

ONTOGENY OF THE IMMUNE RESPONSE

I. THE DEVELOPMENT OF THE FOLLICULAR ANTIGEN-TRAPPING MECHANISM*

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PLATES 3 TO 8

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Previous studies from this laboratory tracing the fate of radioactively labeled antigens in lymph nodes have demonstrated the existence of specialized medullary and cortical structures which take up and retain antigens (1-3). Phagocytosis by macrophages lining the medullary sinuses is an established phenomenon and these cells take up a variety of substances including dyes, inert particles, bacteria, and soluble antigens. The follicular antigen-trapping system of the spleen and cortex of lymph nodes is more fastidious, and the degree of follicular retention of a substance appears to correlate with its antigenic potency (4). Both of these retention systems concentrate antigen in areas of immunological activity, there being close proximity of retained antigen to germinal centers in the cortex, and to developing plasma cells in the medulla. The superior *in vitro* antibody production by primed lymph node fragments compared with suspensions (5) also suggests that structural relationships are important.

One approach toward understanding these structural relationships and their relevance to antibody formation is developmental, and the present studies describe the sequential changes leading to mature antigen localization patterns in rats. We have found that the newborn rat lacks organized phagocytic structures; that the development of lymphoid follicles is preceded by the differentiation of the primitive cortical reticulum into continuous and then spherical antigen-trapping zones; and that the ability to rapidly localize antigen in specific areas develops prior to the ability efficiently to *retain* antigen in these same areas.

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Materials and Methods

Antigen.—Polymerized flagellin from *Salmonella adelaide* was prepared and iodinated with carrier-free I^{125} as described previously (6). Dosages of radioactivity were kept to a minimum in experiments designed to study the long-term fate of antigens, and no animal received more than 300 μC of I^{125} . As there was some variation in the antibody responses of young animals to different batches of antigen, the same batch was used for a given experiment.

Animals.—Wistar albino rats were used. These were bred randomly and littermates were used as controls and for all work correlating antigen distribution with antibody response. All animals receiving iodinated antigens were given added inorganic iodide in the drinking water (7), and under the dosage conditions of these experiments no animals displayed ill effects. Antibody responses were not detectably different from those in littermates receiving non-iodinated antigen.

Processing of Tissue.—Lymph nodes prepared for autoradiographic studies were excised with a considerable amount of adjacent tissue in order to preserve the architecture of the circular sinus. We term aortic lymph nodes those elongated nodes that lie on either side of the aorta at its bifurcation. Tissues were fixed in 10% buffered formalin, and conventional sections of 5 μ thickness were prepared for autoradiography.

Autoradiography.—The method of Messier and Leblond (8) was used. Slides were dipped in Kodak NTB-2 emulsion, dried, and stored at 4°C. One set of sections was exposed to obtain light labeling and a second set was always exposed for the 60 day half-life of I^{125} . Slides were developed in Kodak D19b and stained at 4°C with methyl green-pyronin.

RESULTS

The Development of Antigen Capturing Structures.—

Experimental design: Two rats from each of six litters aged 0, 2, 10, 16, 28, and 42 days were injected into both hind foot pads with 100 μg of polymerized flagellin labeled with 50 μC of I^{125} . One animal from each litter was killed at 1 day following injection and the second was killed at 6 days following injection. Lymphoid tissues were excised and studied to determine antigen uptake and retention patterns, at various ages as well as for the study of the over-all developmental anatomy of lymphoid structures at frequent intervals (1, 3, 6, 8, 11, 17, 22, 29, 34, 43, 48 days of age). Lymphoid structures from rats aged 14 to 16 days localized antigen in a transitional pattern, and additional studies were carried out on 16 animals of this age.

Antigen Distribution within Primitive Lymphoid Tissues.—The popliteal lymph node from animals 1 to 3 days old was difficult to distinguish macroscopically from the areolar tissue of the popliteal fossa. The node did not have the characteristic tan color of mature lymph nodes, and considerable adjacent tissue was often removed to ensure inclusion of the node. Microscopically both the aortic and popliteal nodes from these animals consisted of loosely arranged reticular cells. There was no differentiation into cortex and medulla. The primitive spleen was homogeneous in appearance, and no differentiation into white and red pulp was apparent.

One day following the injection of antigen into the hind foot pads of rats aged 0 and 2 days, autoradiographs revealed label widely distributed in high

concentration throughout the primitive lymph node reticulum (Fig. 1 *a*). The diffuse spread of label was also present in the spleen. Occasional cells were especially heavily labeled, but these cells were randomly distributed, and no organized phagocytic structural units (like adult medullary sinuses) were present.

Despite the high initial concentration of antigen found in the primitive nodes, very little antigen persisted 6 days after injections at ages of 0 and 2 days (Fig. 1 *b*). The only label present at this time was associated with isolated heavily labeled cells. The spleen contained virtually no label 6 days after antigen injection.

