HISTOCHEMICAL CHANGES IN LYMPHOCYTES DURING THE PRODUCTION OF ANTIBODIES IN LYMPH NODES OF RABBITS*

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Plate 8

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The studies of McMaster *et al.* in 1936 (1, 2) provided the first experimental evidence that the lymphatic system might be a site of formation of antibodies, in contrast to the view generally held at the time that the reticuloendothelial system formed these substances (3). Ehrich and Harris (4) confirmed and amplified these findings, including studies of the cell content and antibodies in the lymph entering and emerging from the sole lymph node draining the site of injection of antigen in the rabbit. Again, Dougherty, Chase, and White (5) minced pooled lymph nodes from mice injected with antigens, and prepared a cell mass rich in lymphocytes from which they extracted antibodies to the antigen used. Harris *et al.* (6) gave further evidence of the association of the lymphocyte with the production of antibodies by finding these substances in higher concentration in lymphocytes collected from lymph as it emerged from a lymph node draining the site of injection of antigen.

Other cells of mesodermal origin have, however, been regarded as possible sources of antibodies. Thus, the macrophages of the reticuloendothelial system were proposed in consequence of the work of Sabin referred to above (3). The plasma cell has been suggested as having such a function by Scandinavian workers. Bjørneboe and Gormsen observed proliferation of plasma cells in the spleen and other organs of rabbits which had received repeated injections of massive doses of bacterial cells (7), and extracted antibodies from some of these sites (8). Fagraeus (9) explanted red and white splenic pulp from rabbits similarly treated, and found more antibody in explants of white pulp, which she observed to be relatively richer in plasma cells.

The recent applications of histochemistry to physiology have offered an approach to the problem of the direct identification of cells concerned with the production of antibodies. It was first shown by Caspersson (10, 11) and Brachet (12) that cells which were actively forming new protein were characterized by large amounts of ribonucleic acid in their cytoplasm. Additional evidence for the association of cytoplasmic ribonucleoprotein with protein synthesis has been reviewed by Greenstein (13) and by Dempsey and Wislocki (14).

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The application of the work just summarized to the present problem is as follows:—

There is a considerable body of evidence for the production of antibodies in the popliteal lymph node of the rabbit after the injection of antigenic material into the tissue drained by that node (4, 15–17). Since a tissue in which antibodies are being formed could be expected to show evidences of protein synthesis in the cells producing those antibodies, it seemed feasible to attempt to identify the cells involved by histochemical methods. Following the injection of various antigens and control materials into the foot-pads of rabbits, histologic study was made of sections stained with methyl green and pyronine, and chemical determinations of ribonucleic acid and desoxyribonucleic acid were carried out. These determinations were correlated with the concentration of antibody in the lymph node.

Materials and Methods

Preparation of Antigens and Other Materials for Injection.—Dysentery bacilli (*Shigella paradysenteriae*) were cultivated overnight, on tryptose agar, washed, killed with 70 per cent ethyl alcohol, and injected at a concentration of 0.5 mg. of dry weight per cc. (15). Erythrocytes derived from sheep and rabbits were also washed in saline solution and suspended at a concentration of 50 per cent for injection (4). Preparations of influenza virus were made by inoculating 10-day-old chick embryos with 0.2 cc. of a 10^{-5} dilution of the respective seed cultures. After 48 hours of incubation at 37°C., the allantoic fluids were harvested, subjected to centrifugation at 20,000 R.P.M. for 20 minutes, and the sediments were resuspended in sterile buffered physiological saline solution in 1/10th of the original volume. The concentrated virus was inactivated by exposure to ultraviolet rays for 10 minutes (17). In the case of all these preparations of antigenic material injections into the hind foot-pads of rabbits were made at a volume of 0.2 cc. The lanolin-mineral oil suspension which was used as a non-antigenic but irritating stimulus was prepared as suggested by Freund and Bonanto (18), using sterile buffered isotonic saline solution as the aqueous phase, rather than a suspension of an antigen. Gelatin was used as a non-antigenic protein substance in a sterile 10 per cent solution.

Injection of Materials.—All injections were made into the pads of the hind feet of rabbits, in volumes of 0.2 cc. of sterile material. Injections were paired for reasons of special interest. Thus a heterologous tissue such as sheep erythrocytes would be injected opposite a similar homologous tissue, rabbit erythrocytes, to add weight to the contrasting results. Again, antigenic stimuli would be given in legs opposite to those receiving non-antigenic material. Finally, to lend additional validity to comparisons, a given antigen was injected opposite different antigens in different experiments. Thus the dysentery bacilli were used opposite streptococcal cytoplasmic particles (19), isotonic saline solution, and sheep erythrocytes, respectively, in the three series of rabbits yielding the results given in Table I. Three series of rabbits were, in general, used for each material injected. In the case of non-antigenic control materials, this number was generally two. At least one of the latter was included in each experiment.

