in mice 2 days after they received endotoxin only (group E mice, Fig. 4).

Still another important difference existed between the appearance of these spleens on the 2nd day and on the 1st day. The red pulp now contained numerous collections of cells with pyroninophilic cytoplasm which were not identifiable as large pyroninophilic cells. Since many of them looked not unlike plasmablasts, a preplasma cell reaction might have already been initiated. As yet there were no active germinal centers in the follicles although they contained many cells in mitosis which stained like activated reticulum cells.

In the nodes, a spectacular increase in cellularity also occurred on this day, which far exceeded that shown by the mice that received antigen only (group A). Some medullary cords were already choked with blue-stained lymphocytes, while others contained cells resembling preplasma cells. The mid-regions of the organs contained

great numbers of typical large pyroninophilic cells. This reaction was like that which took place in the animals given antigen only, the group A mice, but it was far more pronounced.

Changes on the 3rd day: The splenic red pulp contained an extraordinarily large number of cells with pyronin-positive cytoplasm. Some of these contained large, pale blue nuclei and had the appearance of activated reticulum cells or, perhaps, they were actually large pyroninophilic cells themselves. Both of these cell types might have moved out from the white pulp since cells with large, pale blue nuclei and pyronin-positive cytoplasm were also found in great numbers around the central arterioles and massed at the outer edges of the follicles. The red pulp also held smaller pyronin-positive cells with darkly stained nuclei, resembling preplasma cells. Since these lay in large clusters in the red pulp it would seem that they, at least, had arisen from units already in that region. This unique appearance of the spleen was altogether different from that seen in the other groups of mice.

The mesenteric nodes on the 3rd day after the injection of both antigen and endotoxin held some small, well-formed germinal centers made up of the usual activated reticulum cells and showing the starry sky appearance. Close to these centers, and extending through a moderate lymphocytic hyperplasia in the mid-regions of the nodes, were great numbers of the large pyroninophilic cells, some appearing as scattered individuals, but others in clusters resembling early forming reaction centers. The nodes of these animals showed much more activity at this time than those of any of the other groups of mice.

Changes on the 4th day: By this day, all the splenic follicles contained well-formed germinal centers usually showing the starry sky appearance. Among the surrounding blue-stained follicular cells were many others with pyronin-positive cytoplasm collected into groups as though they were forming new centers. There were many mitoses among them, and, when stained with hemotoxylin and eosin, these cells showed pronounced basophilia. The red pulp was literally stuffed with pyronin-positive cells, the majority of which had the morphological characteristics of plasmablasts. No mature plasma cells were to be seen.

Clearly the combined endotoxin and antigen had stimulated an early preplasma cell response following the initial appearance of the large pyroninophilic cells in the spleens. By contrast, the red pulp in the spleens of the mice that received endotoxin 2 days prior to sheep cells contained far fewer cells, and the vigorous reaction just described was absent at this time.

On this day, the nodes of the mice simultaneously injected with antigen and endotoxin exhibited a more intense hyperplasia than on the previous day. Although the germinal centers had not greatly enlarged, many large pyroninophilic cells appeared in the mid-regions and among the blue-stained lymphocytes in the medullary cords.

These histological changes in the spleens and nodes coincided with a sharp rise in the hemolysin titer of the blood which reached its peak on the 5th and 6th days (1).

Changes on the 5th day: In the spleens the germinal centers, which were still filled with macrophages containing intracellular debris, had developed prominent mantles. Some of these had become attenuated, as also noted by Congdon and Makinodan (12), in mice given large doses of sheep cell antigen. Rows of pyronin-positive cells appeared to be streaming directly out from the follicles into the red pulp. The latter

still contained large clusters of plasmablasts, preplasma cells, and some mature forms, occurring in such large numbers that these sections actually appeared like those usually seen in secondary reactions. Clearly this represented a very exaggerated primary response.

In the 6th and 7th days: A striking decrease in the cellularity of the splenic red pulp took place, the ratio of lymphocytes to pyronin-positive cells increased, the reaction centers enlarged to such proportions as to constitute the major part of the spleens.

The mesenteric nodes, on these days, showed an increasing lymphocytic hyperplasia. The medullary cords, jammed with blue-stained lymphocytes, contained mature plasma cells, a far more rapid production of these cells than had been seen in the other groups of mice. Large collections of cells, having the morphological characteristics of preplasma cells, made their appearance throughout the nodes.

Subsequent changes: In the spleens, during the following days, the germinal centers enlarged still further and developed thick mantles of lymphocytes. The red pulp, crammed with unidentifiable pyronin-positive cells, also contained collections of preplasma cells and mature plasma cells.

The nodes continued to show a strong lymphocytic hyperplasia. Some of the cords were filled with blue-stained lymphocytes, but others held loose collections of preplasma cells and mature forms. A second peak of circulating antibody occurred at this time (1). It should be stressed that the medullary cords of the nodes of this group of mice held many more cells of the plasma cell series than those of the mice that formed antibody after receiving only the sheep red blood cell antigen.

The cellularity of the lymphatic tissues of these mice, given endotoxin and antigen together, waned steadily from the 12th day to the end of the experiment on the 24th day. Nevertheless, the spleens still retained clusters of plasma and preplasma cells in the red pulp, and the nodes contained a few plasma cells in their cords.

