CELLULAR LOCALIZATION AND QUANTITATION OF TRITIATED ANTIGEN IN MOUSE LYMPH NODES DURING EARLY PRIMARY IMMUNE RESPONSE

AUDREY N. ROBERTS, PH.D.*

From the Department of Preventive Medicine, University of Tennessee College of Medicine, Memphis, Tenn.

The mechanism by which antigen elicits antibody formation and the characteristics of lymphoid tissues associated with immunologic phenomena have received excellent discussion in recent reviews.[1-5] Although numerous investigations have advanced our knowledge of the mechanisms of antibody formation, the role of antigen in the immune response has remained elusive. Using ^{125}I and ^{131}I labeled flagellar antigens, Nossal, Ada and Austin[6-9] observed radioactivity in macrophages in regional popliteal nodes following primary response stimulation in the footpads of rats. Autoradiographic investigations with tritium (^{3}H) labeled soluble azoprotein antigens[10,11] and ^{3}H-tetanus toxin[12-14] in mice have emphasized the efficient incorporation of antigen by macrophages. In addition, ^{3}H-tetanus toxin or labeled fragments of the toxin molecules have been shown to persist in mouse macrophages for intervals up to 9 months.[12,14] These results have supported a possible direct or indirect function of the macrophage in mechanisms of antibody synthesis.[15-18]

Studies on the primary immune response have provided little evidence for the association of antigen with potential precursors of immunologically competent cells. Electron microscopic investigations have demonstrated ferritin in the cytoplasm and nuclei of phagocytic sinus and reticular cells in rabbit lymph nodes within 3 to 12 hours after primary footpad stimulation.[19] Following secondary response stimulation, ferritin also was detected in the cytoplasm and nuclei of plasmoblasts and plasma cells. Other studies on the response of rabbits to single or multiple injections of ferritin have demonstrated antigen in macrophages and reticular cells, but not in blast cells or immature plasma cells.[20,21]

Evidence for the direct role of antigen in the mechanisms of antibody synthesis[3,4,22,23] and for the early synthesis of antibody (24 to 72 hours)

This research was supported by United States Public Health Service Grant AI-04217 from the National Institute of Allergy and Infectious Diseases.

Presented in part at the Sixty-sixth Annual Meeting of the American Society for Microbiology, May, 1966.

Accepted for publication, May 27, 1966.

* Recipient of Research Career Development Award No. 1-K3-AI-21,517 from the National Institute of Allergy and Infectious Diseases.

following primary antigenic stimulation[24-27] has suggested that the initial instructive interaction of antigen with immunologically competent cells occurs during the first few hours of a primary immune response. In the present investigation, autoradiography and liquid scintillation counting techniques were employed to quantitate the early localization of a soluble tritiated azoprotein antigen in mouse lymph nodes after primary response stimulation. The results have demonstrated the incorporation of ^{3}H-antigen or antigenic fragments by macrophages, eosinophils, mast cells and large immature mononuclear cells in draining lymph nodes through the first 24 hours after footpad injection. Quantitative data on the rapid incorporation of ^{3}H-antigen by mouse eosinophils have been presented in part elsewhere.[28] Immature mononuclear cells with heavy intracytoplasmic and intranuclear ^{3}H concentrations were detected in all labeled lymph nodes, suggesting an antigen-chromatin contact in possible precursors of immunologically competent cells.

Material and Methods

Soluble ^{3}H labeled sulfanilazo porcine gamma globulin (^{3}H-S-PGG) and unlabeled sulfanilazo PGG (S-PGG) were employed as antigens. Sulfanilic acid was tritiated by the catalytic exchange method (New England Nuclear Corp., Boston, Mass.) to a final ^{3}H concentration of 2 mc per mg. The preparation of ^{3}H-S-PGG and S-PGG has been described.[28] The final protein concentration of the ^{3}H-S-PGG was 8 mg per ml, and the concentration of ^{3}H was 175 μc per mg. Radioactivity determinations were performed in a Chicago Nuclear liquid scintillation system. The S-PGG was prepared to a final protein concentration of 8 mg per ml.

Adult male albino mice (NLW-CFW-Webster-Swiss origin) were used in this investigation and were fed a diet of Purina® Mouse Breeder Blox and tap water *ad libitum*. Fifty-two mice in group I and 14 mice in group II each received a subcutaneous injection of 80 μg (14 μc per 0.01 ml) of ^{3}H-S-PGG in the right hind footpad. Each of 13 mice in control group III received an injection of 80 μg (0.01 ml) of S-PGG in the right hind footpad.

In group I, 4 mice were studied at each of 13 intervals following injection: 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16 and 24 hours. Six lymph nodes (right and left popliteal, right and left inguinal, right ventral and right dorsal axillary) were removed from each mouse and prepared for autoradiography. Lymph nodes from 2 of the 4 mice were fixed in neutral formalin for hematoxylin and eosin staining of sections, and nodes from the remaining 2 mice were fixed in Bouin-Hollande solution for sections treated with the Dominici stain.[29,30] The above 6 lymph nodes also were obtained from 1 mouse in group III at each of the 13 intervals scheduled for the group I study. Tissue sections from these control nodes were used to establish the level of background grain formation in autoradiographs.

The techniques employed for tissue embedding, sectioning and autoradiography have been described.[11] All tissue sections were stained following development of the autoradiographs. Replicate sections were used for autoradiographs of each tissue in mouse groups I and III; these were then exposed for 2-, 4-, 6- and 8-week periods respectively. Quantitative observations of ^{3}H-exposed emulsion grains over tissue cells were performed on 6-week autoradiographs unless otherwise specified. Intracellular ^{3}H concentrations were graded as light (5 to 10 grains per cell), moderate (10 to 20 grains) and heavy (>20 grains). All grain counts were made at × 1,250

magnification (× 100 oil immersion and × 12.5 eyepiece). The distribution of ^3H in macrophages was determined by microscopically counting 500 labeled cells per section. The total number of labeled and unlabeled eosinophils and mast cells was counted in each section. The mean background over unlabeled control tissue sections (mouse group III) and emulsion-coated, non-tissue areas of experimental slides was 1 grain per 1,000 μ^2.[11]

The ^3H content of the lymph nodes obtained from mouse groups I and III was determined after sectioning and autoradiography as follows: the lymph node pieces which remained after sectioning were deparaffinized in six 5-minute changes of xylene, dehydrated through five 5-minute changes of absolute ethanol, placed in weighed vials, vacuum desiccated over Drierite® for 2 weeks and weighed. All weights were determined with a model M5 Mettler microbalance. The tissues were dissolved in 1.0 ml of methanol-hyamine-hydroxide solution for 2 to 5 days at 52° C.[10] The volume of each tissue solution was measured, and 0.05 ml was added per vial of scintillation solution. The samples were counted in a Nuclear Chicago liquid scintillation system at −2° C with automatic background subtraction. The counting efficiency of the instrument for standard ^3H samples was 34.5 per cent. Quench correction for the experimental samples was determined by the channels ratio method. Results were expressed as mμc of ^3H, from which the μg of ^3H-antigen per mg of dry tissue was calculated.

Serums were collected from all mice in groups I and III which were sacrificed during the first 24 hours following antigenic stimulation. Group II mice were observed for serum antibody formation throughout the first 14 days after immunization. Serums were collected from 2 mice in group II on each of days 1, 3, 5, 7, 9, 12 and 14. Prior to antibody titrations, the serums were heat inactivated at 56° C for 30 minutes and stored at −20° C.

Serum antibodies specific for the sulfanilazo determinant were titrated with the passive hemagglutination (HA) technique using formalinized rabbit erythrocytes (Difco) coupled with diazotized sulfanilic acid.[31] Serum antibodies specific for PGG were titrated with the passive tannic acid HA method.[11] All serums were tested with the 2 HA techniques.

