Marginal Zone and Germinal Center Development in the Spleens of Neonatally Thymectomized and Nonthymectomized Young Rats [1]

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ABSTRACT Spleens from neonatally thymectomized and nonthymectomized young rats were studied histologically and histochemically to elucidate the development of the splenic immune system with and without thymus.

In intact animals primitive germinal center activity could be elicited with antigen as early as 13 days of age. More definitive germinal centers lacking tingible body macrophages were observed at 18 days of age. Germinal centers containing tingible body macrophages did not develop until 35 days of age in response to antigenic stimulation. This coincided with maximal development of the marginal zone of medium-sized lymphocytes and the mature development of nodular macrophages possessing strong acid phosphatase activity.

Neonatally thymectomized rats developed marginal zones and germinal centers similar to control littermates when the young animals were maintained on tetracycline. Thymectomized animals not given tetracycline showed disturbances in splenic development. These are discussed.

The results suggest that the thymus may be critical to the immune system in rats from birth to about 30 days of age but is not essential to its function beyond this period. Marginal zone lymphocytes and germinal center cells proliferate normally and mature to the plasma cell stage in the absence of a thymus if the animals are maintained on tetracycline beyond this critical age.

Histological and histochemical evidence from our laboratory implicates the medium-sized, marginal zone lymphocytes as a major source of germinal center cells following antigenic stimulation (Pettersen, Borgen and Graupner, '67).

The present study was undertaken to determine when marginal zone lymphocytes migrate to the spleen, when they respond to antigenic stimulation and whether the thymus influences their development or maintenance.

MATERIALS AND METHODS

Fifty young Holtzman rats were used to study the development of the marginal zone and the time of appearance of germinal center activity in the spleen. Animals ranged from one hour to 64 days of age at autopsy and include some which received lateral tail vein injections of typhoid-paratyphoid vaccine (Eli Lilly and Co.) or of 055:B5 *E. coli* endotoxin (Difco) at representative ages from 13 to 60 days. Intraperitoneal or intramediastinal injections were given to animals younger than 13 days of age.

Another series of rats was thymectomized on the day of birth. They were anesthetized by cooling in a freezer, the sternums were split with small surgical scissors and the thymic lobes were removed with forceps using clean but not sterile technique. Thirty percent of the animals died during or shortly following surgery. Forty-four animals survived the initial procedure.

At autopsy 12 animals were free of thymus and 13 had portions remaining. Nineteen died of runt disease or were victims of maternal cannabalism. Tetracycline hydrochloride (Pfizer Corp.) in the drinking water eliminated runting and was used routinely following a high mortality in our first four litters. Histological material was obtained from only three thymectomized animals not receiving the antibiotic. Two of these were runted animals 27 days of

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age and the third was a 25 day old nonrunted animal.

The 12 totally thymectomized animals were grouped with littermate controls and partially thymectomized animals of the same age. Some of the animals received typhoid-paratyphoid vaccine via the lateral tail vein. The injections were generally given five days before autopsy to allow a maximum cellular response to develop. Groups of animals were killed at 25, 27, 35, 41 and 64 days of age. One thymectomized animal was killed at 11 months of age five hours following antigenic stimulation. Histological material was obtained from 40 animals in this portion of the study.

All animals were killed with ether, spleens were rapidly removed and pieces fixed in Carnoy's fluid, in Helly's fluid and in 15% cold, neutral formalin. All suspicious mediastinal tissue was removed from the surgical animals and fixed in 15% formalin. The thorax was then held under running tap water to wash it free of blood for further inspection under a hand lens.

Tissue fixed in formalin was stained for acid phosphatase activity as previously described (Pettersen, '64). These sections were studied for macrophage distribution and general splenic topography. Mediastinal tissue stained in this manner was used to identify thymic remnants.

Tissue fixed in Helly's fluid was sectioned in paraffin at 5 μ and stained with hematoxylin-eosin-azure II or with hematoxylin and eosin. Carnoy-fixed tissue was sectioned in paraffin at 5 μ and stained with methyl green-pyronin.