DISCUSSION

As shown in the accompanying paper (1) and discussed above, mice given 50 μg of endotoxin 2 days prior to an injection of sheep red blood cells (group E_2A) failed to produce hemolysins, while those simultaneously injected with endotoxin and antigen (group AE) formed large amounts of this antibody. A comparison of the cellular responses in the spleens and mesenteric nodes of these animals with those of mice given the sheep red cell antigens only and those of a control group (E), herein described and given endotoxin only, has provided new insight into the cellular basis of the primary antibody response in this species.

The present work showed that a profound depletion of the lymphoid elements of spleens and mesenteric lymph nodes occurred in *all* mice given the endotoxin regardless of whether it was injected alone (group E), or 2 days before the antigen (group E_2A), or with it (group AE). This effect lasted for about 6 days in groups E and E_2A, both of which formed no hemolysin. It endured for only 24 hr in the mice that formed large amounts of antibody, the group AE mice. It seems clear from these findings that the early depletion of the lymphoid tissues did not prevent the subsequent synthesis of antibody when the regeneration of

the lymphoid tissue was rapid, as in mice that received antigen and endotoxin simultaneously (group AE). By contrast, a prolongation of the depleted condition of the lymphoid tissues by endotoxin treatment prior to antigen injection (group E_2A) seems to have brought about a blocking of the cytological events normally leading to hemolysin production.

In the mice given antigen and endotoxin simultaneously, the animals that formed huge amounts of antibody, there appeared exceptionally large numbers of polymorphonuclear cells in the spleens on the first day after the injections, whereas far fewer presented themselves in the spleens of the other two groups of animals. Whether or not these cells were active in rapidly overcoming the lymphocytolysis by the endotoxin and in stimulating the subsequent enhanced immune response in the group AE mice cannot be established from our data.

In correlating the cellular changes in the lymphoid tissues with the titers of serum hemolysin, at various times after giving antigen (1), our studies indicate that the appearance of large pyroninophilic cells always preceded the first detectable hemolytic antibody in the sera of both of the groups of mice that formed it. Further, by far the most striking histological effect seen in the mice that formed the greatest amount of antibody, the group AE animals given simultaneous injections of endotoxin and antigen, was the appearance of many more large pyroninophilic cells both in the spleens and in the mesenteric nodes than were to be seen in the same organs of the other group of mice that formed hemolysin after receiving the sheep red blood cell antigen, the group A mice. It seems likely, therefore, that the early and very great increase in the number of large pyroninophilic cells in the follicles of the former group can be held responsible for the augmented production of antibody. On the other hand, the scarcity of the large pyroninophilic cells as well as their inability to migrate or multiply in the lymphoid tissues of both groups of mice that failed to form specific hemolysin (groups E and E_2A) seems to point to at least one of the ways in which the endotoxin prevented the formation of antibody. These various findings taken with those of the accompanying paper (1) provide additional support, in agreement with others (3, 12–14, 17–23), for the idea that the large pyroninophilic cells are precursors of antibody-synthesizing cells.

It was also very evident from our studies that far more pyronin-positive cells occupied the red pulp of the spleens of the mice that formed the most antibody (group AE) than those of the other groups of mice. These cells, which in this instance appeared on the 2nd day after the injections of antigen, were replaced by a vigorous preplasma cell response on the 3rd and 4th day and later with a mature plasma cell reaction which, from its intensity, resembled a secondary response to antigen. A similar, but much weaker and less rapid, reaction occurred in the spleens and lymph nodes of the mice given antigen only, while neither a preplasma cell nor a mature plasma cell reaction was exhibited by the mice that failed to form antibody (group E_2A). From these observations, it

would appear that some pyronin-positive cells of the red pulp seem to be the immediate precursors of the plasma cell series. However, their origin still remains in doubt since they may have stemmed either from large pyroninophilic cells that had migrated to the red pulp or they might have arisen from cells already present in the red pulp itself.

Since preplasma cells were invariably present in large numbers in the lymphoid tissues at the time of the initial appearance of serum antibody in both groups of mice that formed it, while mature plasma cells were apparent only after the time of peak antibody titers, it seems justified to assume with others (5, 4, 19) that the major portion of the immune protein is produced by the former cell type.

Although germinal centers in lymph nodes and spleens have been repeatedly observed to react to antigenic stimuli (see reference 13), their role in the production of antibody is still open to controversy. Be that as it may, further studies, made on the group A mice that received only antigen—but not detailed here for lack of space—have indicated that the earliest "activation" of centers in lymph nodes and spleens, following the injection of antigen, usually occurred on the day before or on the same day that antibody first appeared in the blood. Further, it should be stressed that mice simultaneously injected with antigen and endotoxin (group AE), those which formed the greatest amounts of antibody, also showed larger centers which held many more activated reticulum cells than those in the other experimental groups. These findings are in general agreement with those of Ward et al. (16) and Langevoort et al. (4, 5) who employed soluble antigens given to rabbits. In contrast, activated germinal centers, in the mice that formed no hemolysin (those given endotoxin only, group E, and those injected with endotoxin 2 days before the antigen, group E_2A), were poorly formed and remained so until the 6th and 7th days. Whether or not partial suppression of the activation of germinal centers, for a limited time in the E_2A mice, under conditions which completely inhibited both the typical cellular response and the production of antibody to sheep erythrocytes over a 14 day period (1), had anything to do with bringing about this result still remains to be seen. Nevertheless, the enhancement of germinal center activation, before the demonstration of antibody synthesis and under conditions leading to an increase in hemolysin production in the mice given antigen and endotoxin simultaneously, indicates strongly that the stimulation of cells in these areas may be closely related to the early phases of the primary response to antigen. This observation is in agreement with the findings of Congdon and Makinodan (12), and of Hanna (23) who also injected sheep red blood cells into mice, and with recent work which has demonstrated the persistence of antigenic components (21, 22) as well as the absence of antibody (5, 13) in these centers of cellular activity during the primary response.