Antigen Distribution during Early Lymphoid Differentiation.—Between the ages of 8 and 16 days popliteal and aortic lymph nodes underwent differentiation into cortical and medullary areas. The processes of differentiation occurred 4 to 6 days earlier in the aortic than in the popliteal lymph nodes. The oval shaped immature popliteal node acquired a kidney shape as loose areolar tissue and lymphatic sinuses appeared centrally to form the hilum. Cords of tissue then protruded into lymphatic sinuses. As these cords enlarged gradually they became surrounded with increasing numbers of phagocytic cells. The architecture of the cortex changed to a lesser extent during this period. The predominant cell was still the reticular cell. These cells became more compactly arranged giving the cortex a denser appearance; but otherwise the cortex retained the appearance seen at the ages of 1 and 3 days. Few small lymphocytes were present. Postcapillary venules with their characteristic high cuboidal endothelium were first distinguished, but in small numbers, at age 10 days.

Over this period, the spleen also differentiated into definable red and white pulp areas. The periarteriolar region expanded, and came to contain densely packed cells including reticular cells and small lymphocytes.

For the first time, specific patterns of antigen capture could be noted in the cortex of the lymph nodes. Autoradiographs displayed label in a continuous rim just deep to the cortical sinus (Fig. 2 *a*). The rim of label varied from 5 to 35 cells in width and extended from one pole of the lymph node to the other. Popliteal nodes from rats injected at age 14 days contained a rim that was thinner and more homogeneous than that of animals injected at age 16 days; at the latter time, antigen injections produced a more segmental cortical antigen distribution in some popliteal nodes and in all aortic nodes. Heavily labeled thick cortical areas were found to alternate with thinner, less labeled areas producing a pattern midway between the "cortical rim" distribution and the follicular localization characteristic of the adult. At this age, no grouping of either reticular cells or lymphocytes could be detected in the heavily labeled zones. In fact, from inspection of sections adjacent to the autoradiographic section but not covered with emulsion it was impossible to predict which areas of the cortex would be labeled and which would not. This is opposite to the

situation in the adult, where primary follicles can readily be recognized histologically.

When rats injected at age 16 days were killed 6 days later, it could be noted that in the popliteal node the antigen which had been localized in a continuous rim had "condensed" into a series of densely labeled zones which alternated with faintly labeled areas (Fig. 2 *b*). Although this pattern resembled adult follicular localization, the densely labeled areas were much smaller than in the mature node.

Antigen localization in the spleen of animals in this age group was predominantly in the marginal zone with some scattered collections of label in the red pulp (Fig. 2 *c*). Six days following injection, the bulk of this label had disappeared, substantiating the poor retention of antigen by the immature reticulo-endothelial system.

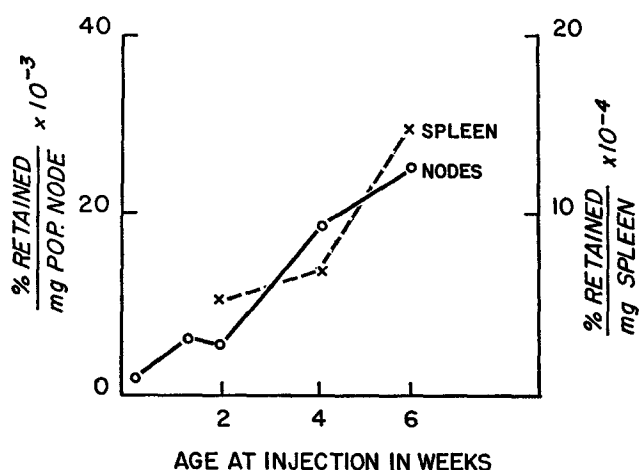
In contrast to the appearance of a new pattern of antigen distribution that occurred in the cortex of lymph nodes between 8 and 16 days, the distribution of antigen in the medulla changed more subtly. As more phagocytic cells lined the sinuses more antigen was localized in the medulla. The distribution of antigen was spotty, some sinus macrophages containing much label whereas others were very lightly labeled. The amount of antigen retained by these cells 6 days after injection was considerably greater than that retained by cells from animals injected at age 1 to 3 days.

Thus between the ages of 8 and 16 days significant anatomical and functional differentiation of antigen processing structures occurred. In the lymph node cortex, follicular rudiments appeared to condense from a less mature continuous rim. Medullary sinuses were formed, and antigen retentive capacity improved, more so in the lymph nodes than in the spleen.

Antigen Distribution during Late Lymphoid Differentiation.—Between the ages of 2 and 4 weeks lymph nodes acquired large numbers of small lymphocytes spread diffusely through the cortex and concentrated in the follicles. Postcapillary venules proliferated extensively. Serial sections showed that these structures often departed from small germinal centers. In fact, the endothelial cells were very similar in their histological appearance to germinal center blast cells. Plasma cells also appeared in the medullary cords and in the splenic red pulp. The medullary sinus macrophages became increasingly more numerous, staining a characteristic salmon color with pyronin. The mean weight of popliteal nodes of 4 week old animals was 7 times greater than that found at 2 weeks. Antigen injected into 4 week old rats was localized 1 day later in primary follicles and sinus macrophages (Fig. 3 *a*), and the follicles grew to almost adult size by 6 weeks (Fig. 3 *b*). In the medulla, the distribution of antigen was patchy at age 4 weeks (Fig. 4 *a*), but much more even at age of 6 weeks (Fig. 4 *b*), when the sinus macrophages contained more cytoplasm, and confluent strands of uniformly labeled cells interdigitated with nonlabeled pyroninophilic cells.