Results

Cellular Localization of ^3H-Antigen in Draining Lymph Nodes

Regional Popliteal Nodes. Autoradiographs revealed the dense accumulation of ^3H labeled macrophages in and around medullary sinuses within 1 hour after injection. These cells were characterized by heavy intracytoplasmic ^3H concentrations and large quantities of extracellular ^3H-antigen at the cytoplasmic membranes. The extracellular ^3H localization was not observed beyond the 1-hour study. Between 2 and 24 hours labeled macrophages were clustered in the medullary regions, interfollicular cortex and polar regions of the nodes (Fig. 1). Within 2 hours over 90 per cent of the labeled macrophages demonstrated heavy intracytoplasmic concentrations of ^3H-antigen (Fig. 2). The proportion of heavily labeled macrophages varied from 82 to 96 per cent during the 24-hour study (Table I).

Eosinophils containing ^3H were observed from 1 through 24 hours. The proportion of labeled eosinophils with heavy intracytoplasmic ^3H concentrations varied from 80 to 98 per cent. The tritiated eosinophils were

scattered singly or in clusters in the medullary regions and interfollicular cortex of the nodes. Many heavily labeled eosinophils were surrounded by unlabeled lymphoid cells, while others were distributed in areas adjacent to groups of labeled macrophages and mast cells. The ^{3}H labeled and unlabeled eosinophils did not encircle or cluster about the other labeled cell types.

TABLE I

^{3}H-ANTIGEN INCORPORATION IN REGIONAL POPLITEAL LYMPH NODES FOLLOWING PRIMARY FOOTPAD STIMULATION *

Hours after injection †	Cell	Mean cells per section ± S.D. ‡	*Per cent tritiated cells* ± S.D. §			
			Per section	Grain count per cell		
				5-10	10-20	>20
1	Macrophage ¶					
	Mast	2 ± 1	87 ± 47	17 ± 16	33 ± 33	50 ± 48
2	Macrophage			2 ± 2	7 ± 6	91 ± 4
	Mast	4 ± 3	61 ± 33	16 ± 16	33 ± 26	51 ± 42
3	Macrophage			2 ± 2	9 ± 5	89 ± 6
	Mast	22 ± 12	74 ± 19	36 ± 19	44 ± 1	20 ± 19
4	Macrophage			1 ± 0	4 ± 2	95 ± 1
	Mast	177 ± 108	99 ± 5	13 ± 7	28 ± 9	59 ± 13
5	Macrophage			2 ± 2	9 ± 2	89 ± 4
	Mast π	6 ± 2	49 ± 36	50 ± 38	18 ± 17	32 ± 32
6	Macrophage			0	4 ± 2	96 ± 2
	Mast	1 ± 1	0			
7	Macrophage			1 ± 1	9 ± 2	90 ± 2
	Mast	113 ± 16	69 ± 24	14 ± 11	21 ± 11	65 ± 12
8	Macrophage			1 ± 1	6 ± 2	93 ± 2
	Mast	9 ± 7	57 ± 11	18 ± 13	26 ± 22	56 ± 30
12	Macrophage			1 ± 0	7 ± 4	92 ± 4
	Mast	4 ± 3	72 ± 13	7 ± 7	54 ± 41	39 ± 35
10	Macrophage			0	9 ± 2	91 ± 3
	Mast	5 ± 3	67 ± 20	0	52 ± 38	48 ± 38
14	Macrophage			5 ± 3	13 ± 5	82 ± 7
	Mast	0				
16	Macrophage			2 ± 1	4 ± 1	94 ± 2
	Mast	1 ± 1	0			
24	Macrophage			2 ± 1	10 ± 4	88 ± 3
	Mast	2 ± 1	91 ± 12	0	33 ± 27	67 ± 27

* Each of the 52 mice received a subcutaneous injection of 80 μg (14 μc per 0.01 ml) ^{3}H-S-PGG in the right hind footpad.

† Four right popliteal nodes were studied at each time interval.

‡ Labeled macrophages, 500 per section, were scored for intracellular ^{3}H concentration (grain count per cell). The total number of mast cells were counted per section.

§ Values were derived from 96 sections (24 sections per lymph node).

¶ Heavy extracellular grain concentrations prevented accurate scoring of labeled macrophages.

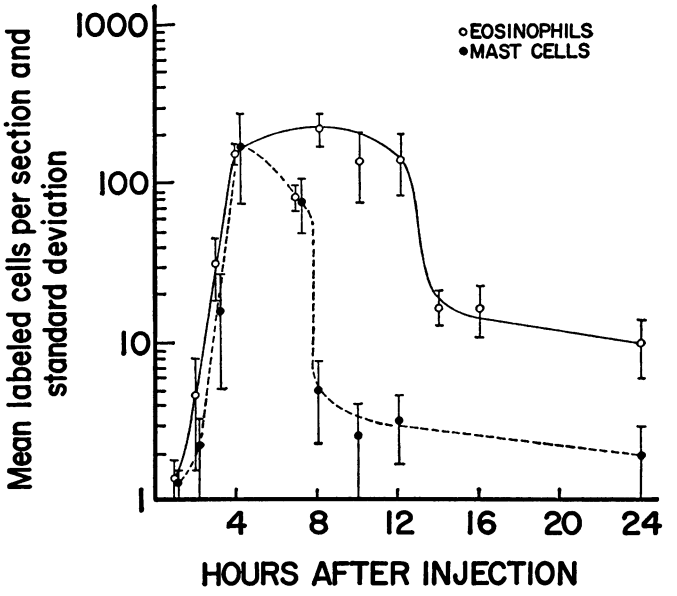
π Only 2 of 4 lymph nodes contained labeled mast cells.

The early incorporation of ^{3}H-antigen by eosinophils preceded an eosinophilia which was first apparent at 4 hours (Text-fig. 1). At each study interval between 4 and 12 hours, the means of total eosinophil

counts and of labeled eosinophil counts per section exceeded by 5 to 29 times the normal mean of 8 eosinophils per section derived from counts of the 1,248 unstimulated contralateral popliteal node sections.

The intracytoplasmic incorporation of ^{3}H-antigen by mast cells was observed 1 hour after primary injection, and the labeled mast cell responses persisted through the 24-hour study (Table I). The tritiated mast cells were localized in the interfollicular cortex and medullary regions of the nodes. Clusters of labeled mast cells usually were surrounded by numerous densely labeled mononuclear cells (Fig. 3). A few mast cells with high intracytoplasmic ^{3}H concentrations were isolated among unlabeled lymphoid cells, but no mast cells were found in lymphoid follicles.

A 135-fold increase in the mean of labeled mast cell counts per section almost paralleled the labeled eosinophil responses between 1 and 4 hours (Text-fig. 1). The peak mast cell response at 4 hours presented a mean

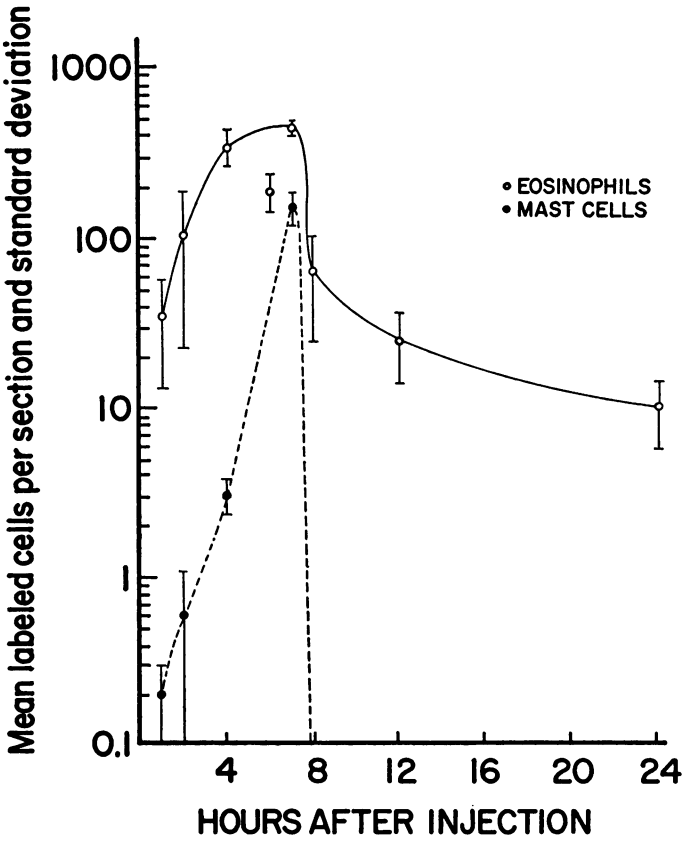


TEXT-FIG. 1. Total number of labeled eosinophils and mast cells in the draining popliteal lymph nodes following primary footpad stimulation with ^{3}H-S-PGG. Each value is the mean count of 4 lymph nodes, 24 sections per node.