SUMMARY

The effects of a single injection of a bacterial endotoxin on the cellular changes of a primary immune response to a standard dose of sheep red blood cells were studied in the spleens and mesenteric lymph nodes of mice. Daily histological comparisons of these organs in mice, injected with endotoxin, or with antigen, or both, showed that endotoxin given simultaneously with sheep red blood cells, as antigen, significantly enhanced all of the cellular changes that appear in the mesenteric lymph nodes and spleens of mice that form antibody when that antigen is given alone. First, in the white pulp of the spleens and cortical regions of the nodes, there appeared an early and excessive proliferation of the large pyroninophilic cells which seems to be responsible for the earliest formation of antibody, as judged by this work and that of others cited in the body of the paper. Polymorphonuclear cells invaded the spleens of these animals early after simultaneous challenge with antigen and endotoxin, and in far greater numbers than have ever been seen in mice given the same antigen without endotoxin. "Activated" germinal centers formed in the lymphoid tissue either 1 day before the appearance of antibody in the blood stream or on the same day, and they became larger than in the mice given antigen only. On the other hand, these specific and characteristic cellular changes failed to appear in mice prevented from forming any antibody at all by injections of endotoxin given 2 days before the antigenic challenge.

These findings are discussed in the light provided by data from recent reports of others as well as in the light of the accompanying paper (1) which demonstrated not only the enhancement of antibody formation following simultaneous injections of antigen and endotoxin, as already known, but a totally unexpected, complete suppression of its formation when endotoxin was given 2 days before antigen.

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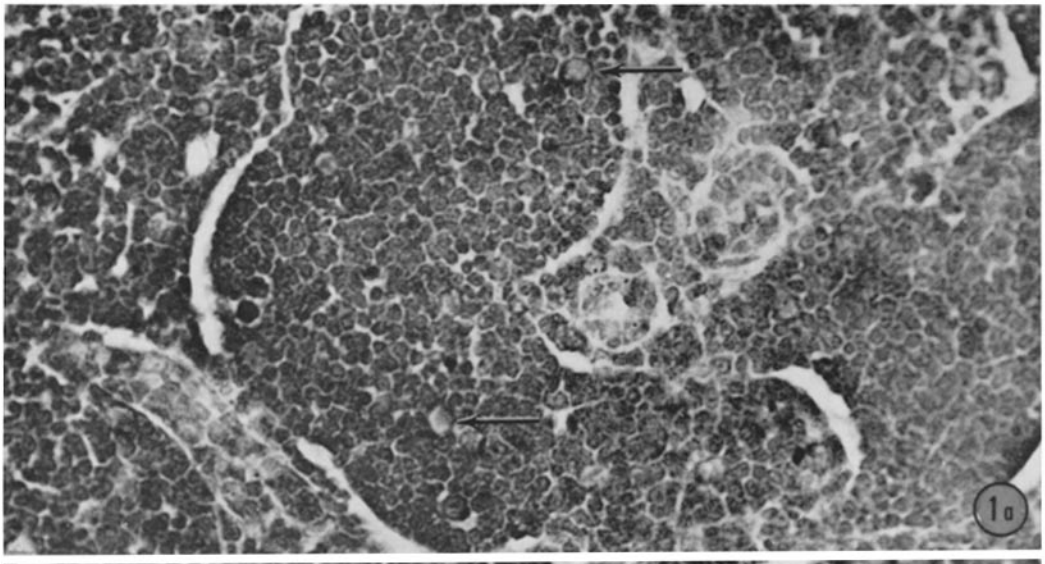
EXPLANATION OF PLATES