Collection of Specimens.—Rabbits were sacrificed at 0, 2, 3, 4, 5, 7, and 9 days after the injection of antigens. Blood was obtained for the preparation of serum and the popliteal lymph nodes were removed immediately. These were weighed and then divided. A central slice of tissue was fixed in Serra solution for 15 minutes, then washed several times in 70 per

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cent alcohol, and stored in the latter solution until histologic preparations were made. The remainder was weighed and frozen for subsequent extraction. The extracts were made by sand-grinding the specimens in a mortar, with 15 volumes of isotonic saline solution, clearing away the debris by centrifugation. This extract was used for serologic and chemical analysis. Sections of all specimens were stained with Azur II-eosin and with methyl green–pyronine. Unstained sections adjoining those studied were prepared from each block for experiments such as those with ribonuclease and hot trichloracetic acid.

TABLE I

Determination of Concentrations of Nucleic Acids and Antibodies in the Popliteal Lymph Node Following Injection of Dysentery Bacilli

Experiment No.	Time after injection	Agglutinin titer	Ribonucleic acid per gm. of fresh node	Desoxyribonucleic acid per gm. of fresh node	Weight of lymph node
	days		*mg.*	*mg.*	*mg.*
HC 8	0	<24	3.7	7.8	184
	2	<24	3.6	7.4	383
	3	48	6.2	6.9	164
	4	384	6.7	8.2	559
	5	1536	7.7	7.9	755
	7	768	5.6	8.9	578
	9	1536	3.9	7.6	737
HC 9	0	<24	3.8	8.3	148
	2	<24	4.3	7.2	327
	3	48	7.0	8.9	399
	4	384	6.9	6.9	598
	5	1536	7.6	7.4	483
	7	768	5.9	7.2	780
	9	768	4.2	6.8	1090
HC 10	0	<24	4.2	6.6	178
	2	<24	4.1	8.4	189
	3	96	6.5	7.7	265
	4	384	7.3	7.7	192
	5	1024	7.4	6.3	394
	7	1024	6.2	8.4	798
	9	512	4.6	7.9	563

Chemical Methods.—The extracts of tissues were prepared according to the scheme suggested by Schneider (20, 21). The hot extract of trichloracetic acid was analyzed for its content of desoxyribonucleic acid by the method of Dische (22) and for ribonucleic acid by Mejbaum's (23) adaptation of the Bial reaction. A few repetitions of fractionations of tissue extracts by the method of Schmidt and Thannhauser (24) yielded results which were sufficiently similar to those by the Schneider method to lead us to use the latter exclusively thereafter. In the standardization of the methods it was found that desoxyribonucleic acid gave a reaction with orcinol equal to the color given by one-tenth that amount of ribonucleic acid (24). Accordingly all concentrations of ribonucleic acid are reported here following the

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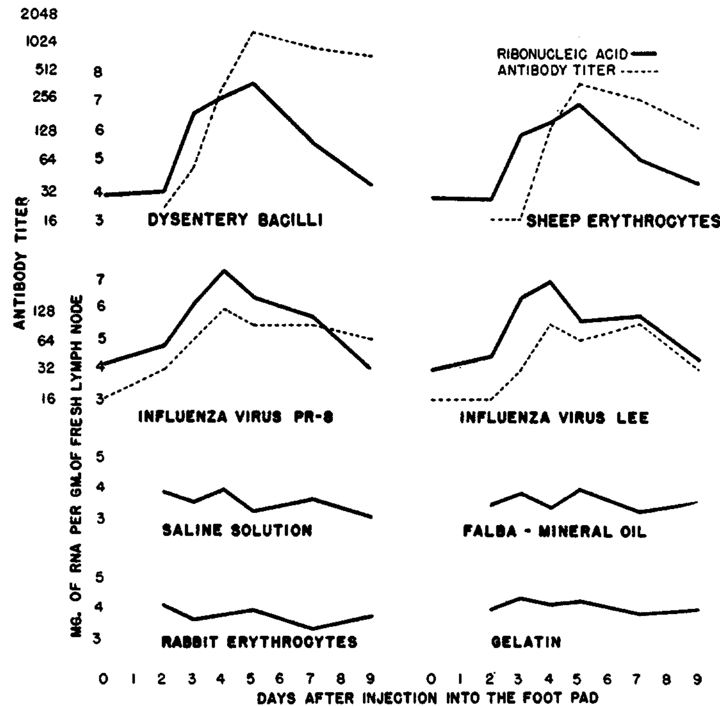
subtraction of one-tenth the concentration of desoxyribonucleic acid found in the same specimen. (There is no analogous interference in the analysis for desoxyribonucleic acid.)