RESULTS

Splenic development in nonthymectomized animals. Spleens of newborns were largely myeloid with an occasional small sheath of lymphocytes surrounding the arterial vessels. Macrophages were dispersed throughout the myelopoietic areas but were not present in the small lymphoid areas (fig. 1). At three days of age several tiers of lymphocytes surrounded the arterioles (fig. 2). These cells resembled the medium-sized lymphocytes found in the marginal zones of adult rats. Suggestions of a marginal zone were present at nine days of age and it was definitive by 13 days of age (fig. 3). Small lymphocytes were then present in a position adjacent to the arterioles with medium-sized lymphocytes peripheral to them (fig. 4). A disturbance in this relationship occurred one day following intravenous injections of antigen in 13 day old animals. The two types of lymphocytes were commingled rather than segregated in these spleens. This phenomenon was suggested in animals eight or nine days of age following intraperitoneal injections but was difficult to assess because segregation was not complete in control spleens of this age. Figures 5 and 6 illustrate splenic nodules from a control and an injected animal 20 days of age.

Germinal centers developed in 18 day old rats following intravenous injections of 0.25 ml typhoid-paratyphoid vaccine at 14 days of age. They consisted of small nests of pyroninophilic cells surrounded by mantle layers of non-pyroninophilic small lymphocytes. Tingible body macrophages were not present and no plasma cells could be identified. About 30% of the lymphoid nodules contained such germinal centers.

The marginal zone was the source of cells for early germinal centers. The width of this rim of cells reached adult size (80–100 μ) by 35 days of age at which time tingible body macrophages were present in germinal centers of those spleens exposed to antigen for five days. About 20% of the nodules contained germinal centers with these macrophages (fig. 15). Up to this age tingible bodies were not present in response to antigen but there were increases in numbers of macrophages possessing acid phosphatase activity in the lymphoid areas of both stimulated and non-stimulated spleens. In 60 day old animals nearly 100% of the lymphoid nodules responded to similar antigenic stimulation by the formation of germinal centers containing tingible body macrophages.

Our data were not complete enough to establish the age of appearance of plasma cells. Sixty day old rats contained large numbers of plasma cells in the splenic red pulp five days following stimulation. Efforts to induce a similar response in 20–30 day old rats were negative. Sufficient numbers of antigenically stimulated ani-

mals were not available in the 30 to 60 day age group.

Thymectomized animals. Neonatal thymectomy retarded the development of the marginal zone in the spleens of animals not maintained on tetracycline. Figure 7 illustrates this in a spleen section stained for acid phosphatase activity from a 25 day old, non-runted, thymectomized animal. Figure 8 is a section from a littermate control. Runted animals lacked marginal zones (fig. 9).

Small lymphocytes were deficient but not entirely lacking in these spleens. Runted animals also showed decreased numbers of medium-sized lymphocytes (fig. 12). These latter cells were present in normal numbers in the spleen of the non-runted animal (fig. 11).

Spleens from thymectomized animals maintained on tetracycline developed marginal zones almost as rapidly as control animals and contained significant although reduced numbers of small lymphocytes. Cellular responses to antigenic stimulation were indistinguishable from control responses except for decreased numbers of mantle layer small lymphocytes. Recovery of the marginal zone population of medium-sized lymphocytes following initial depletion in response to antigenic stimulation was normal in thymectomized animals. Plasma cell proliferation was unaffected.

The 11 month old thymectomized animal responded like its control to antigen administered five hours before autopsy. Figure 16 shows a partial collapse of the marginal zone on a section from this spleen stained for macrophages. An additional thymectomized animal of this age was not available to test whether repopulation of the marginal zone occurs by four days following stimulation.