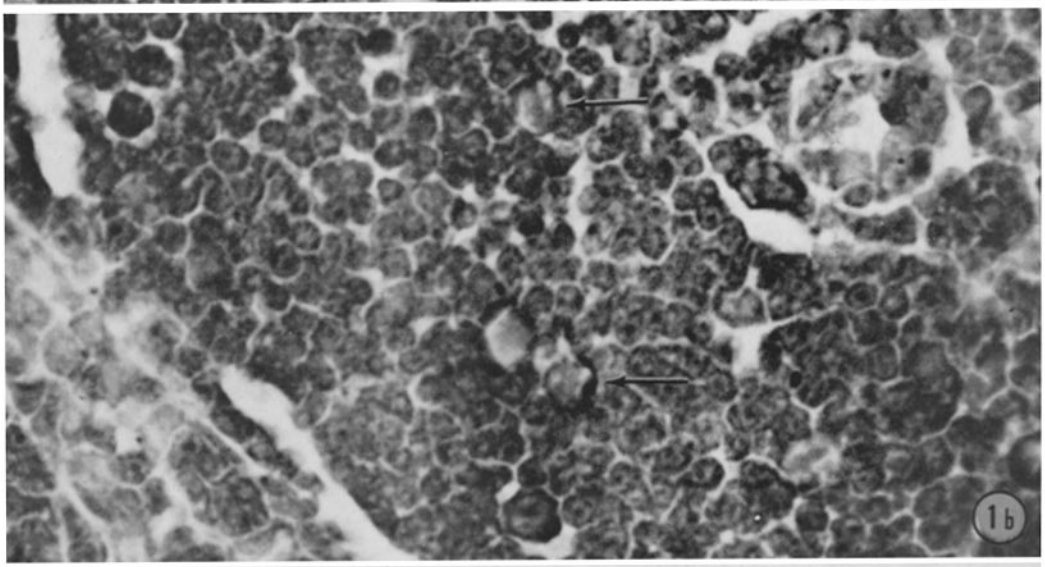
PLATE 106

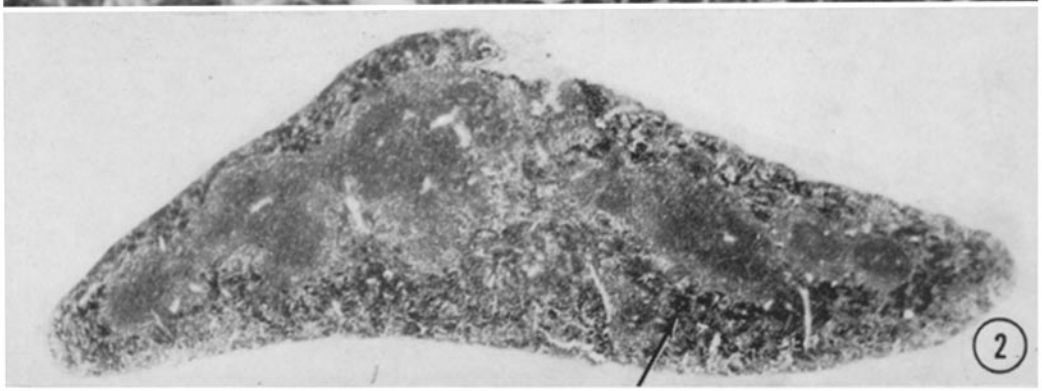
FIG. 1 *a*. Part of a medullary cord of a mesenteric node, taken 4 days after an intraperitoneal injection of the standard dose of sheep red blood cells and stained with methyl green and pyronin. The photomicrograph was taken on orthochromatic film to demonstrate the presence of large pyroninophilic cells within the cord, since, by the use of this film, the red, pyronin-positive cytoplasm of each of these cells, two of which are indicated by arrows, stands out clearly as a dark, black ring. These cells are surrounded by densely packed, blue-stained, small lymphocytes which are dark gray in the picture. × 400.

FIG. 1 *b*. A portion of the same medullary cord shown in Fig. 1 *a* at a higher magnification. Two large pyroninophilic cells are also indicated by arrows in this picture, which is presented to bring out the characteristics of these large cells. As mentioned in the text, all the cells in the cord would, in life, be carried to the efferent lymphatics and thence to the blood to be distributed throughout the body. × 1000.

FIG. 2. A transverse section of the spleen of one of the normal control mice. It was stained with methyl green and pyronin and photographed on orthochromatic film. In prints made from this film, all red-stained, pyronin-positive portions of cells appear black. The red pulp of this spleen contained many cells possessing strongly pyronin-positive cytoplasm, one group of which is indicated in Fig. 2 by an arrow. The pulp was normally cellular, and the follicles were normal in appearance and size. They showed no active germinal centers. × 30.