of 176 labeled cells per section, 59 per cent of which had incorporated heavy concentrations of ^{3}H-antigen. The mean of total mast cell counts per section in the 4- and 7-hour regional nodes (Table I) exceeded the normal mean of 21 mast cells per section, determined from counts of the 1,248 unlabeled contralateral node sections. The normal range in mast cells per section was 1 to 46, with only 52 sections containing over 40

cells each. The mast cell response was not a consistent observation in all experimental animals. The 12 lymph nodes studied at 6, 14 and 16 hours contained no labeled mast cells and demonstrated very low total mast cell counts (Table I).

From 1 to 3 hours after injection, the regional popliteal nodes contained numerous mononuclear cells with heavy ^{3}H concentrations in the nucleus and at the cytoplasmic membrane. These cells first appeared in areas of heavily labeled macrophages. All lymph nodes studied between 4 and 24 hours contained large numbers of the mononuclear cells with heavy concentrations of ^{3}H in both nucleus and cytoplasm (Fig. 4).



TEXT-FIG. 2. Total number of labeled eosinophils and mast cells in the draining inguinal lymph nodes following primary footpad stimulation with ^{3}H-S-PGG. Each value is the mean count of 4 lymph nodes, 24 sections per node.

The characteristic features of these cells were observed with difficulty due to their dense grain formations in autoradiographs. The round to oval-shaped cells varied from 12 to 20 μ in diameter and appeared to

have large, vacuolated nuclei with nucleoli and thin to moderate amounts of cytoplasm.

^{3}H-antigen was not incorporated by neutrophils or small lymphocytes. Both cell types were distributed generously among densely labeled

TABLE II

^{3}H-ANTIGEN INCORPORATION IN DRAINING INGUINAL LYMPH NODES FOLLOWING PRIMARY FOOTPAD STIMULATION *

Hours after injection †	Cell	Mean cells per section ± S.D. ‡	*Per cent tritiated cells* ± S.D. §			
			Per section	Grain count per cell		
				5-10	10-20	>20
1	Macrophage			6 ± 8	15 ± 12	79 ± 19
	Mast	2 ± 1	10 ± 7	0	50 ± 50	50 ± 50
2	Macrophage			2 ± 2	8 ± 4	90 ± 12
	Mast	1 ± 1	60 ± 22	50 ± 41	50 ± 41	0
3	Macrophage			3 ± 3	9 ± 8	88 ± 10
	Mast ¶	5 ± 3	7 ± 6	100	0	0
4	Macrophage			1 ± 1	8 ± 5	91 ± 6
	Mast	39 ± 7	80 ± 8	14 ± 9	36 ± 8	50 ± 3
6	Macrophage			2 ± 2	6 ± 4	92 ± 5
	Mast ¶	3 ± 2	36 ± 36	33 ± 33	9 ± 8	58 ± 42
7	Macrophage			1 ± 0	7 ± 3	92 ± 4
	Mast	156 ± 31	99 ± 2	6 ± 4	20 ± 3	74 ± 6
8	Macrophage			2 ± 1	6 ± 3	92 ± 4
	Mast	2 ± 1	0			
12	Macrophage			3 ± 2	9 ± 4	88 ± 5
	Mast	0				
24	Macrophage			1 ± 1	17 ± 3	82 ± 2
	Mast	0				

* Each of 52 mice received a subcutaneous injection of 80 μg (14 μc per 0.01 ml) of ^{3}H-S-PGG in the right hind footpad.

† Four inguinal nodes were studied at each time interval. Lymph nodes obtained at 5, 10, 14 and 16 hours contained no labeled macrophages or mast cells.

‡ Labeled macrophages, 500 per section, were scored for intracellular ^{3}H concentration (grain count per cell). The total number of mast cells were counted per section.

§ Values were derived from 96 sections (24 sections per lymph node).

¶ Only 2 of 4 lymph nodes contained labeled mast cells.

macrophages and mast cells, but remained unlabeled throughout the study.

Right Inguinal Nodes. Thirty-six of the 52 group 1 mice responded with ^{3}H incorporation by macrophages in the draining inguinal nodes. Many of the labeled nodes also demonstrated ^{3}H in eosinophils, mast cells and large mononuclear cells. All labeled cells were confined in the interfollicular medullary areas, interfollicular cortex and polar regions of the nodes. Between 1 and 24 hours, from 79 to 92 per cent of the labeled macrophages had incorporated heavy concentrations of ^{3}H-antigen (Table II). These macrophages demonstrated specific intracytoplasmic ^{3}H localization.

The 36 inguinal nodes which contained labeled macrophages also demonstrated the efficient uptake of ^{3}H-antigen by eosinophils. The means of labeled eosinophil counts per section increased from 36 to 348 between 1 and 4 hours after injection (Text-fig. 2). A peak of 444 labeled eosinophils per section was recorded at 7 hours. The means of eosinophil counts per section between 2 and 7 hours greatly exceeded the normal mean of 11 (range 1 to 44) determined from counts of 1,248 sections of the left (uninjected side) inguinal lymph nodes. The proportion of labeled eosinophils which incorporated heavy concentrations of ^{3}H-antigen varied from 55 to 84 per cent. While many eosinophils were localized in large groups of heavily labeled macrophages, others were distributed individually or in clusters in areas of unlabeled lymphoid cells. Most of the labeled eosinophils were mature cells with bilobed nuclei, and all demonstrated intracytoplasmic ^{3}H localization.

Autoradiographs revealed ^{3}H incorporation by mast cells in 20 of the 36 labeled inguinal nodes (Table II). The uptake of ^{3}H-antigen by mast cells stimulated an almost 1,000-fold increase in the mean of labeled mast cell counts per section between 1 and 7 hours (Text-fig. 2). The sharp peak response at 7 hours was followed by a very rapid disappearance of labeled mast cells by 8 hours. With the exception of the 4- and 7-hour means, the mast cell counts in draining inguinal nodes were below the normal mean of 33 (range 8 to 62) derived from counts of the 1,248 left inguinal node sections.

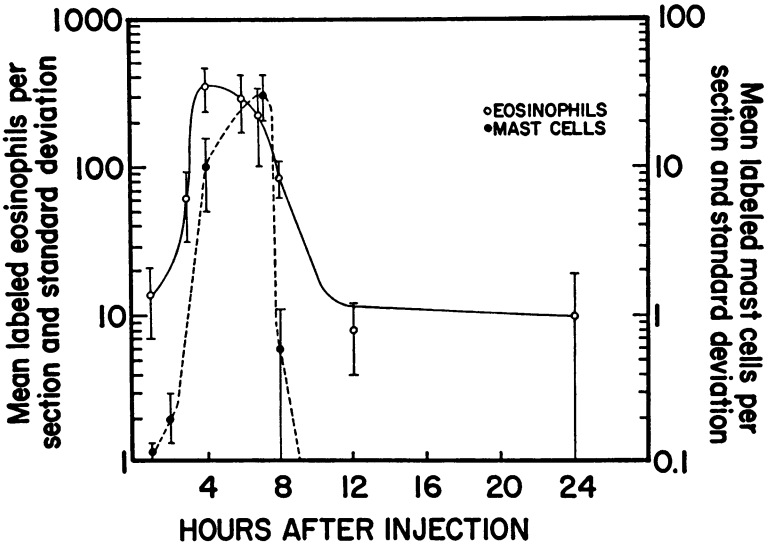
From 4 to 7 hours following injection, heavy intracytoplasmic concentrations of ^{3}H-antigen were demonstrated in 50 to 74 per cent of the labeled mast cells (Table II). As in the regional popliteal nodes, mast cells frequently were surrounded by groups of labeled macrophages and eosinophils. The incorporation of ^{3}H-antigen by mast cells was associated with a decrease in basophilia of the cytoplasmic granules, until only faint basophilic ringlets were detectable (Figs. 5 and 6). Unlabeled mast cells localized in close association with labeled mast cells retained their normal intense basophilia (Fig. 5). At peak response, clusters of 10 to 15 labeled mast cells with diminished granule basophilia often were isolated among unlabeled lymphoid cells (Fig. 6). A bursting degranulation of labeled mast cells was not observed.