DISCUSSION

Two types of lymphocytes can be distinguished in the young lymphoid areas of the rat spleen. The first of these to enter the spleen is a medium-sized, slightly pyroninophilic lymphocyte that initially occupies a position adjacent to the arterial vessels (fig. 2). These are later located more peripherally as the second type, a small non-pyroninophilic lymphocyte, replaces them around the vessesl (fig. 4). There is some intermingling of the two types in spleen sections from animals less than ten days of age. By 13 days of age there is a definitive marginal zone (figs. 3, 4) of medium-sized lymphocytes enveloping an inner core of small lymphocytes. This relationship is disturbed within one day following the intravenous administration of antigen with suggestions of immature germinal center formation. Medium-sized lymphocytes invade the small lymphocyte mass and differentiation into hemocytoblasts occurs. Figures 5 and 6 illustrate splenic nodules from a control and an experimental animal 20 days of age. No plasma cell formation occurred at this age when animals were killed at later stages following antigenic stimulation.

Halliday ('56) reported an abrupt cessation of the ability of young rats to absorb antibodies through the gut wall at 20 days of age. This phenomenon was unrelated to the uptake of solid food. It was also shown (Halliday, '57) that young rats could produce antibodies in response to active immunization prior to the loss in absorptive capacity. Evidence indicated that these antibodies were located in the β-globulin fraction. Our results in young animals show that cellular changes occur in rats younger than 20 days of age following active immunization. These changes are similar to the response in adult spleens in certain respects. Marginal zone lymphocytes are the source of germinal center cells in both young and adult spleens and mitotic activity and hemocytoblast formation occur in both. Germinal centers in young spleens, however, did not contain tingible body macrophages and did not produce plasma cells. Additional studies in the 10–30 day age group employing immunochemical techniques would be desirable to determine what cell type produces antibodies during this stage of development. Our sections stained histochemically for macrophage distribution suggest that a follicular antigen trapping mechanism as described by Miller and Nossal ('64) in rat popliteal lymph nodes and by Hunter ('66) in the rat spleen may not be functional in young rats because of the paucity of macrophages in the white pulp at this age. MacFadden ('68) has studied the

phagocytic function of macrophages in the spleens of late fetal (17–18 days of gestation) to old (475 days) rats. Increased numbers of white pulp macrophages possessing phagocytic ability and hydrolytic enzyme activity were reported as the animals aged. Our results support his conclusions but also suggest that antigenic stimulation enhances the maturation of white pulp macrophages. Bauer et al. ('66) reported that the intracellular digestion of antigen was slower in macrophages of germfree versus conventional mice. Spleen sections from several germfree rats were stained for acid phosphatase activity in our laboratory. The white pulp contained relatively few cells exhibiting enzyme activity compared to spleen sections from untreated adult control rats. The red pulp did not differ from that of the controls. This suggests that ageing is not necessarily the variable in the maturation of white pulp macrophages but that some exogenous stimulation is required. Red pulp macrophages possess hydrolytic enzyme activity in the late fetal stages of development (MacFadden, '68) before such stimulation is likely to occur.

Williams and Nossal ('66) studied the antigen-trapping ability of the lymphoid tissues in young rats following footpad injections of I^{125}-labelled polymerized flagellin from *Salmonella adalaide*. Autoradiographs showed initial signs of cortical localization in lymph nodes from animals between 10–14 days of age. True follicular retention did not occur until animals were four to six days older at the time of injection. Antigen was not retained by the lymphoid nodules of the spleen six days following an injection given at four weeks of age. An adult pattern of retention was observed in the spleen in animals injected at six weeks of age. The ability to retain antigen increased five fold per unit weight of lymphoid tissue between two and six weeks of age. Williams ('66) demonstrated a parallel development of the antigen-trapping mechanism and the ability to form antibodies.

White pulp macrophages may mature from reticular cells already present in the splenic anlage at the time lymphoid immigration begins. Their specialization into antigen-processing cells may be influenced by their lymphoid environment and triggered upon initial exposure to antigen. Pettersen ('64) reported that the number of cells in the white pulp reactive to Marshall's silver impregnation stain for macrophages does not increase following antigenic stimulation but the number of cells with active hydrolytic enzyme systems does increase following such treatment. This indicates that increases in macrophages represent maturation of existing cells. It is difficult to explain how macrophages get into the developing lymphoid areas. They may be incorporated from the red pulp as the lymphoid cells migrate into the spleen. This explanation is inconsistent with the following observations: (1) during development macrophages pile up at the marginal zone-red pulp junction but are rarely found in the marginal zone (fig. 3); (2) the marginal metalophils (Snook, '64) appear to migrate outward as the small lymphocytes move into the periarteriolar regions; (3) white pulp macrophages in newborns are not selectively localized near the periphery of the lymphoid masses; (4) macrophages in the red pulp possess hydrolytic enzyme activity at or before birth, whereas white pulp macrophages acquire this activity later in development.