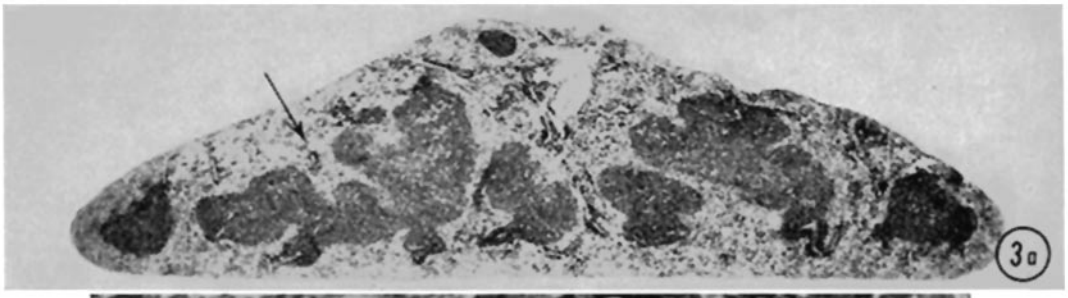


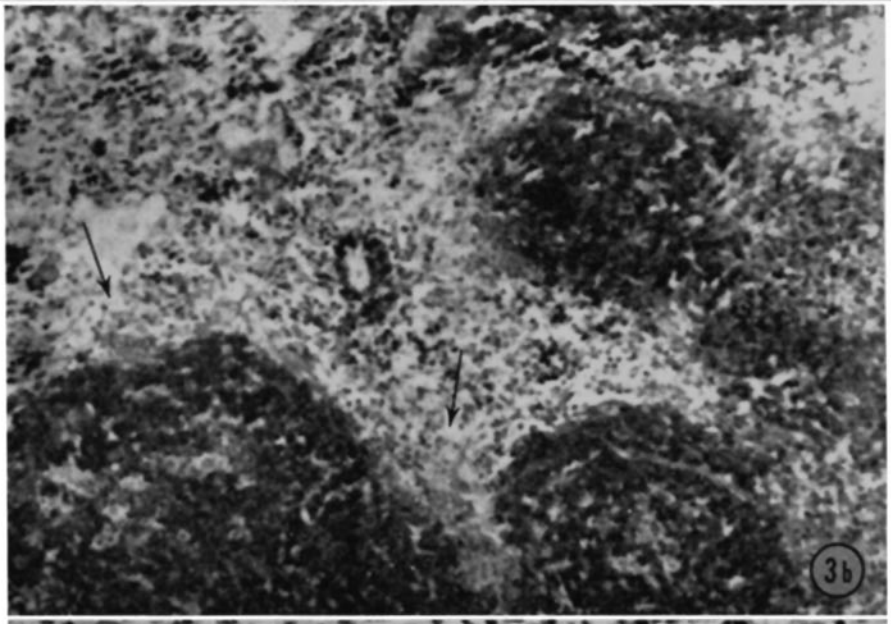


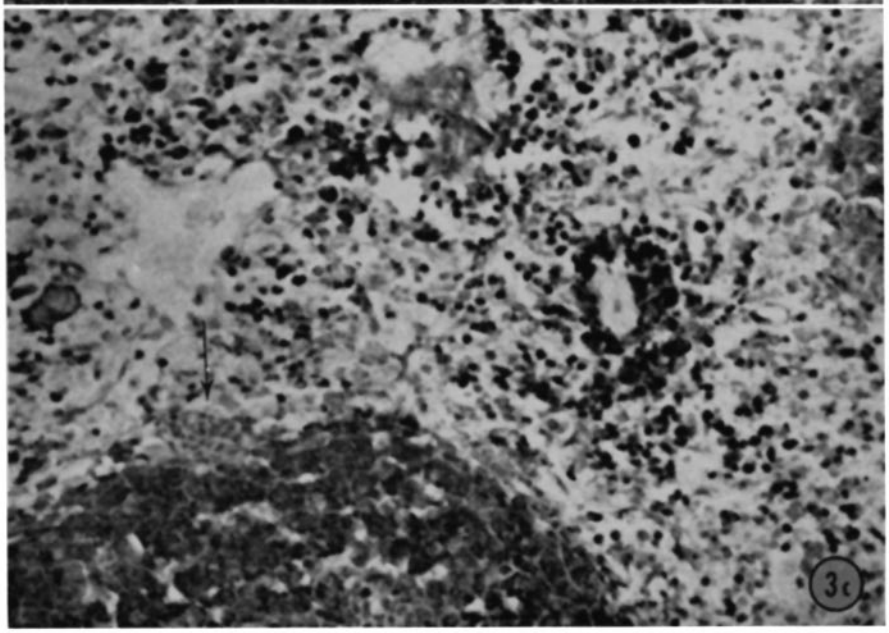
(McMaster and Franzl: Primary immune response in mice. II)

PLATE 107

FIGS. 3 *a*, 3 *b*, and 3 *c*. Three views of a transverse section of the spleen of a group E mouse that received 50 μg of endotoxin by intraperitoneal injection about 24 hr previously. The section was stained with methyl green and pyronin, and photographed on panchromatic film. The arrow in Fig. 3 *a* indicates the area depicted in Figs. 3 *b* and 3 *c*. Very few lymphocytes remained in either the red or the white pulp. The atypical follicles had no germinal centers. The sparsely populated red pulp contained many polymorphonuclear cells, two groups of which are indicated by the arrows in Fig. 3 *b*. The left hand arrow in Fig. 3 *b* points to the same group of cells as the arrow in Fig. 3 *c*. Many polymorphonuclears were also present within the empty spaces of the central portions of the follicles. Such cells are not identified clearly in the figures because they stain poorly with methyl green and pyronin. × 30, 200, and 400.







(McMaster and Franzl: Primary immune response in mice. II)