The amount of antigen retained in phagocytic structures 6 days after injection appeared to increase steadily during late lymphoid differentiation. The increased ability to retain antigen was best demonstrated by the spleen which had less intense labeling than regional lymph nodes. The spleen from animals injected at 4 weeks contained little antigen 6 days later (Fig. 5 *a*), whereas the follicles of 6 week old rats retained antigen in high concentration (Fig. 5 *b*) in the characteristic "cap" region typical of adult follicles (3).

Whole organ radioactivity counts confirmed the increased ability of lymphoid tissue from older rats to retain antigen. The percentage of the dose injected retained per milligram of lymphoid tissue is plotted in Text-fig. 1. It is ap-



TEXT-FIG. 1. Antigen retention in popliteal nodes and spleen 6 days after injection.

parent that on the basis of unit weight there is a steady increase in the amount of antigen retention with maturation.

Table I summarizes the findings presented. The very young animal lacks antigen-capturing units and the primitive reticular cells are exposed to high concentrations of antigen. Late in the second week of life early differentiation occurs. Antigen is localized in the cortex of lymph nodes first in a continuous zone then in segments. Antigen is also taken up by cells surrounding the primitive splenic follicles. Development of phagocytic structures continues to the age of 6 weeks, but later changes are more quantitative than qualitative.

The Long Term Fate of Antigen Injected into the Foot Pads on The Day of Birth.—It is possible that the small amounts of antigen present in scattered phagocytic cells 1 week after neonatal injection might have moved to defined antigen retaining areas some time later. Two litters of rats were injected into the hind foot pads within 8 hr of birth with 20 μ g of polymerized flagellin containing 150 μ c of I^{125} . Animals from each litter were killed at weekly intervals up to the

TABLE I
The Development of Antigen-Capturing Structures

Age of rat	Organ	Histological features	Antigen distribution	
			1 day after injection	6 days after injection
<i>days</i> 0-2	Lymph nodes	Undifferentiated mass of reticular cells	Diffuse	Occasional heavily labeled cells.
	Spleen	Undifferentiated organ with erythromyelopoiesis and other blast cells	Diffuse	Occasional heavily labeled cells. Poor over-all retention.
8-16	Lymph nodes	Corticomedullary differentiation. Tissue still poor in lymphocytes. Appearance of postcapillary venules.	Cortical rim progressing to rudimentary follicular localization. Commencing medullary macrophages.	Condensation of label to cortical segments (rudimentary follicles)
	Spleen	Commencing appearance of periarterial lymphocyte sheath and marginal zone. Red pulp becoming less cellular.	Predominately marginal zone. Little white pulp retention.	Poor over-all retention. Occasional scattered heavily labeled cells.
16-42	Lymph nodes	Progressive assumption of adult histology. Increase in size, number of lymphocytes and plasma cells. Formation of primary follicles and later of germinal centers.	Follicles; rare germinal centers, medullary macrophages.	Follicles; rare germinal centers, medullary macrophages.
	Spleen		Follicular "crescents." Some in marginal zone and red pulp.	Follicular "crescents." Some in marginal zone and red pulp. Improved retention.

age of 6 weeks, and the regional lymph nodes were studied for antigen distribution.

All lymph nodes showed declining amounts of label with time after injection. Sinus macrophages contained label as late as 6 weeks following neonatal injection.

tions. However, lymphoid follicles were never detectably labeled. The amount of antigen retained by sinus macrophages can be estimated from specific activity of the antigen, its dose, the exposure time of autoradiographic sections, and the grain-producing efficiency of I^{125} (9). Under the conditions of this experiment, occasional sinus macrophages contained as much as 1000 monomeric units of flagellin 4 to 6 weeks following injection. The follicle antigen concentration never reached the threshold of detectability—75 molecules of flagellin per $16 \mu^2$ area—in this experiment. Thus we were not able to detect “migration” of antigen into cortical areas with progressive maturation following neonatal injection.

The Effects of Antigen Injections on the Formation of Germinal Centers.—As we have noted previously, most lymphoid follicles of young (4 week old) rat

TABLE II
Stimulation of Germinal Center Formation by Antigen Injections at 2 and 6 Weeks of Age

Lymph node	Side	Mean number of germinal centers per entire lymph node		
		Age, 4 weeks	Age, 6 weeks	Age, 8 weeks
Popliteal	Injected	4	7	20
	Control	0.5	2	10
Aortic	Injected	21	18	39
	Control	8	11	24

popliteal nodes are primary follicles; *i.e.*, contain no germinal centers. After intentional antigenic stimulation, germinal centers rapidly appear. To document whether injections of antigen at a very young age also caused germinal center formation, we embarked on a study involving serial sectioning of entire lymph nodes from rats given antigen at age 14 days. Each animal was used as its own control, receiving $5 \mu\text{g}$ ($300 \mu\text{c}$) of I^{125} labeled polymerized flagellin into *one* hind foot pad and no injection into the contralateral side. Unfortunately, some “spillover” of antigen did occur in these young animals and nodes from the un-injected side contained about 10% of the radioactivity present in nodes from the injected side. However, this difference was great enough to allow us to establish a clear-cut effect of antigen on germinal center formation rate. Ten rats (forty lymph nodes) were involved in the study. After the initial injection at age 2 weeks, some rats were killed at age 4 and 6 weeks; the remainder were given a second unilateral injection of $50 \mu\text{g}$ of antigen into the same foot pad at 6 weeks and killed at age 8 weeks. The results (Table II) showed a clear-cut acceleration of the rate of germinal center formation on the injected side.