Alternatively, one can postulate that the white pulp macrophages represent a breed that is different from those in the red pulp. They may migrate in with the lymphoid cells during development or may be products of maturation of certain of these cells. The data available suggest some form of this alternative hypothesis.

The retarded development of the marginal zone in young neonatally thymectomized rats not maintained on tetracycline may be related to deficiencies of small lymphocytes of thymic origin or to infectious processes that keep the marginal zone lymphocytes in a state of disorganization. There were no germinal centers and no hemocytoblasts in the white pulp of these animals to suggest a cellular response to invading organisms. Azar ('64) substantially reduced the incidence of runting in neonatally thymectomized rats by adding oxytetracycline HCl (Pfizer Corp.) to the drinking water. He believed that bronchopulmonary infections were the cause of

death in runted animals. Non-hemolytic streptococcus, a Hemophilus organism and diphtheroids were cultured from bronchopulmonary tissue. The spleens were atrophic, lacked well-formed follicles and contained no plasma cells. Plasma cells were present in the bronchopulmonary lesions and the serum gamma globulin level was increased. This suggests that the spleen was not involved in an immunological process in runted animals but that cells that normally would develop within the spleen were employed at the site of infection. This may be a possible explanation for the deficiency of medium-sized lymphocytes in spleens of our runted animals.

Schriever, Hsu and Azar ('67) found normal plasma cell formation and normal numbers of cells containing gamma globulin in the mesenteric lymph nodes of neonatally thymectomized animals. They concluded that, in their experience, "should neonatally thymectomized rats survive the first critical eight to ten weeks of life without succumbing to infection, they will then adequately cope with subsequent infections and compensate for the loss of thymus function."

Waksman, Arnason and Jankovic ('64) reported a complete absence of small lymphocytes in spleens of neonatally thymectomized rats. None of our spleens, with the exception of those from the two runted animals, showed small lymphocyte deficiencies of this magnitude. Tetracycline may have potentiated the development of small lymphocytes or may have permitted their development under less antigenically stressful conditions.

It is clear that the thymus is not essential in maintaining a marginal zone cell population in young adult rats once the splenic immune system has developed. It cannot be concluded from our data that the thymus is not the stem source of marginal zone medium-sized lymphocytes. The following observations are consistent with a thymic origin for these cells: (1) medium-sized lymphocytes appear in the spleen before small lymphocytes and can be found in spleens of one hour old rats corresponding to the time of our earliest thymectomies; (2) a 25 day old non-runted thymectomized animal showed retarded development of the marginal zone and two 27 day old runted animals showed near absence of this region; (3) 35 day old neonatally thymectomized animals maintained on tetracycline showed subtle but distinguishable differences in the ability to repopulate the marginal zone following antigenic stimulation; (4) small lymphocytes entered the spleen in significant numbers in neonatally thymectomized animals maintained on tetracycline suggesting a potentiating influence.

The following are more consistent with a nonthymic origin: (1) a spleen from a non-runted thymectomized animal not maintained on tetracycline contained a sizable population of medium-sized lymphocytes although the deficiency of small lymphocytes disturbed the usual organization of the marginal zone; (2) marginal zone lymphocytes were present in normal numbers in the neonatally thymectomized animals maintained on tetracycline; (3) the marginal zone was reconstituted after the usual depletion following antigenic stimulation.