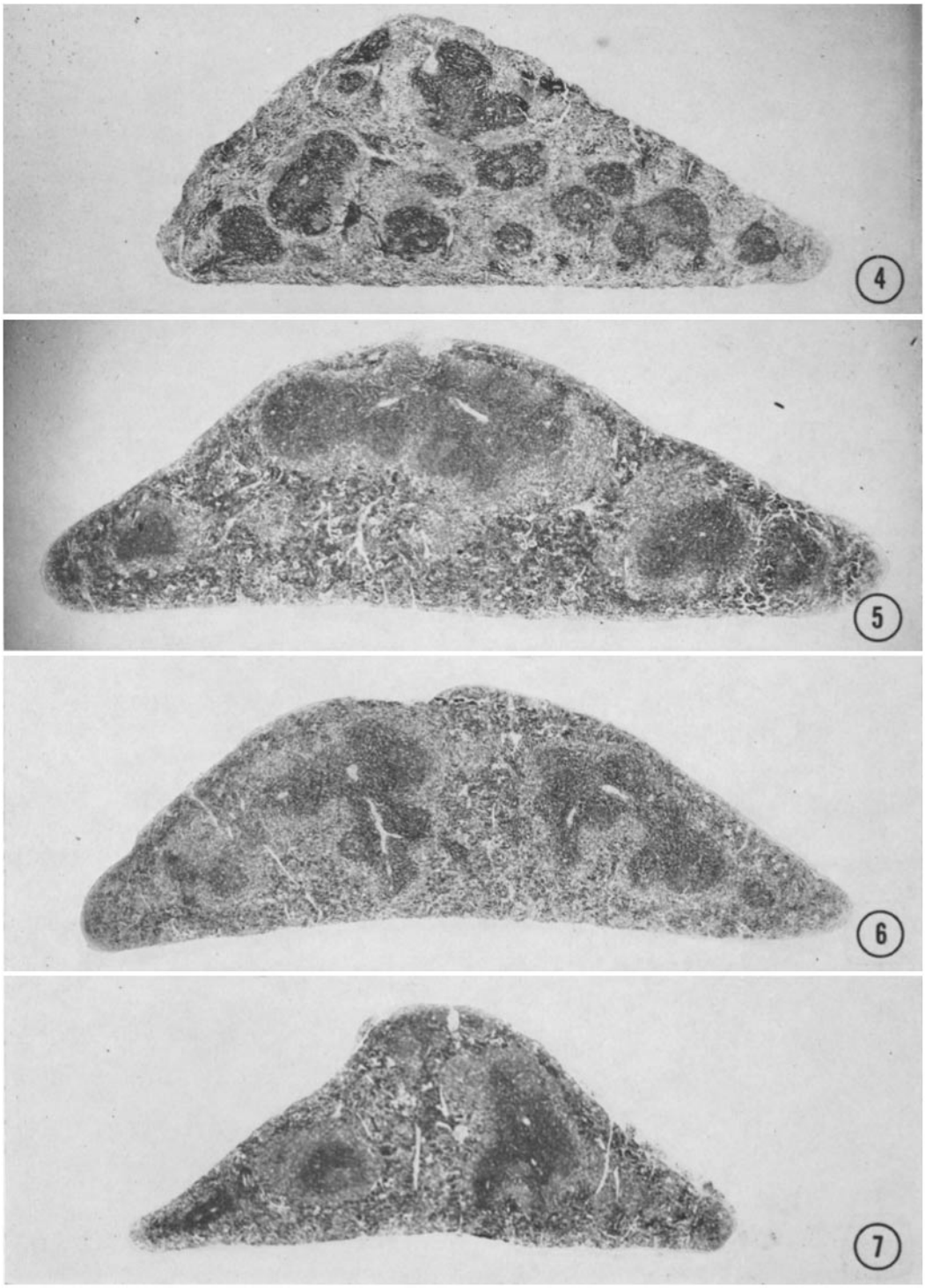
PLATE 108

FIG. 4. A transverse section of the spleen of a mouse 2 days after a single injection of 50 μg of endotoxin (a group E mouse). The section was stained, as usual, with methyl green and pyronin and photographed on panchromatic film. The red pulp is relatively empty, as in the spleens taken 1 day after giving endotoxin (see Fig. 3 *a*). In this respect, the reaction to endotoxin was still present. × 30.

FIG. 5. Obtained by the same technique 3 days after injecting endotoxin, this figure shows an increase in the number of cells in the red pulp and beginning recovery. The spleens of these mice began to look more like normal ones. × 30.

FIG. 6. A transverse section of a typical spleen from a mouse given 50 μg of endotoxin intraperitoneally 2 days before an injection of the standard dose of sheep red blood cells (a group E_2A mouse). The section was taken 1 day after the antigen injection, that is on the 3rd day after giving the dose of endotoxin. It was stained with methyl green and pyronin and photographed on panchromatic film. The section resembles those taken at the same time interval after injections of endotoxin only, as illustrated in Fig. 5. There were no active germinal centers. When examined under higher power than that used for taking the photograph, there was no apparent increase in the number of large pyroninophilic cells. Fig. 6 does show, however, that the cellular content of the red pulp was no greater than normal, as shown in Fig. 2. × 30.

FIG. 7. This section taken from a mouse injected 2 days previously with the antigen, 4 days after the mouse had been given endotoxin, was obtained by the same techniques as Fig. 6. The red pulp of the spleens of this group of animals had increased only a little in cellularity from that of the preceding day. There was no evidence of a beginning immune response to the antigen. Fig. 7 looks somewhat like a photograph of a normal splenic section, like that shown in Fig. 2. × 30.