Serologic Methods.—Serologic tests were performed to measure the concentration of antibodies to each antigen, according to procedures described previously (4, 15, 17). These tests were controlled by simultaneous tests of the extracts of lymph nodes with the antigens injected into the opposite legs. Of course no serologic tests were possible in the case of the



TEXT-FIG. 1. Ribonucleic acid concentration and antibody titer in lymph nodes draining the site of injection of materials noted. Each point represents a mean of specimens obtained from two or three rabbits. Falba is the name of the lanolin preparation used with mineral oil.

materials indicated in the lower half of Text-fig. 1. Hemolysin for rabbit erythrocytes was sought in extracts of lymph nodes injected with these cells, and a complement fixation test was carried out between gelatin and the corresponding lymph node extract. These were, of course, entirely negative.

RESULTS

Histologic Observations.—Preliminary observations were made, for orientation, on sections stained with Azur II and eosin. It was found that lymph nodes excised after successively increasing numbers of days following the injection of antigen distal to them were progressively larger. This increase in size was due almost entirely to an increase in the width of the cortex, the me-

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dulla of the node remaining approximately constant in size. Two days after the injection of antigen in the distal tissue the cortex of the lymph node presented a picture of intense, diffuse hyperplasia by young lymphocytes interspersed with reticulum cells. This hyperplasia was so intense as to obscure the organization of the lymph node, and continued with even greater intensity into the 3rd day after the injection. On the 4th day, a few mature, small, lymphocytes were seen, as well as the beginnings of organization of the cortex of the lymph node into follicles. Both of these changes continued into the 5th day, while the number of free reticulum cells became progressively smaller. On the 6th day, the follicular structure of the node was clearly established and the majority of the lymphocytes were of the small, mature type.

Similar series of sections were examined after being stained with methyl green and pyronine.

In sections of lymph nodes excised from uninjected animals, the tissue was stained almost entirely with methyl green. The nuclei of the lymphocytes in the cortex were most deeply stained, although these structures presented a less dense appearance than with Azur II-eosin stain. The cytoplasm of all cells was seen to have absorbed hardly any of either methyl green or pyronine, and was scarcely discernible. Occasional cells exhibited a rather pale pink cytoplasm, indicating some absorption of pyronine, and a very few cells contained granules stained with pyronine. Masses of cellular debris faintly stained with pyronine were also found occasionally.

In the specimens of lymph nodes obtained 2 days after the injection of antigen into the distal tissue, a number of cells could be found scattered throughout the masses of diffusely proliferating cells whose cytoplasm contained numbers of sharply defined granules deeply stained with pyronine. These cells had large vesicular nuclei and resembled the reticulum cells stained by this technic which had been seen in sections of normal lymph nodes. On the 3rd day after the injection of antigen these large cells were quite numerous and were again scattered throughout the as yet unorganized mass of young lymphocytes which comprised the cortex of the lymph node at this time. A number of these cells had crescents or rings of cytoplasm filled with red granules. In some cases these had fused into a solid crescent of the deeply red-stained material. Many of these cells showed sharply outlined nucleoli, also deeply stained with pyronine. On the 4th day, the number of cells with the pyronine-stained elements mentioned above had increased, and many of these cells had nuclei sufficiently smaller and less vesicular than the cells described on earlier days as to be indistinguishable from the young, or large, lymphocytes about them. By the 5th day after injection almost all the reticulum cell-like cells with red-staining structures had disappeared, and the great majority of cells with red nuclei and red cytoplasmic granules and crescents were indistinguishable from the lymphocytes about them, although the nuclei of the former were, in many cases, somewhat larger. At this time, as noted above, the follicular organization of the enlarged cortex was quite distinct, and under low magnification it could be seen that the cells with pyronine-stained structures were rather uniformly scattered throughout these new follicles, in greater or less profusion. At this day, some cells could be seen near which lay a red-stained cytoplasmic mass either granular or solid. These masses appeared to have become detached from their parent cells. On the 6th day, more of the cells with red nucleoli and granules had the smaller, more pyknotic nuclei characteristic of mature lymphocytes, and more of the pyronine-stained material was lying free of its parent cell. The total amount of pyronine-stained material in the section was, however, smaller, and this decrease continued

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until the 9th day, when the lymph node, except for the greater width and more distinct follicular structure of its cortex, resembled the picture found in uninjected animals. Some examples of these histologic findings have been illustrated in Figs. 1 to 4.