Moore and Owen ('67) recently presented evidence that the yolk sac in the chick furnishes blood-borne stem cells which enter the thymic rudiment. A sex chromosome marker and histological techniques used to study parabiosed chick embryos showed that a sizeable proportion of thymocytes came from the partner. Evidence suggested that the same type of cell migrates into the thymic rudiment of the mouse. The cell type shown on their illustrations closely resembles those we see in young germinal centers one day following antigenic stimulation (fig. 6, arrow). We have called this cell a hemocytoblast but it is believed by us to be a transitional form of the medium-sized marginal zone lymphocytes which migrate into the germinal centers in response to antigenic stimulation.

Several conclusions seem reasonable from our data: 1. The thymus plays some essential role in survival in young conventional rats. 2. Tetracycline permits normal development of the lymphoid structure in spleens of neonatally thymectomized rats except for relative deficiencies of small lymphocytes. 3. An intact thymus is not essential in maintaining these lymphoid structures once adult development has

been attained. 4. Two types of lymphocytes migrate to the spleen during development of the lymphoid structures neither of which is entirely thymus dependent following birth.

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PLATES

PLATE 1

EXPLANATION OF FIGURES

1 Section of spleen stained for acid phosphatase activity from a rat killed one hour after birth. Macrophages are dispersed throughout the red pulp which is entirely myeloid. Arrow identifies a small lymphoid area free of macrophages. × 70.

2 Section of spleen from a three day old rat showing a sheath of medium-sized lymphocytes surrounding an obliquely sectioned arteriole. Methyl green-pyronin. × 270.

3 Spleen section from a 13-day old rat showing developing marginal zone, MZ. A few macrophages are present in the nodules. RP, red pulp; LN, lymphoid nodule; MZ, marginal zone. Arrow identifies marginal metalophils. Acid phosphatase. × 70.

4 Spleen section from a 16 day old rat showing an inner core of small lymphocytes surrounding an arteriole and a peripheral rim of medium-sized lymphocytes occupying the marginal zone. MZ, marginal zone. Methyl green-pyronin. × 170.

5 A nodule in a spleen section from a 20 day old rat showing a core of small lymphocytes surrounding an arteriole. Methyl green-pyronin. × 400.

6 A nodule in a spleen section of a 20 day old rat one day following an intravenous injection of 0.25 ml typhoid-paratyphoid vaccine. Medium-sized lymphocytes have invaded the mass of small lymphocytes. Arrow identifies a hemocytblast. Methyl green-pyronin. × 400.

7 Section of spleen from a 25 day old, neonatally thymectomized, nonrunted rat showing retarded development of the marginal zone. WP, white pulp. Acid phosphatase. × 70.

8 Spleen section from a 25 day old littermate control. MZ, marginal zone; RP, red pulp. Arrow identifies marginal metalophils. Acid phosphatase. × 70.