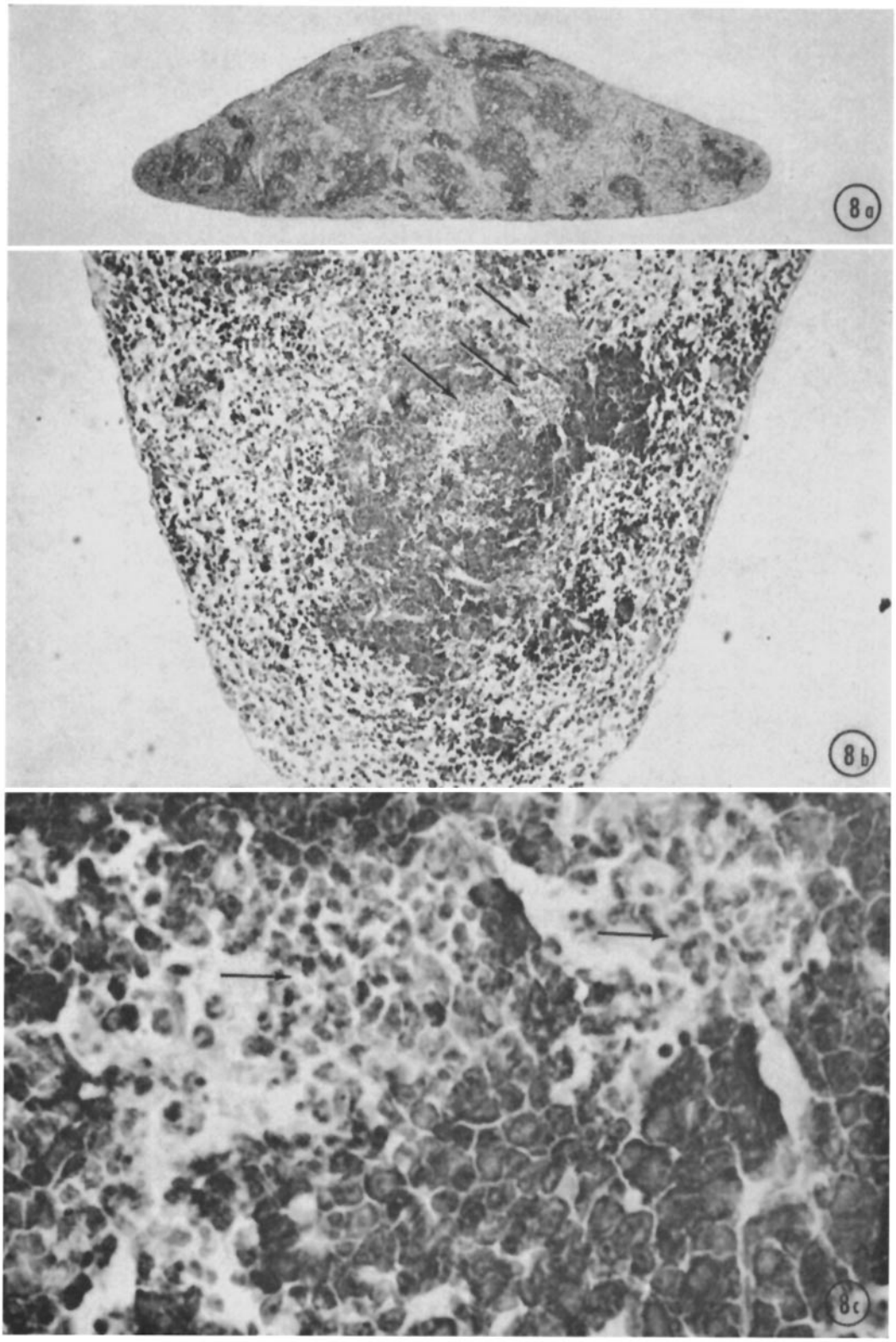
(McMaster and Franzl: Primary immune response in mice. II)

PLATE 109

FIG. 8. *a*. A transverse section of a spleen stained with methyl green and pyronin, photographed on panchromatic film, and taken 1 day after injecting a mouse with endotoxin and sheep red blood cell antigen simultaneously. The empty red pulp, deprived of many of its cells, is clearly evident. The follicles were atypical too, being deprived of most of their lymphocytes, a fact not shown at this magnification. This section was similar in appearance to the one shown in Fig. 3 *a*, which was taken from a mouse injected 1 day previously with endotoxin only (group E). Both of these sections should be compared with Fig. 2, a photograph of a normal spleen section. × 30.

FIG. 8 *b*. A higher power view of the right hand tip of the section shown in Fig. 8 *a*, rotated 90° to the right and photographed on orthochromatic film with a K-2 Eastman yellow filter. In this picture, therefore, the red, pyronin-stained parts of cells appear black. The relatively empty red pulp is obvious. The large, atypical follicle shows collections of polymorphonuclear cells, three of which are indicated by arrows, two in its central portion and one above and to the right. The loss of cells is striking when this figure is compared with that of a normal spleen presented in Fig. 2. × 150.

FIG. 8 *c*. Another photomicrograph taken on orthochromatic film at a still higher magnification, which includes the areas in Fig. 8 *b* indicated by the two lower arrows there. In Fig. 8 *c*, two other arrows indicate collections of polymorphonuclear cells which do not show well because these cells stained poorly with the methyl green and pyronin used in this work. × 700.