Large mononuclear cells with heavy ^{3}H concentrations in the nucleus and at the cytoplasmic membrane were detected in autoradiographs of the draining inguinal nodes 1 hour after injection. These cells were comparable in appearance and localization with those observed in regional popliteal nodes. Within 4 hours, the labeled mononuclear cells demonstrated heavy grain localization over both the nucleus and cytoplasm. These cells were found in all of the labeled inguinal node autoradio-

graphs, but were less numerous than in the corresponding regional popliteal nodes.

Right Ventral Axillary Nodes. Autoradiographs revealed no ^{3}H incorporation by cells of the dorsally-located right axillary lymph nodes. Thirty-two of the 52 ventral nodes, however, were found to drain ^{3}H-antigen from the right hind footpad injection site (Table III). Autoradiographs of these nodes yielded results very similar to the inguinal node observations. Between 1 and 24 hours, the proportion of labeled macrophages with heavy ^{3}H concentrations varied from 59 to 95 per cent. Most of the axillary nodes which contained labeled macrophages also demonstrated ^{3}H incorporation by eosinophils, mast cells and large, immature mononuclear cells. All labeled cells were localized in the interfollicular areas and polar regions of the nodes.

The uptake of ^{3}H-antigen by eosinophils was demonstrated in all of the labeled axillary nodes. A 25-fold increase in the mean of labeled eosinophils per section was recorded between 1 and 4 hours (Text-fig. 3). Mature eosinophils with heavy intracytoplasmic concentrations of



TEXT-FIG. 3. Total number of labeled eosinophils and mast cells in the right ventral axillary nodes following primary right hind footpad stimulation with ^{3}H-S-PGG. Each value is the mean count of 4 lymph nodes, 24 sections per node.

^{3}H were especially prominent at 4, 6 and 7 hours. Eosinophilia persisted from 4 through 7 hours. During eosinophilia the mean cell counts per section exceeded by 17 to 33 times the normal mean of 14 (range 1 to 49) eosinophils established from counts of 1,248 right dorsal axillary node sections. Although many of the labeled eosinophils were localized in areas

of labeled macrophages, cell-to-cell contact between eosinophils and macrophages was not prevalent.

The incorporation of ^{3}H-antigen by mast cells was observed in 23 of the 24 labeled lymph nodes studied between 1 and 8 hours (Table III).

TABLE III

^{3}H-ANTIGEN INCORPORATION IN DRAINING VENTRAL AXILLARY LYMPH NODES FOLLOWING PRIMARY FOOTPAD STIMULATION *

Hours after injection †	Cell	Mean cells per section ± S.D. ‡	*Per cent tritiated cells* ± S.D. §			
			Per section	Grain count per cell		
				5-10	10-20	>20
1	Macrophage			19 ± 22	22 ± 17	59 ± 35
	Mast	4 ± 3	3 ± 2	0	0	100
3	Macrophage			3 ± 2	14 ± 4	83 ± 5
	Mast	11 ± 3	6 ± 5	0	100	0
4	Macrophage			3 ± 1	17 ± 4	80 ± 4
	Mast	24 ± 7	43 ± 10	48 ± 22	30 ± 14	22 ± 17
6	Macrophage			2 ± 1	8 ± 4	90 ± 4
	Mast ¶	2 ± 2	22 ± 22	0	78 ± 31	22 ± 22
7	Macrophage			0	6 ± 4	94 ± 4
	Mast	40 ± 13	9 ± 4	14 ± 7	23 ± 11	63 ± 16
8	Macrophage			1 ± 0	6 ± 2	93 ± 2
	Mast	1 ± 1	40 ± 33	100	0	0
12	Macrophage			3 ± 2	8 ± 3	89 ± 5
	Mast	1 ± 0	0			
24	Macrophage			1 ± 0	4 ± 0	95 ± 1
	Mast	1 ± 0	0			

* Each of 52 mice received a subcutaneous injection of 80 μg (14 μc per 0.01 ml) of ^{3}H-S-PGG in the right hind footpad.

† Four right ventral axillary nodes were studied at each time interval. Lymph nodes obtained at 2, 5, 10, 14 and 16 hours contained no labeled macrophages or mast cells.

‡ Labeled macrophages, 500 per section, were scored for intracellular ^{3}H concentration (grain count per cell). The total number of mast cells were counted per section.

§ Values were derived from 96 sections (24 sections per lymph node).

¶ Three of the 4 lymph nodes contained labeled mast cells.

With the exception of the 1- and 7-hour lymph nodes, 78 to 100 per cent of the labeled mast cells possessed light to moderate quantities of intracytoplasmic ^{3}H. The labeled mast cells were similar in appearance and distribution to those observed in the inguinal node autoradiographs. The mean of labeled mast cells per section increased from $<$ 1 at 1 hour to a peak of 31 at 7 hours (Text-fig. 3). The mean of mast cells per section recorded for each of the labeled and unlabeled ventral axillary nodes was either below or comparable with the normal mean of 35 (range 1 to 57) established from counts of the 1,248 unlabeled dorsal axillary node sections.

As in the labeled inguinal nodes, large mononuclear cells with ^{3}H localized in the nucleus and at the cytoplasmic membrane were demonstrated in the ventral axillary nodes at 1 hour. From 3 to 24 hours, all of

the labeled axillary node sections contained a small number of mononuclear cells with heavy ^3H concentrations in both nucleus and cytoplasm. These cells usually were localized in clusters of labeled macrophages and were comparable in appearance with those observed in the draining popliteal and inguinal nodes.

Quantitation of ^3H-Antigen in Draining Lymph Nodes

The quantitative distribution of ^3H-antigen in the 312 lymph nodes in group 1 was determined in a liquid scintillation system. Following autoradiograph preparations, each remaining lymph node fragment was deparaffinized, dried, weighed, dissolved and counted. Since only fragments of the tissues remained after sectioning, the per cent of injected ^3H antigen localized in each lymph node could not be determined. After quantitation of the lymph node radioactivities, however, comparative results were expressed as mμc of ^3H per mg dry weight of tissue. Assuming that each tissue radioactivity represented ^3H-antigen, the amount of ^3H-S-PGG was calculated from the ^3H concentration (Table IV).

TABLE IV

QUANTITATIVE DISTRIBUTION OF ^3H-ANTIGEN IN DRAINING LYMPH NODES FOLLOWING PRIMARY RESPONSE STIMULATION IN RIGHT HIND FOOTPADS *

Hours after injection	Amount of ^3H-S-PGG per mg dry weight of tissue †		
	Popliteal node	Inguinal node	Ventral axillary node
	($\mu g \times 10^{-2}$)	($\mu g \times 10^{-2}$)	($\mu g \times 10^{-2}$)
1	674.42	8.43	30.54
2	99.72	1.46	0
3	155.31	3.98	3.84
4	72.96	7.36	0.87
5	161.85	0	0
6	562.14	5.12	14.10
7	140.76	13.11	7.20
8	17.89	1.89	0.63
10	124.79	0.17	0.06
12	52.49	1.40	1.00
14	326.48	0.04	0.07
16	20.29	0	0
24	39.50	5.74	9.65

* Each of 52 mice received a subcutaneous injection of 80 μg (14 μc per 0.01 ml) of ^3H-S-PGG.