Autoradiographic study showed that this acceleration had been caused by very low local antigen concentration. In fact, at the time of killing, the amount of antigen per germinal center varied from <10 to 40 monomeric units per lymphocyte area.

DISCUSSION

The present studies revealed clear-cut qualitative and quantitative deficiencies in the processing of antigens by the young rat. The newborn rat possessed phagocytic cells but was devoid of organized phagocytic structures in lymphoid organs. Although the newborn rat can clear certain bacteria and inert particles rapidly from the blood, the structural components characteristic of adult lymph nodes (1) and spleen (3), which sequester and retain protein antigen, were absent. At the age of 10 days, the cortex of the aortic nodes became modified and localized antigen in a continuous "rim." This continuous zone was subsequently broken into active spherical areas which subsequently developed into follicles. The popliteal lymph nodes developed the ability to take up antigen in the cortex 4 to 6 days later. Germinal centers and plasma cell development were usually more extensive in aortic than popliteal nodes (Table II), suggesting that in an unimmunized rat, the aortic nodes were subject to more intense antigenic stimulation. It thus appears that the development of cortical antigen-trapping structures in lymph nodes is, to a degree, conditioned by local factors, possibly in response to specific local demands.

It is of interest that antigen is localized in spherical cortical areas before these structures contain many lymphocytes. In fact we could not demonstrate any definite cell type aggregating in the cortex that could account for the accumulation of antigen in these areas. Rather, these zones appear to represent some functional change in the reticular cells of the cortex. Perhaps the appearance of postcapillary venules is a related development; as soon as these arise lymphocytes appear to migrate to areas that have been adapted previously to take up and retain antigens, thereby forming the primary lymphoid follicle.

After the age of 2 weeks, the chief differences between young and adult rats are quantitative. Antigen uptake and retention by lymphoid tissue becomes increasingly greater as measured by whole organ counts and by autoradiography. Increased antigen retention is not related to increased size of the nodes and spleen alone as retention per unit weight increased fivefold between the ages of 2 and 6 weeks (Text-fig. 1). Studies tracing the long-range fate of antigen injected at birth or at 2 weeks of age revealed poor persistence. Thus even though antigen-trapping structures had appeared they had not developed the same degree of retentive capacity as their adult counterparts. Initial antigen localization and long-range antigen retention may depend on somewhat different mechanisms.

The role played by antigen-retaining cells in antibody formation remains unknown. Elsewhere (10) we have discussed the notion that inefficient antigen capture is one common link between many model systems of induction of tolerance. In the accompanying paper, we will attempt to link the progressive changes in cortical antigen localization with the development of antibody formation and memory. An intriguing defect of young animals is their inability to act as hosts for adult cells transferred during the early inductive phase of antibody formation (11, 12). If interactions between antigen-trapping cells and lymphocytes are involved in induction, the poor quality of antigen capture in young animals may be a contributory factor. Further, if it is true that contact between recirculating lymphocytes and antigen retained on the follicle web is the inductive trigger, then the absence of postcapillary venules in rats aged less than 10 days may be important, as these play a key role in lymphocyte traffic (13). It is noteworthy that cortical antigen localization, postcapillary venule formation and progressive lymphocyte accumulation all develop more or less in parallel from the tenth day of life. This may reflect coincidental maturation of the afferent, central, and efferent systems of immunity.

SUMMARY

Polymerized flagellin from *Salmonella adelaide* was labeled with I^{125} and injected into rats varying in age from 0 to 42 days. Lymphoid organs were removed at various intervals and the progressive development of antigen-capturing structures was studied using autoradiography. The chief findings were as follows:

1. Newborn rats lack the follicular and medullary antigen-trapping structures characteristic of adult animals.
2. At the age of 10 to 14 days, the first signs of specific cortical antigen localization appear in lymph nodes. This initially takes the form of a continuous "cortical rim" of antigen localization.
3. Within a further 4 to 6 days, the *Anlagen* of true follicular antigen-capturing structures appear, the continuous rim being only a transitional mechanism.
4. The antigen-capturing part of the follicle appears before the lymphoid component; follicle *Anlagen* can be defined only on autoradiographs and cannot be seen on ordinary histological sections.
5. The system of medullary macrophages develops gradually over the period 2 to 6 weeks of age.
6. The ability of lymph nodes to retain antigen increases progressively, there being a fivefold increase in the amount of antigen retained per unit weight of lymphoid tissue between 2 and 6 wk of age.