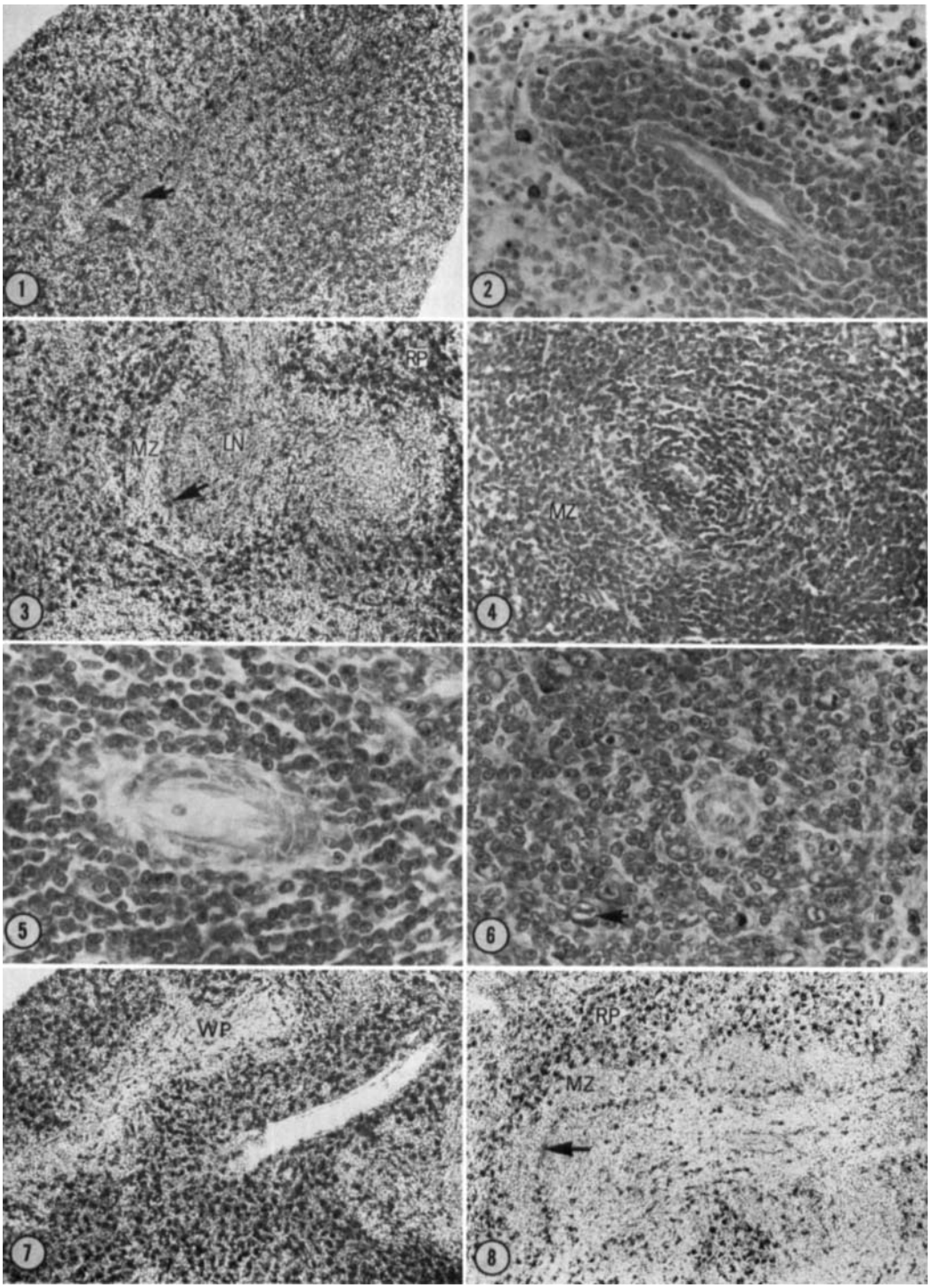
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PLATE 2

EXPLANATION OF FIGURES

9 Spleen section from a 27 day old, thymectomized, runted rat showing poor development of lymphoid structures. Acid phosphatase. × 70.

10 Spleen section from a 27 day old littermate control. MZ, marginal zone; RP, red pulp. Acid phosphatase. × 70.

11 Splenic nodule from a 25 day old, thymectomized rat showing deficiency of small lymphocytes. Medium-sized lymphocytes show a normal population density in the marginal zone, MZ. H. and E. × 170.