† Calculated from scintillation counts of ^3H in dissolved tissues and from the activity of the ^3H-antigen (175 mμc per μg). Each value is the mean of 4 lymph node determinations.

Highest quantities of ^3H-antigen were incorporated by cells of the regional popliteal nodes between 1 and 14 hours after injection. During this interval the mean amount of localized antigen varied over a wide range, but usually measured over 1 μg per mg of tissue. Notable exceptions occurred at 4, 8 and 12 hours. The mean quantities of antigen re-

tained in the popliteal nodes at 16 and 24 hours had decreased to 3.0 and 5.8 per cent, respectively, of the 1-hour mean.

All regional popliteal nodes possessed greater quantities of ^{3}H-antigen than the corresponding right inguinal and ventral axillary nodes (Table IV). The amounts of antigen localized in the inguinal nodes usually exceeded the antigen concentrations in the corresponding ventral axillary nodes, with exceptions at 1, 6, 14 and 24 hours. The mean amounts of antigen retained in the 24-hour inguinal and axillary nodes were 68.0 and 31.6 per cent, respectively, of their 1-hour mean concentrations. Some mice failed to localize ^{3}H-antigen in the right inguinal-axillary lymphatic system. These results probably reflected anatomic variation in the route of antigen transport from the footpad injection site. No ^{3}H was detected in the control contralateral popliteal nodes, the left inguinal nodes or the right dorsal axillary nodes in the 52 group I mice.

Serum Antibody Formation Following Primary Response Stimulation

Serums collected from all mice in groups I and III contained no antibodies specific for the sulfanilazo determinant or PGG when titrated with the 2 passive HA techniques. The 14 mice in group II were studied for serum antibody formation from days 1 through 14 following footpad stimulation with ^{3}H-S-PGG. No antibodies specific for the sulfanilazo determinant or PGG were detected prior to day 9. The 6 mice studied on days 9, 12 and 14 demonstrated titers of 1:2 against PGG. Four of these 6 mice yielded titers of 1:4 and 2 mice exhibited titers of 1:8 against the sulfanilazo determinant. These results were indicative of primary responses in the 3 experimental mouse groups.

Discussion

The present experiments have employed a soluble protein conjugate possessing heavily tritiated sulfanilazo determinants (175 μc of ^{3}H per mg) to quantitate the incorporation of antigen or antigenic fragments into lymphoid cells. The localization of high ^{3}H concentrations in macrophages, eosinophils, mast cells and immature mononuclear cells was observed in draining lymph nodes between 1 and 24 hours following primary stimulation in the footpads of mice. Assuming that the incorporation of antigen or active antigenic fragments by immunologically competent cells initiates antibody formation,[3,4] the present results have implicated one or more of these 4 cell types in mechanisms of antibody synthesis.

Nossal and co-workers[6-8] demonstrated the rapid uptake of ^{125}I and ^{131}I labeled flagellar antigens by macrophages of medullary sinuses and lymphoid follicles following primary footpad immunization of rats. In

contrast with the present results, their autoradiographs did not detect the labeled soluble and particulate antigens in eosinophils, mast cells and immature mononuclear cells during the primary immune response. These differing lymphoid cell activities may have been due in part to the high immunogenicity of the flagellar antigens.[6–8] In addition, although minute quantities of ^{125}I and ^{131}I labeled flagellar antigens have provided very high sensitivity in autoradiographs, the association of radioactivity with antigenic determinants has not been defined.[6–9] The presence or absence of radioactivity, therefore, may not have reflected the fate of all immunogenic fragments.

Early observations by Leduc, Coons and Connolly[32] using fluorescence methods detected soluble protein antigens in interfollicular medullary phagocytes of draining popliteal nodes after primary stimulation of rabbit footpads. The localization of soluble, polymerized and particulate antigens in macrophages during primary, secondary and hyperimmune responses has been described repeatedly.[1,6–14,17–20] In the present investigation, the efficient incorporation of ^{3}H-S-PGG (or fragments containing the ^{3}H-determinants) by draining lymph node macrophages was an expected result. The localization of labeled macrophages in the interfollicular medullary areas, interfollicular cortex and polar regions of the nodes corresponded with previously reported observations.[6–8,19,32] *In vitro* and *in vivo* studies have suggested both direct and indirect functions of macrophages in mechanisms of antibody synthesis.[15–18] Fishman and Adler[15] defined a critical role of macrophage RNA in the initiation of antibody formation. Askonas and Rhodes[18] demonstrated that antigen-sensitized mouse macrophages did not form specific new informational RNA, but yielded RNA-antigen complexes which were more highly immunogenic than the antigen alone. The function of macrophages in the degradation of antigens and in the preparation and transfer of immunogenic fragments has been suggested by several investigators.[12–14,17,18] The present experimental results are compatible with these views. In contrast, recent evidence has indicated that macrophages are not essential for antibody formation, and that their phagocytic properties destructively compete with an immune response.[33]

In the present study, ^{3}H-antigen was detected in eosinophils in draining lymph nodes 1 hour following primary stimulation. Subsequent evidence of eosinophilia was observed consistently in all labeled lymph nodes. Previous investigations emphasized the role of antigen-antibody complexes in eosinophil responses in guinea pigs.[34,35] Recent evidence has indicated that soluble antigen (or antigenic fragments) also is incorporated by mouse lymph node eosinophils, stimulating eosinophilia in the absence of specific antibodies.[28] The present data yield further

support for the role of antigen in the eosinophil response. Autoradiographs did not reveal a prevalent association between ^{3}H-labeled eosinophils and macrophages. In addition, no evidence was obtained for the phagocytosis of labeled eosinophils or eosinophil fragments by macrophages. Both phenomena were demonstrated in autoradiographs of peritoneal exudate smears from immunized mice after restimulation with ^{3}H-tetanus toxin.[13,14] It is probable that the peritoneal exudate cell interactions occurred in response to the defined experimental conditions, and did not reflect mechanisms of primary antigenic stimulation in lymphoid tissues. Because of the deficiency of RNA in eosinophils, it is difficult to propose a function of these cells in mechanisms of antibody synthesis. As in the function of neutrophils in the phagocytosis of foreign particulate antigens, however, the present results have emphasized the importance of eosinophils in the rapid uptake of a soluble antigen during the primary response.

Mast cells have not been implicated previously in mechanisms of the primary response. The present autoradiographic studies revealed the intracytoplasmic localization of ^{3}H-antigen in lymph node mast cells within 1 hour post-injection. A subsequent increase in mast cells occurred with 99, 99 and 79 per cent of cells labeled at peak response in the draining popliteal, inguinal and ventral axillary nodes, respectively. In the popliteal nodes, labeled mast cells were concentrated within clusters of heavily labeled macrophages, suggesting the transformation of macrophage to mast cell. The mast cells could have accumulated, however, through migratory processes. Burnet[36] has emphasized the possibility that mast cells arise by direct transformation from small or medium lymphocytes. He also proposed that the stimulus for mast cell accumulation is a secondary result of lymphocyte-antigen contact, and that mast cells remain immunologically competent. In the present study, small lymphocytes were consistently unlabeled in all labeled lymph nodes, indicating the absence of small lymphocyte-antigen contact. Thus, it is unlikely that the small lymphocytes were transformed into mast cells. The possibility remains that the numerous labeled immature mononuclear cells were primitive stem cells capable of transformation into mast cells. Ginsburg and Sachs[37] have observed the change of mouse thymus stem cells to mast cells in *in vitro* cultures.

The loss of basophilia in labeled mast cells indicated an alteration in the granule RNA in response to the intracytoplasmic localization of immunogen. Although the labeled mast cell responses were brief, these results suggest a possible function of mast cells in mechanisms of antibody synthesis. The bursting degranulation of mast cells characteristic of antigen-antibody reaction[38] was not observed, correlating with the absence of specific circulating antibodies during this 24-hour study.