For further identification of the material responsible for the pyronine-staining of the red nucleoli and cytoplasmic granules, adjacent sections to those examined were cut from the same blocks of tissue in the case of specimens which exhibited the greatest profusion of such pyronine-stained structures. These were treated for 20 minutes at 37°C. with a solution of ribonuclease which had been freed of proteases by treatment with heat and ammonium sulfate. The slides were then stained with methyl green and pyronine, back-to-back with slides bearing adjoining sections cut from the same block of tissue. The control slides showed the same pyronine-stained structures as described above, whereas the ribonuclease-treated slides presented only the methyl green–stained structures, with no trace whatever of red-stained cellular elements.

The treatment with ribonuclease also affected to some extent the structures predominantly stained with methyl green. These, primarily nuclei of lymphocytes, were of a clearer green color than in the control sections in the nodes of both injected and uninjected animals, so that on reviewing slides of sections not treated with the enzyme, in direct comparison with those so treated, it was seen that a lavender tinge existed in most of the methyl green–stained structures in the tissue. This was in all probability due to ribonucleic acid which was present in the tissue but was not organized into specific structures rich in this substance. That this loss of pyronine-staining was not due merely to overdecolorization following the application of that stain was indicated incidentally by the fact that traces of pink color could often be seen even in the subcapsular connective tissue of the node in both ribonuclease-treated and control sections.

Other sections cut from these blocks were treated for 15 minutes in a 5 per cent solution of trichloracetic acid at a temperature of 90°C. in order to remove nucleic acids. On subsequent treatment with methyl green–pyronine stain, neither the green nor the red elements of the control sections could be found.

Chemical Analyses.—The histologic observations reported above made it possible to identify the cells containing the ribonucleic acid granules, but allowed only a subjective impression of the relative amounts of ribonucleic acid in the various specimens of lymph nodes. For chemical measurement of this material, and of desoxyribonucleic acid, portions of the same nodes were subjected to analysis as described above. It was found that the concentration of desoxyribonucleic acid in the tissue of the lymph node did not vary over a wide range, or in any pattern, among the specimens analyzed. The extent of this variation is illustrated in Table I, which shows the results of such analyses in a series of twenty-one specimens involving lymph nodes collected from 0

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up to 9 days after the injection of dysentery bacilli. The concentration of ribonucleic acid in the lymph node, on the other hand, showed considerable variation, involving increases up to more than twice the normal values. These fell in a definite pattern, as indicated in Table I and Text-fig. 1, involving a high rate of change between the 2nd and 4th days after the injection of antigen, and a maximum value on the 4th or 5th day, followed by a decline in later days toward the values found in uninjected rabbits.

The changes in concentration of ribonucleic acid of the lymph node were studied in relation to the concentration of antibodies to the antigen injected contained in the aqueous extracts of that node. The results of such studies are shown in the case of four antigens in Text-fig. 1.

The figure shows that the ribonucleic acid content of the node rises somewhat in advance of the titer of antibody, and that both attain their maximum value at about the same time. Thereafter the concentration of ribonucleic acid declines more rapidly than does that of the antibody. It can be noted that in the case of the viral antigens, where the concentration of antibody in the lymph node is maximal somewhat earlier than is true of cellular antigens, the rise in ribonucleic acid concentration is correspondingly earlier.

The lower half of Text-fig. 1 presents the results of experiments involving the injection of non-antigenic materials or materials not antigenic to rabbits as a species. It is seen that the changes in ribonucleic acid reported above are not found in these cases. For example, the changes in ribonucleic acid content of the lymph nodes are markedly different in the case of injections of erythrocytes of rabbits and sheep, respectively. The series involving the injection of lanolin-mineral oil was also noteworthy because of the marked local reaction at the site of injection. Despite this severe inflammation, no increase of ribonucleic acid was found in the node draining that site, compared with that following the injection of antigenic material.