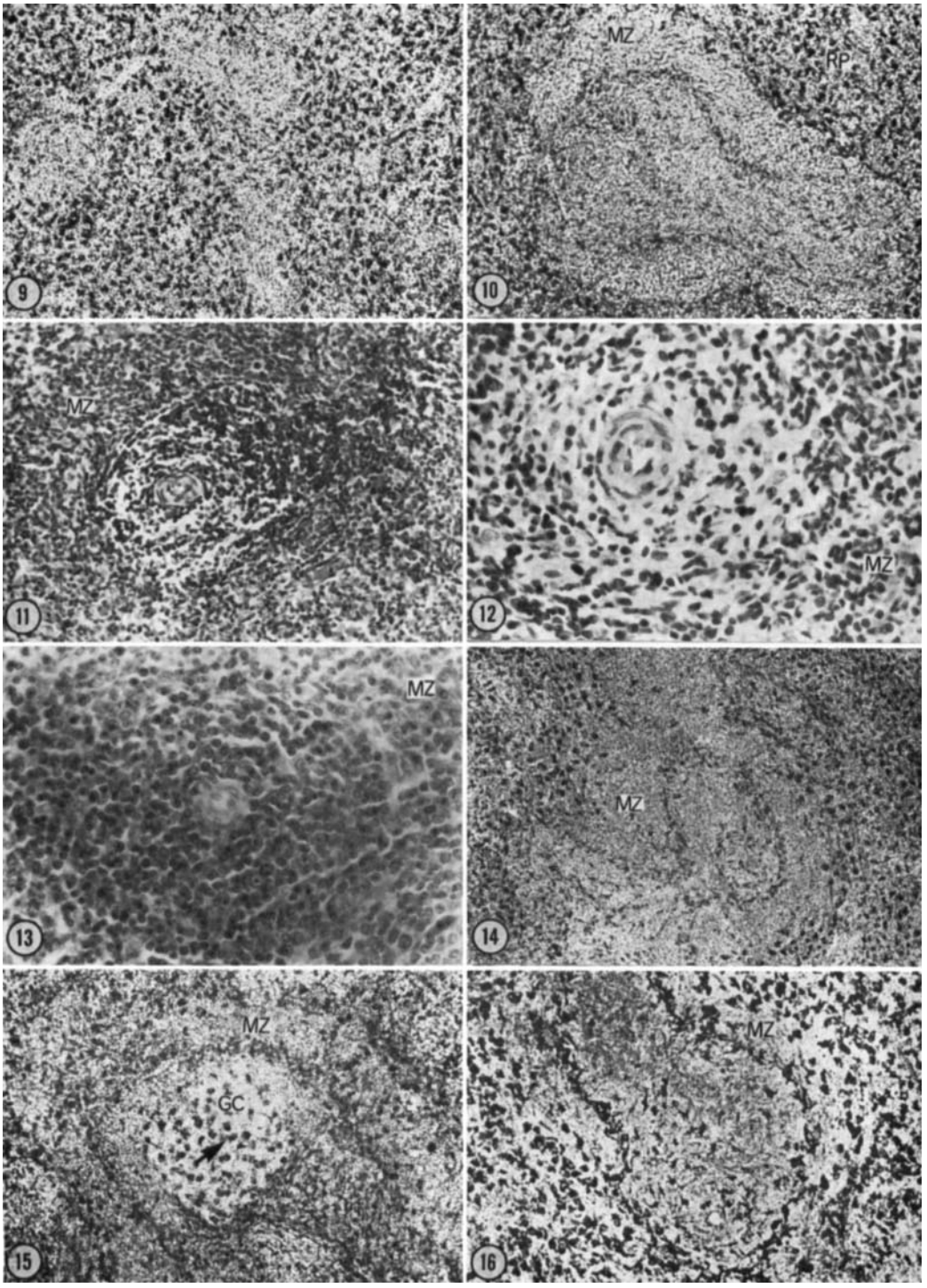
12 Splenic nodule from a 27 day old, thymectomized, runted rat showing marked depletion of small lymphocytes and a marginal zone, MZ, with a low population density of medium-sized lymphocytes. H. and E. × 330.

13 Splenic nodule from a 27 day old control rat. MZ, marginal zone. Methyl greenpronin. × 330.

14 Spleen section from a 32 day old, neonatally thymectomized rat maintained on tetracycline. Marginal zone, MZ, is well developed although slightly smaller than in controls. Compare to figure 10. Acid phophatase. × 70.

15 Spleen section from a 35 day old, neonatally thymectomized rat killed five days following an intravenous injection of typhoid-paratyphoid vaccine. The marginal zone, MZ, is reconstituted, a germinal center, GC, is present in the nodule and tingible body macrophages (arrow) have developed. Acid phosphatase. × 70.

16 Ammoniacal silver-stained section of spleen from a neonatally thymectomized, 11 month old rat killed five hours after an intravenous injection of 1.0 ml typhoid-paratyphoid vaccine. The marginal zone, MZ, is constricted which is a characteristic response observed in spleens of nonthymectomized animals within one day following antigenic stimulation. × 70.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