Mouse footpad stimulation with ^{3}H-S-PGG led to the rapid appearance in draining lymph nodes of large immature mononuclear cells possessing heavy intracytoplasmic and intranuclear ^{3}H concentrations. Their intranuclear antigen-chromatin contact indicated a highly probable association of these cells with early mechanisms of antibody formation. These results also suggested that the immature cells were primitive precursors (stem cells) of immunologically competent cells. The present observations of intranuclear antigen localization agree closely with the results of Wellensiek and Coons,[19] who demonstrated intact antigen (ferritin) in the nuclei and cytoplasm of phagocytic sinus and reticular cells of rabbits during the primary response.

In addition to the regional popliteal nodes, the draining inguinal and ventral axillary nodes in many experimental mice functioned importantly in antigen localization. Although the popliteal nodes trapped much higher concentrations of antigen than the corresponding inguinal and axillary nodes, the latter 2 nodes demonstrated higher percentage levels of antigen retention. Patterns of intracellular antigen localization and labeled cell distribution in the 3 lymph nodes were comparable, with only slight time variations in the peak eosinophil and mast cell responses. Small and medium lymphocytes and neutrophils were consistently unlabeled throughout the study interval.

Summary

Autoradiography and scintillation counting techniques were used to investigate the intracellular and tissue localization of a soluble ^{3}H-azoprotein antigen in mouse lymph nodes following primary response stimulation. Each of 52 mice received a single right hind footpad injection of 80 μg(14 μc) of ^{3}H-sulfanilazo porcine gamma globulin. The intracellular localization of ^{3}H-antigen was quantitated in the draining popliteal, inguinal and ventral axillary lymph nodes at 13 intervals during the first 24 hours post-injection. Since only the sulfanilazo determinants were tritiated, the localized radioactivity represented intact antigen or immunogenic antigen fragments.

Intracellular ^{3}H concentrations were quantitated in 12,000 labeled macrophages per lymph node and in all labeled eosinophils and mast cells observed in the lymph node autoradiographs. The labeled cells were localized primarily in the medullary regions, interfollicular cortex and polar regions of the nodes. Macrophages incorporated heavy intracytoplasmic concentrations of ^{3}H throughout the study interval. Almost all labeled lymph nodes demonstrated ^{3}H in the cytoplasm of eosinophils and mast cells. The early incorporation of ^{3}H-antigen by eosinophils induced subsequent eosinophilia with peak responses at 8, 7 and 4 hours in the popliteal, inguinal and ventral axillary nodes, respectively. The up-

take of ^{3}H-antigen by mast cells was associated with a pronounced increase in mast cell counts per section in the popliteal and inguinal nodes. The mast cells containing ^{3}H demonstrated a decreased basophilia, indicating an alteration of granule RNA in response to the intracytoplasmic localization of immunogen. Large immature mononuclear cells possessing heavy intracytoplasmic and intranuclear ^{3}H concentrations were also observed in all labeled lymph nodes. The intranuclear localization of ^{3}H indicated an antigen-chromatin contact and suggested the participation of these cells in early mechanisms of antibody formation.

The quantitation of radioactivity per mg dry weight of each tissue detected highest concentrations of ^{3}H-antigen in the regional popliteal nodes. The draining inguinal and ventral axillary nodes in many mice also functioned importantly in antigen uptake and retention. The significance of these observations and the probable functions of the labeled cell types in mechanisms of the primary immune response have been discussed.

References

1. Nossal, G.J.V. Cellular genetics of immune responses. *Advances Immun.*, 1962, 2, 163–204.
2. Thorbecke, G. J., and Benacerraf, B. The reticulo-endothelial system and immunological phenomena. *Progr. Allerg.*, 1962, 6, 559–598.
3. Campbell, D. H., and Garvey, J. S. Nature of retained antigen and its role in immune mechanisms. *Adv. Immun.*, 1963, 3, 261–313.
4. Haurowitz, F. Antibody formation. *Physiol. Rev.*, 1965, 45, 1–47.
5. Gowans, J. L., and McGregor, D. D. The immunological activities of lymphocytes. *Progr. Allerg.*, 1965, 9, 1–78.
6. Nossal, G.J.V.; Ada, G. L., and Austin, C. M. Behavior of active bacterial antigens during the induction of the immune response. II. Cellular distribution of flagellar antigens labelled with iodine-131. *Nature (London)*, 1963, 199, 1259–1262.
7. Nossal, G.J.V.; Ada, G. L., and Austin, C. M. Antigens in immunity. IV. Cellular localization of ^{125}I- and ^{131}I-labelled flagella in lymph nodes. *Aust. J. Exp. Biol. Med. Sci.*, 1964, 42, 311–330.
8. Ada, G. L.; Nossal, G.J.V., and Austin, C. M. Antigens in immunity. V. The ability of cells in lymphoid follicles to recognize foreignness. *Aust. J. Exp. Biol. Med. Sci.*, 1964, 42, 331–346.
9. Nossal, G.J.V.; Ada, G. L., and Austin, C. M. Antigens in immunity. IX. The antigen content of single antibody-forming cells. *J. Exp. Med.*, 1965, 121, 945–954.
10. Roberts, A. N., and Haurowitz, F. Intracellular localization and quantitation of tritiated antigens in reticuloendothelial tissues of mice during secondary and hyperimmune responses. *J. Exp. Med.*, 1962, 116, 407–422.
11. Roberts, A. N. Quantitative cellular distribution of tritiated antigen in immunized mice. *Amer. J. Path.*, 1964, 44, 411–430.
12. Speirs, R. S. Antigenic material: persistence in hypersensitive cells. *Science*, 1963, 140, 71–72.

13. SPEIRS, R. S., and SPEIRS, E. E. Cellular localization of radioactive antigen in immunized and nonimmunized mice. *J. Immun.*, 1963, **90**, 561–575.

14. SPEIRS, R. S., and SPEIRS, E. E. Cellular reactions to reinjection of antigen. *J. Immun.*, 1964, **92**, 540–549.

15. FISHMAN, M., and ADLER, F. L. Antibody formation initiated *in vitro*. II. Antibody synthesis in x-irradiated recipients of diffusion chambers containing nucleic acid derived from macrophages incubated with antigen. *J. Exp. Med.*, 1963, **117**, 595–602.

16. HULLEY, S. B.; DVORAK, H. F., and WAKSMAN, B. H. Effect of macrophages on primary immunization of lymph node cells against homografts in diffusion chambers. *Proc. Soc. Exp. Biol. Med.*, 1964, **117**, 110–115.

17. GILL, F. A., and COLE, R. M. The fate of a bacterial antigen (streptococcal M protein) after phagocytosis by macrophages. *J. Immun.*, 1965, **94**, 898–915.

18. ASKONAS, B. A., and RHODES, J. M. Immunogenicity of antigen-containing ribonucleic acid preparations from macrophages. *Nature (London)*, 1965, **205**, 470–474.

19. WELLENSIEK, H. J., and COONS, A. H. Studies on antibody production. IX. The cellular localization of antigen molecules (ferritin) in the secondary response. *J. Exp. Med.*, 1964, **119**, 685–696.

20. DE PETRIS, S., and KARLSBAD, G. Localization of antibodies by electron microscopy in developing antibody-producing cells. *J. Cell Biol.*, 1965, **26**, 759–778.

21. MOVAT, H. Z., and FERNANDO, N.V.P. The fine structure of the lymphoid tissue during antibody formation. *Exp. Molec. Path.*, 1965, **4**, 155–188.

22. UHR, J. W., and FINKELSTEIN, M. S. Antibody formation. IV. Formation of rapidly and slowly sedimenting antibodies and immunological memory to bacteriophage ϕX 174. *J. Exp. Med.*, 1963, **117**, 457–477.

23. SCHECHTER, I. Competition of antigenic determinants. *Biochim. Biophys. Acta*, 1965, **104**, 303–305.

24. NOSSAL, G.J.V.; MITCHELL, J., and MCDONALD, W. Autoradiographic studies on the immune response. IV. Single cell studies on the primary response. *Aust. J. Exp. Biol. Med. Sci.*, 1963, **41**, suppl., 423–436.

25. UHR, J. W.; FINKELSTEIN, M. S., and BAUMANN, J. B. Antibody formation, III. The primary and secondary antibody response to bacteriophage ϕX 174 in guinea pigs. *J. Exp. Med.*, 1962, **115**, 655–670.