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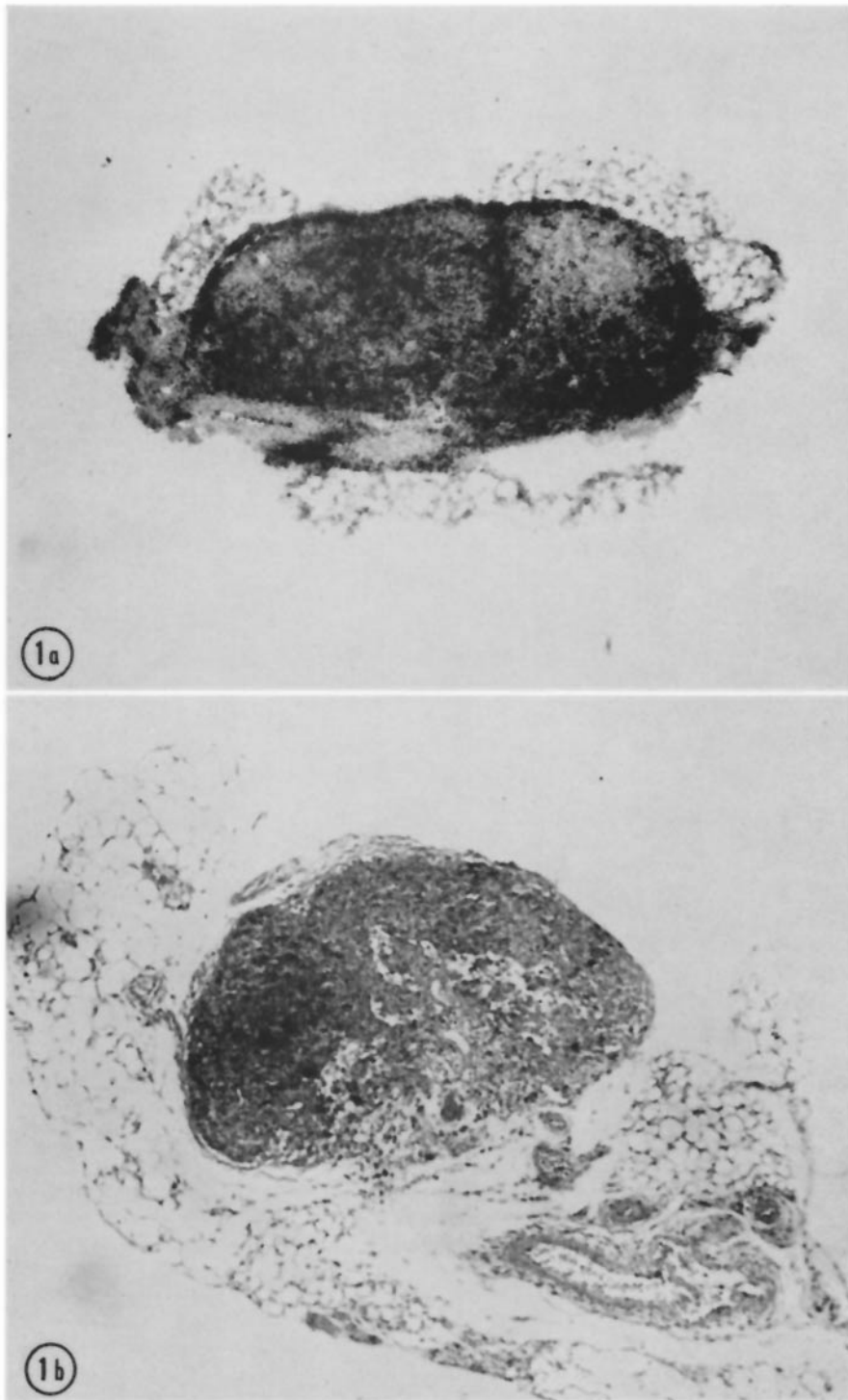
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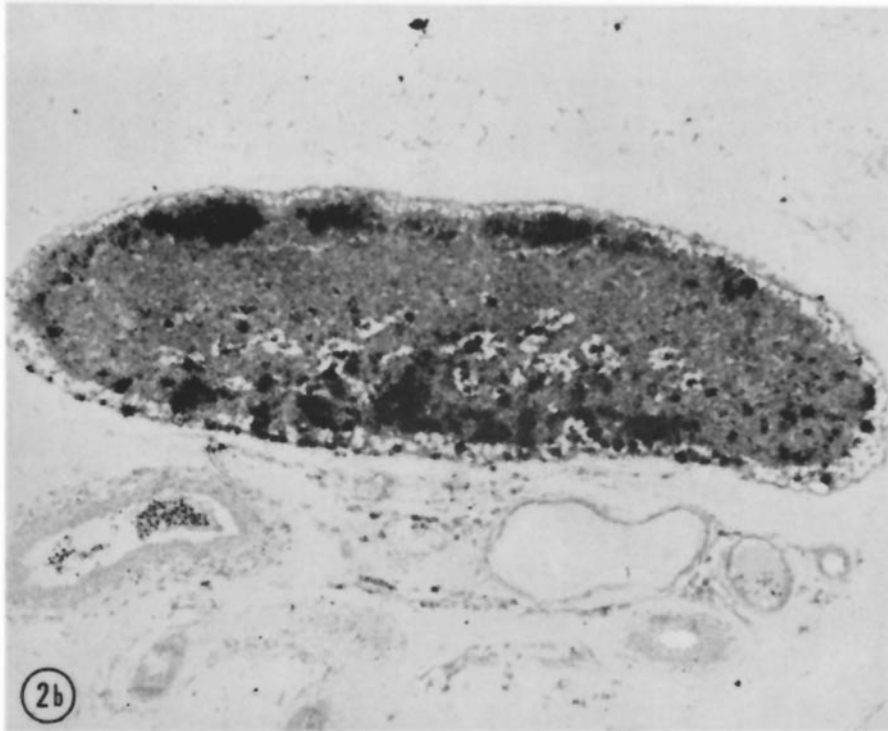
EXPLANATION OF PLATES