(McMaster and Franzl: Primary immune response in mice. II)

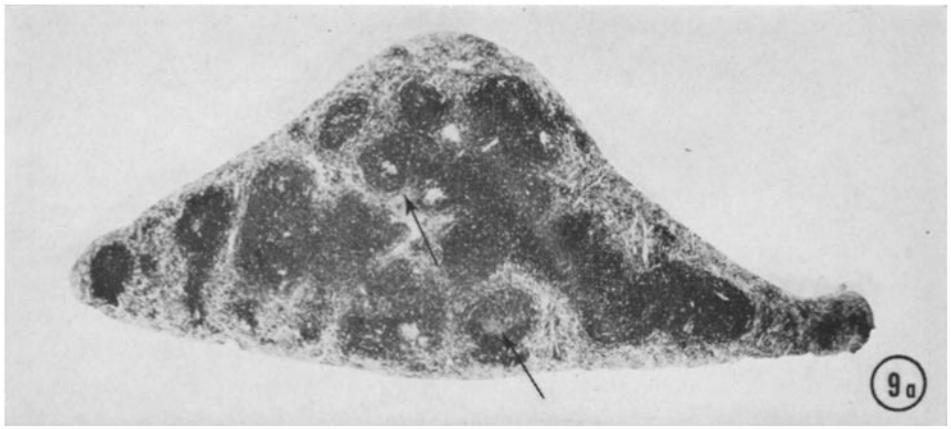
PLATE 110

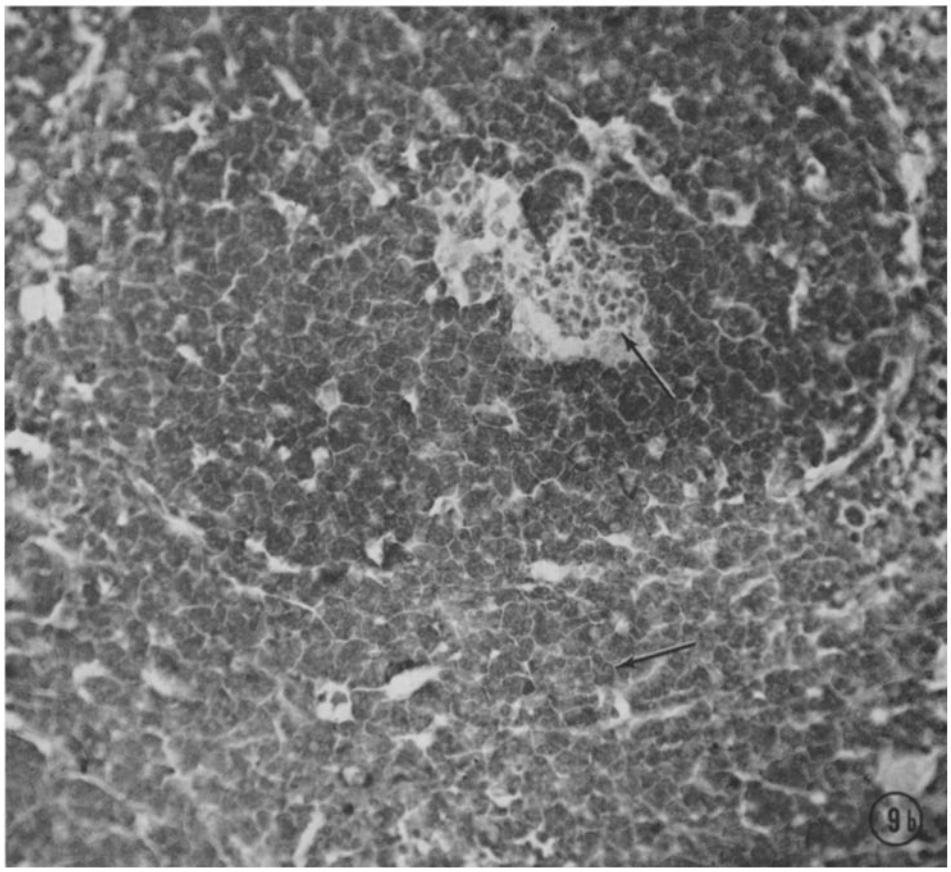
FIG. 9 *a*. A picture of a transverse section of a spleen from a mouse taken 2 days after the simultaneous injection of endotoxin and antigen. The section was stained with methyl green and pyronin and photographed on orthochromatic film. The follicles appeared red, not the usual blue, since most of the cells were pyronin-positive. Hence the orthochromatic film rendered these follicles darker than in the other pictures. Several follicles showed pale centers because they contained either fewer cells or cells taking a more bluish tint. The lower arrow in the figure points to one of the former with fewer cells.

Open spaces appeared in the centers of other follicles. These were partially filled with collections of polymorphonuclear cells, which also took a more bluish tint than the follicle cells. The upper arrow in the figure points to one of these. The striking increase in the cell population of the red pulp as compared to that seen on the previous day, in these mice, is obvious (Fig. 8 *a*). × 30.

FIG. 9. *b*. This picture, taken by the same technique as Fig. 9 *a* but at a higher magnification, shows part of the follicle indicated by the upper arrow in Fig. 9 *a* and its surrounding red pulp. The orthochromatic film, on which this picture was taken, renders the pyronin-stained cells of the follicle darker than those of the red pulp surrounding it, which are seen in the lower half of the picture and indicated by the lower of the two arrows. The collection of leukocytes in the open space in the follicle, and indicated by the upper arrow, shows poorly because these cells do not stain well with the methyl green and pyronin technique used in this work.

In this picture, the increased cell density both of the follicle and of the red pulp speaks for the exceedingly great proliferation of cells which had taken place by the 2nd day. This is in contrast to the lack of cellular increase seen in the mice 2 days after they got endotoxin only (group E mice, Fig. 4) and with those given endotoxin 2 days before the antigen (group E_2A, Figs. 6 and 7). × 600.





(McMaster and Franzl: Primary immune response in mice. II)