26. NOSSAL, G.J.V.; SZENBERG, A.; ADA, G. L., and AUSTIN, C. M. Single cell studies on 19S antibody production. *J. Exp. Med.*, 1964, **119**, 485–502.

27. LANDY, M.; SANDERSON, R. P., and JACKSON, A. L. Humoral and cellular aspects of the immune response to the somatic antigen of *Salmonella enteritidis*. *J. Exp. Med.*, 1965, **122**, 483–504.

28. ROBERTS, A. N. Rapid uptake of tritiated antigen by mouse eosinophils. *Nature (London)*, 1966, **210**, 266–269.

29. SAINTE-MARIE, G., and COONS, A. H. Studies on antibody production. X. Mode of formation of plasmocytes in cell transfer experiments. *J. Exp. Med.*, 1964, **119**, 743–760.

30. LITT, M. Studies in experimental eosinophilia. V. Eosinophils in lymph nodes of guinea pigs following primary antigenic stimulation. *Amer. J. Path.*, 1963, **42**, 529–549.

31. NACHTIGAL, D., and FELDMAN, M. The immune response to azo-protein conjugates in rabbits unresponsive to the protein carriers. *Immun.*, 1964, **7**, 616–625.

32. LEDUC, E. H.; COONS, A. H., and CONNOLLY, J. M. Studies on antibody production. II. The primary and secondary responses in the popliteal lymph node of the rabbit. *J. Exp. Med.*, 1955, **102**, 61–72.

33. PERKINS, E. H., and MAKINODAN, T. The suppressive role of mouse peritoneal phagocytes in agglutinin response. *J. Immun.*, 1965, **94**, 765–777.

34. LITT, M. Studies in experimental eosinophilia. III. The induction of peritoneal eosinophilia by the passive transfer of serum antibody. *J. Immun.*, 1961, **87**, 522–529.

35. LITT, M. Studies in experimental eosinophilia. VI. Uptake of immune complexes by eosinophils. *J. Cell Biol.*, 1964, **23**, 355–361.

36. BURNET, F. M. Mast cells in the thymus of NZB mice. *J. Path. Bact.*, 1965, **89**, 271–284.

37. GINSBURG, H., and SACHS, L. Formation of pure suspensions of mast cells in tissue culture by differentiation of lymphoid cells from the mouse thymus. *J. Nat. Cancer Inst.*, 1963, **31**, 1–39.

38. CARTER, P. B.; HIGGINBOTHAM, R. D., and DOUGHERTY, T. F. The local response of tissue mast cells to antigen in sensitized mice. *J. Immun.*, 1957, **79**, 259–264.

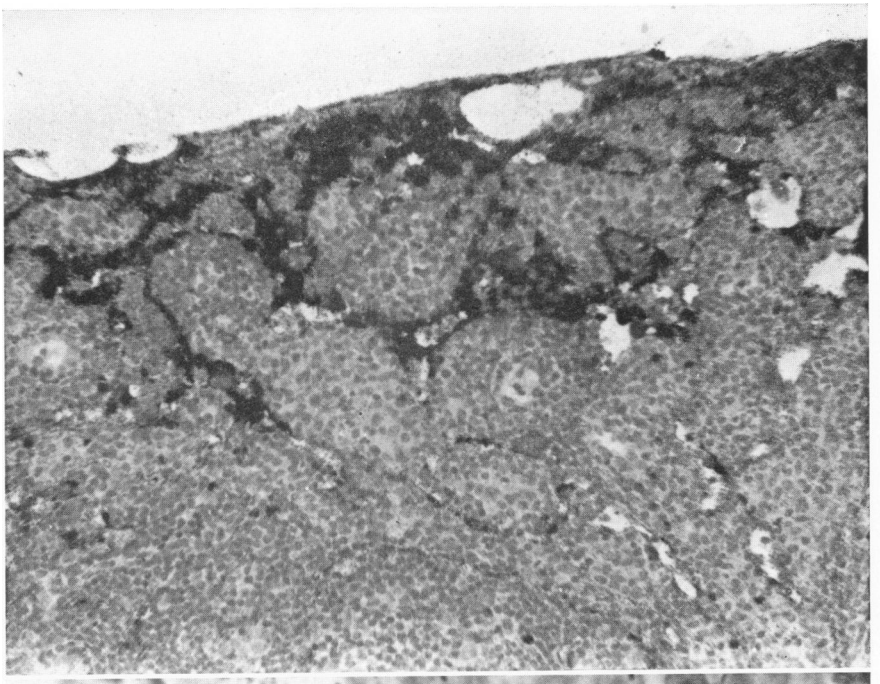
It is a pleasure to acknowledge my gratitude to Mrs. Janelle Shaw Hunt and Mrs. Louise Boring for excellent and devoted technical assistance.

LEGENDS FOR FIGURES

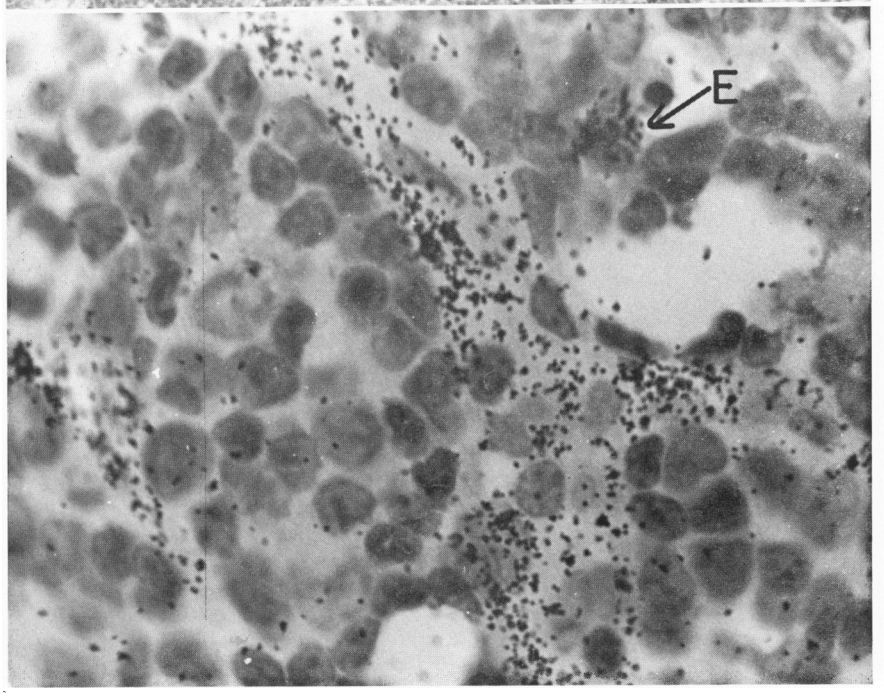
Autoradiographs were prepared from mouse lymph nodes at various intervals following primary response stimulation with ^{3}H-antigen. All lymph nodes drained the right hind footpad injection site, and the times cited below are the intervals between immunization and tissue collection. Except where otherwise indicated, the autoradiographs are stained with hematoxylin and eosin and magnifications are × 1,000.

FIG. 1. Right popliteal node 7 hours after injection. Heavily labeled cells exhibit an interfollicular distribution. × 160.

FIG. 2. A right popliteal node at 2 hours demonstrates macrophages with heavy intracytoplasmic ^{3}H concentrations. A single heavily labeled eosinophil (E) is noteworthy.