PLATE 3

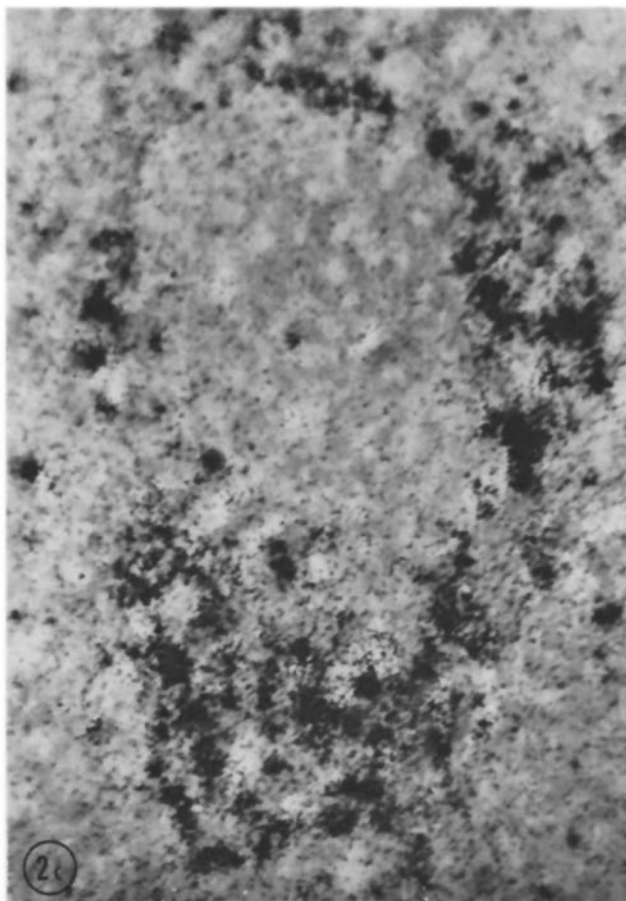
FIGS. 1 *a* and 1 *b*. Autoradiographs from popliteal lymph nodes of rats injected with I^{125} -labeled polymerized flagellin on the day of birth Fig. 1 *a*, killed 1 day later, Fig. 1 *b*, killed 6 days later. Note the diffuse labeling pattern and the poor retention. $\times 30$.



(Williams and Nossal: Development of follicular localization)



(Williams and Nossal: Development of follicular localization)



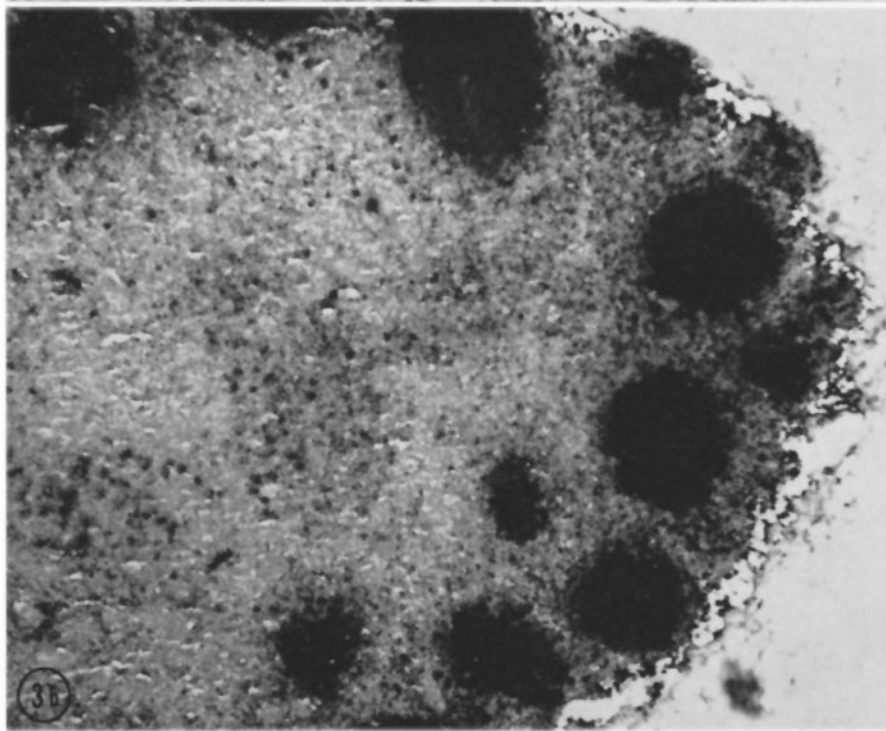
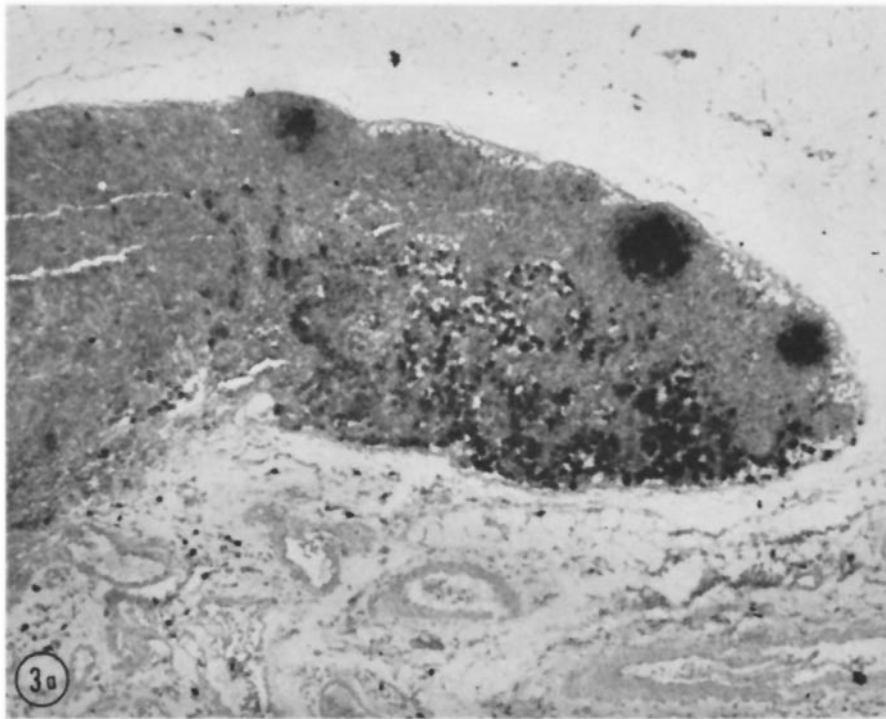
(Williams and Nossal: Development of follicular localization)