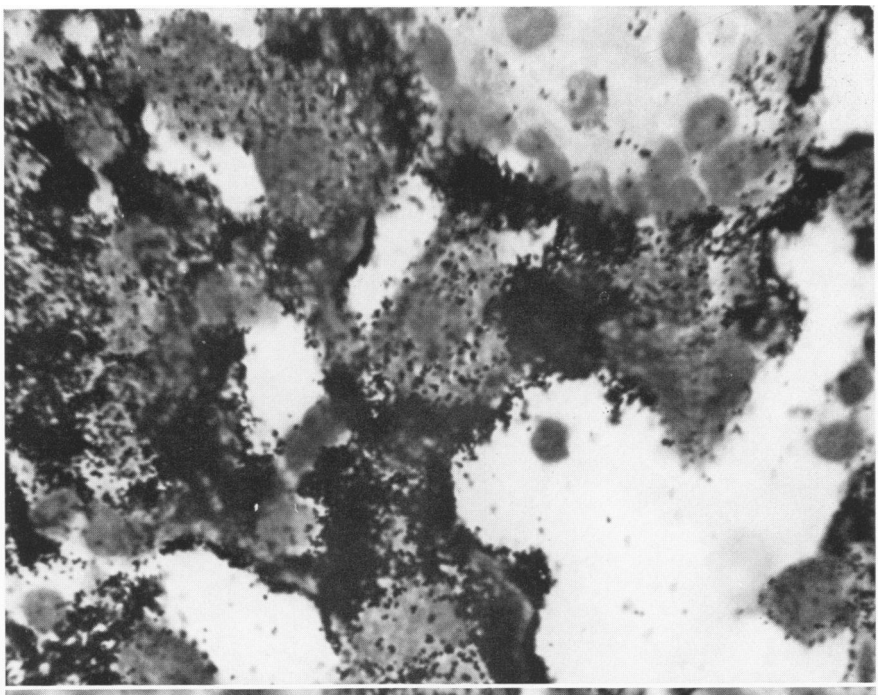


1



2

3



4

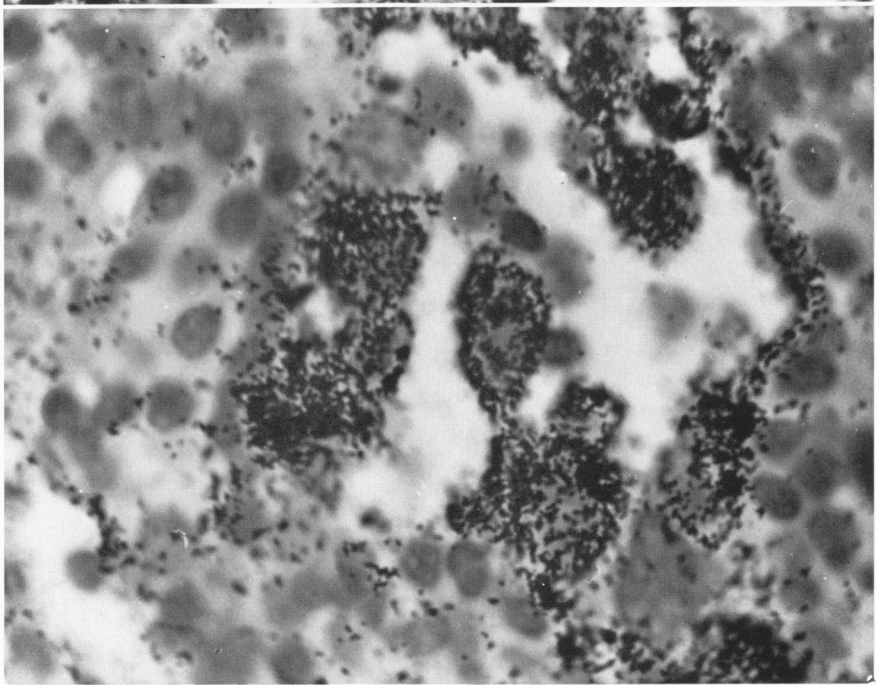
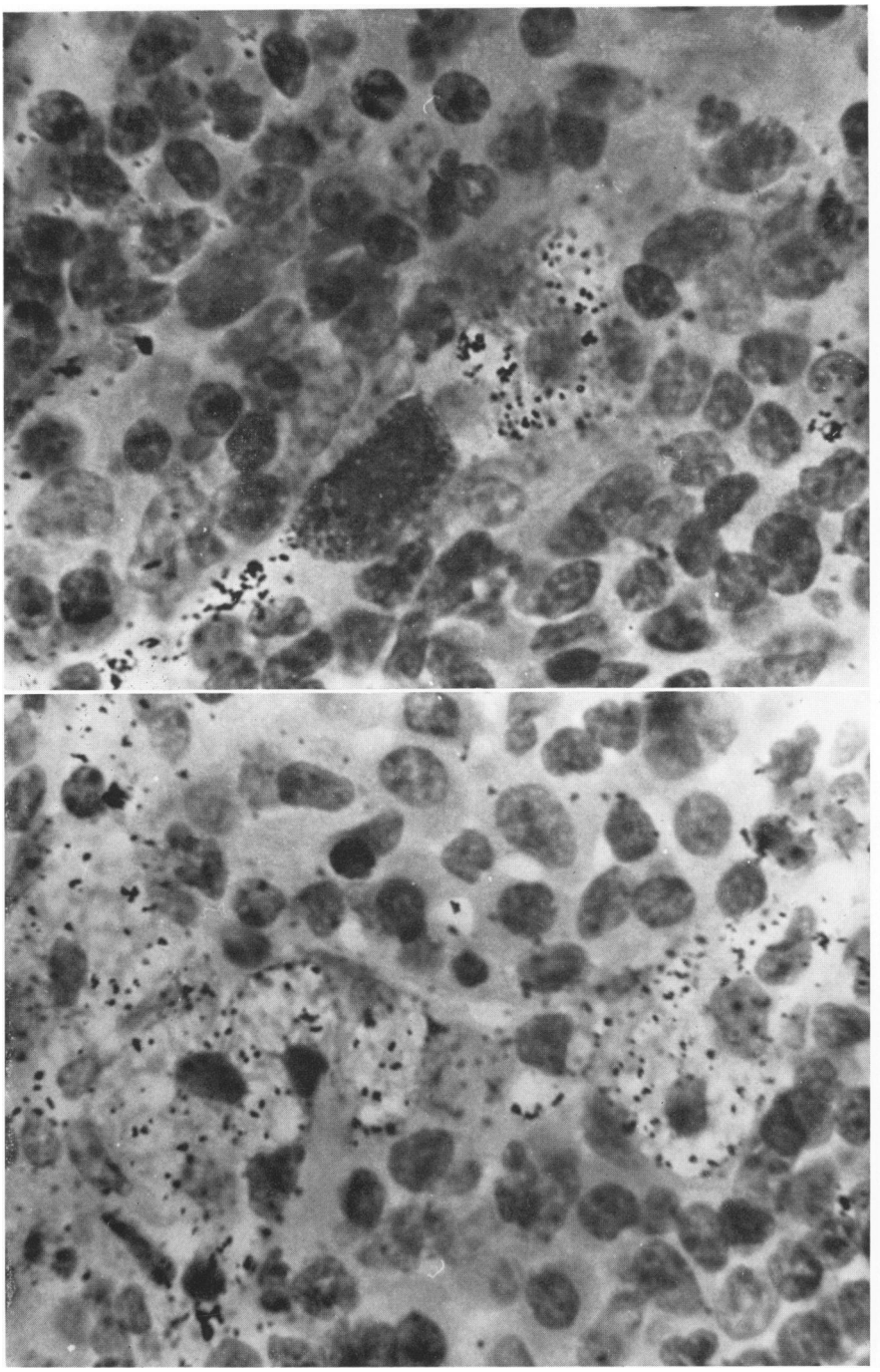


FIG. 3. Labeled mast cells cluster in contact with heavily labeled macrophages in a popliteal node section 7 hours after injection.

FIG. 4. Numerous mononuclear cells contain heavy intracytoplasmic and intranuclear ^{3}H concentrations. Popliteal node, 7 hours after injection.



5

6

FIG. 5. A heavily labeled mast cell demonstrates loss of granule basophilia. The cell lies adjacent to an unlabeled mast cell with normal appearance. Inguinal node, 7 hours after injection.

FIG. 6. A group of heavily labeled mast cells is distributed in an otherwise unlabeled field of lymphoid cells. All labeled mast cells exhibit a loss of granule basophilia. Right inguinal node, 7 hours after injection.