PLATES 4 AND 5

FIGS. 2 *a* to 2 *c*. Autoradiographs from rats similarly injected at age 16 days. Fig. 2 *a*, Popliteal node 1 day after injection; Fig. 2 *b*, popliteal node 6 days after injection, Fig. 2 *c*, spleen 1 day after injection. Note the "cortical rim," Fig. 2 *a*, $\times 30$; its condensation into rudimentary follicles, Fig. 2 *b*, $\times 30$; and marginal zone localization in the spleen Fig. 2 *c*, $\times 125$.

PLATE 6

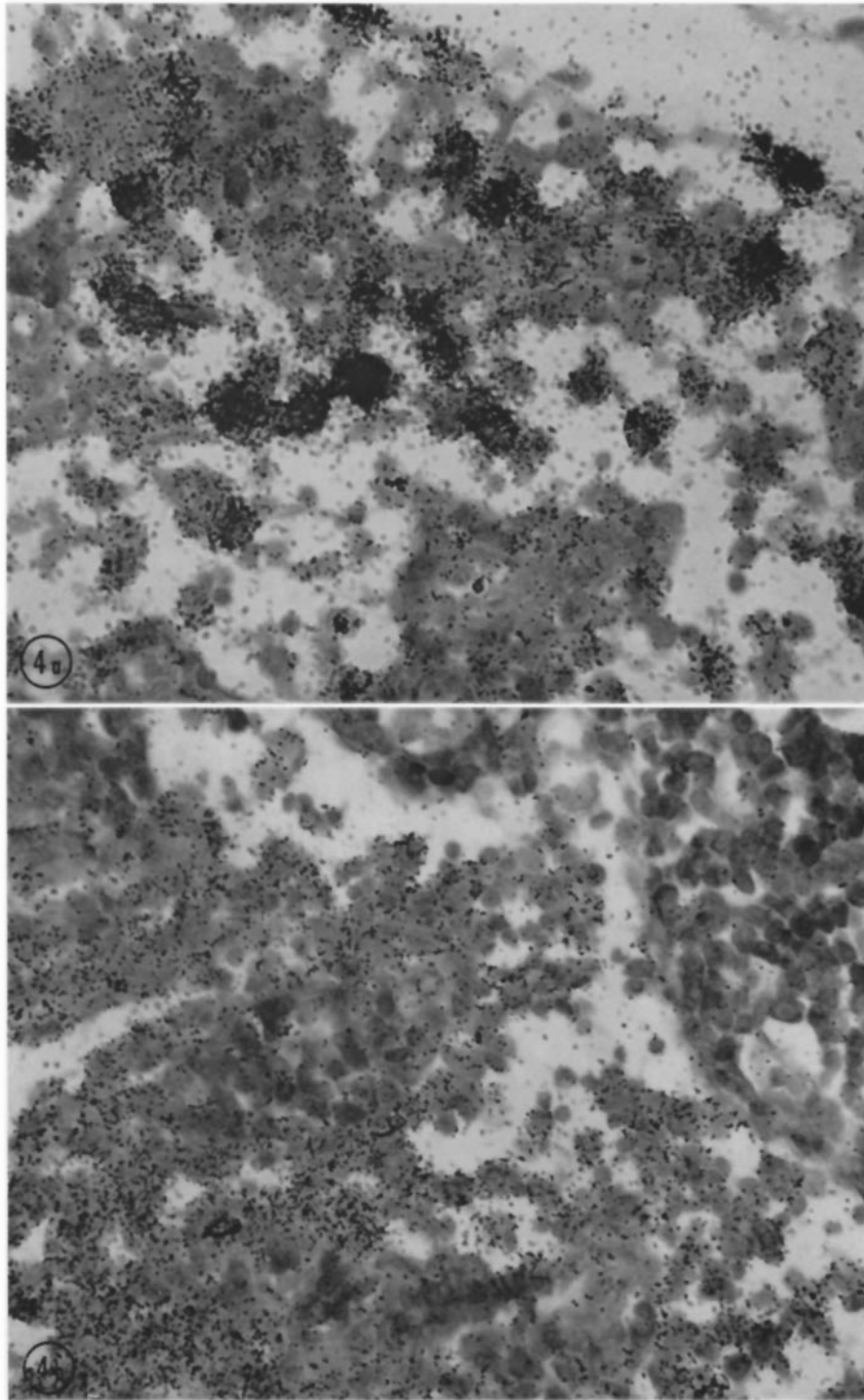
FIGS. 3 *a* and 3 *b*. Progressive maturation of the follicular antigen-trapping mechanism of popliteal lymph nodes. Rats killed 1 day after antigen injection. Fig. 3 *a*, aged 4 weeks; Fig. 3 *b*, aged 6 weeks. $\times 30$.



(Williams and Nossal: Development of follicular localization)

PLATE 7

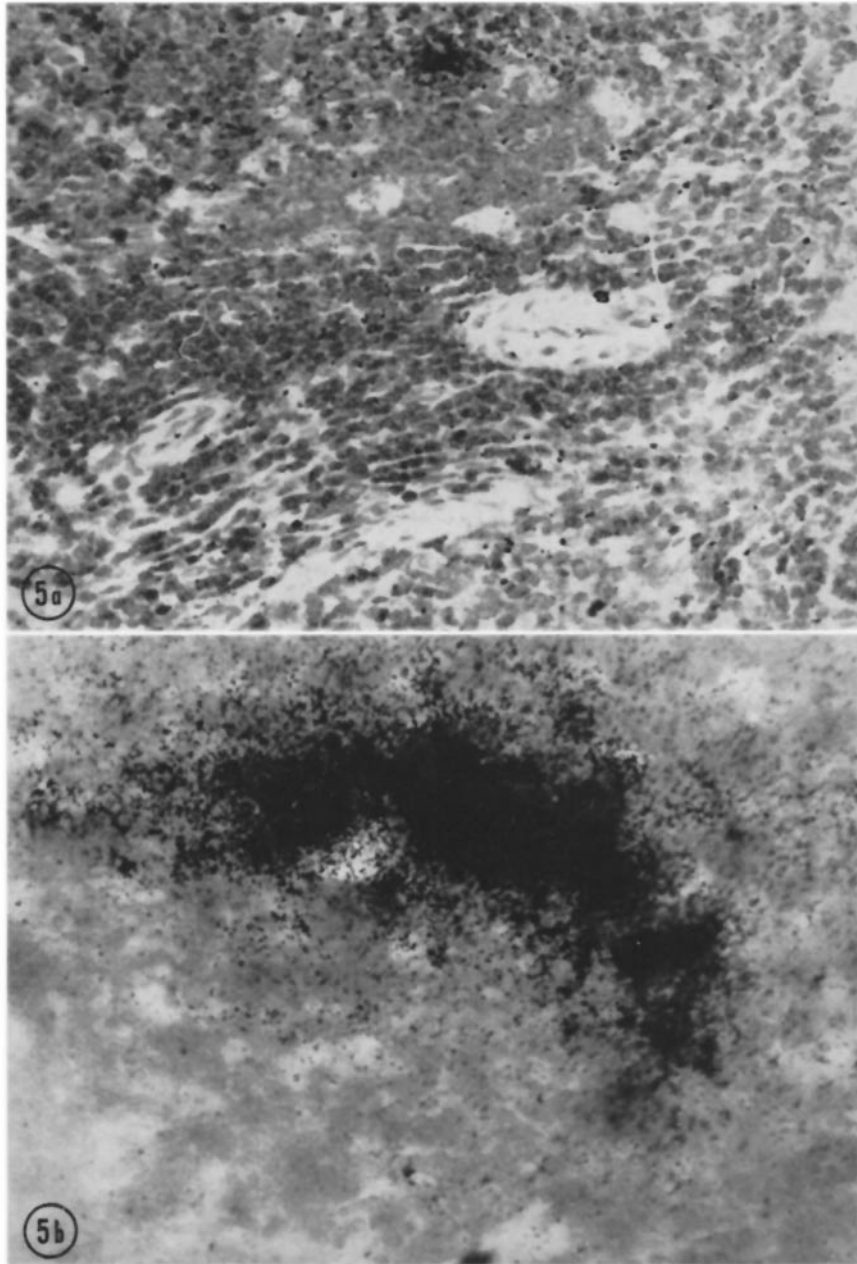
FIGS. 4 *a* and 4 *b*. Maturation of medullary phagocytosis in popliteal nodes. Rats killed 1 day after antigen. Fig. 4 *a*, aged 4 weeks. Note the great degree of variation in labeling intensity amongst individual cells; Fig. 4 *b*, aged 6 weeks. The macrophages are more evenly labeled and cords of plasma cells are appearing between them. $\times 250$.



(Williams and Nossal: Development of follicular localization)

PLATE 8

FIGS. 5 *a* and 5 *b*. Maturation of antigen retention in the spleen. Rats killed 6 days after foot pad injection of antigen. Fig. 5 *a*, aged 4 weeks. One small patch of label is present as well as some scattered material just above background; Fig. 5 *b*, aged 6 weeks. The crescentic cap of label in the follicle is characteristic of the adult distribution. Over-all retention is much greater. $\times 250$.



(Williams and Nossal: Development of Follicular localization)