Other Cell Types.—Histologic observations involving cells other than lymphocytes and transitional forms of reticulum cells agreed with findings made in earlier studies in this laboratory. Macrophages were found in the earliest specimens of lymph nodes at the periphery of the cortex, but then disappeared from the cortex of the node. They were found in abundance at the site of injection of antigen after the 2nd day following the injection. Plasma cells were found in the medulla of the lymph node. In the cortex of the nodes of uninjected rabbits a few plasma cells could be found along the sinuses. Following the injection of antigenic material in the foot, however, the diffuse lymphocytic hyperplasia of the cortex was such that no plasma cells were noted in such sections, except in the medulla. Here the concentration of plasma cells did not give a definite impression of being greater than in uninjected rabbits.

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DISCUSSION

The Histologic Picture of the Young Lymphocytes.—The pyronine-stained structures in the transitional forms of the reticulum cells and in the young lymphocytes were quite similar to those described by Caspersson and by Brachet in embryonal tissue which is the site of synthesis of protein. Of some interest for the work of the former is the frequent association of one or two clearly red-stained nucleoli in cells having pyronine-staining cytoplasmic crescents or granules, since Caspersson has speculated on a possible relationship between these intranuclear and extranuclear structures.

That the pyronine-stained material was ribonucleoprotein would appear to be quite well established inasmuch as the staining reaction of these granules was abolished by specific treatment with ribonuclease free of proteases, and by treatment with hot trichloracetic acid solution. Since the latter is a solvent for both types of nucleic acid, the desoxyribonucleic acid was removed by this procedure as well.

The fact that the color of methyl green-stained structures was changed to a clearer green color by the treatment with ribonuclease implies in all probability the removal of the ribonucleic acid which exists in a low concentration in a diffuse fashion in normal tissue, as well as that organized in the cellular elements described in this paper. The presence of ribonucleoprotein in low concentrations in such structures as nuclei, in which desoxyribonucleoprotein predominates, is consistent with the chemical finding of some ribonucleic acid in lymph nodes containing very few ribonucleic acid granules, such as lymph nodes of uninjected animals.

The Correlation in Time of Chemical and Serologic Changes.—The average values of the concentration of ribonucleic acid and of antibodies presented in Text-fig. 1 show that the two substances reached their maximal concentration at about the same time, with an apparently earlier beginning in the rise of ribonucleic acid content. In a number of series of individual rabbits the peak of ribonucleic acid content was actually a day earlier than the peak of antibody titer. This tendency toward an earlier rise in ribonucleic acid concentration is quite consistent with an assumption that the pyronine is presumably not staining the antibodies themselves. Thus on the 6th and 7th days, as the majority of lymphocytes in the node have reached the mature stage, the number of ribonucleic acid structures, and the chemical concentration of this substance have begun to fall off progressively. Again, a number of determinations of ribonucleic acid content on lymphocytes collected from lymph emerging from the antibody-producing lymph node, too few to include in the section of Results, failed to show increased ribonucleic acid chemically or histochemically. These cells, it should be recalled, are mature lymphocytes, and contain relatively the highest concentration of antibody of any tissue studied in all these investigations (6).

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The Association of Cytoplasmic Ribonucleic Acid with Protein Synthesis.—The implication of protein synthesis in relation to the lymphocytes containing structures rich in ribonucleic acid rests in large measure on the work of Caspersson and of Brachet. These workers found a high degree of association between the presence of such cellular structures and the synthesis of protein in embryonal tissue at points of rapid growth. The assumption that this high degree of association represents a physiologic mechanism in protein synthesis by these granules would involve a function of ribonucleic acid or a derivative thereof as an enzyme with protein-synthetic and perhaps proteolytic properties. There is, in fact, some evidence that such may be the case. Proteolytic functions have been ascribed to such particles, together with other functions as enzymes (25). Again, it is known that washed preparations of cytoplasmic particles from normal mammalian tissue can cause massive intravascular coagulation on intravenous injection into animals (26). This property can be shown *in vitro* to be due to conversion of prothrombin into thrombin, in the presence of suspensions of such particles, and this conversion can be duplicated with ordinary trypsin (27).

In the case of protein synthesis there has been some experimental approach to the question of whether an intermediate metabolite of ribonucleic acid might contain a high-energy phosphorus bond to provide energy for peptide synthesis, although this question is at present quite open (28).

Histologic Aspects of These Observations.—It will be recalled that the pyronine-stained nucleoli and cytoplasmic structures were first seen 2 days after the injection of antigen, in a group of cells which were scattered throughout the enlarged cortex. These cells resembled quite closely the reticulum cells of the lymph node and among them mitotic figures were found. A day or so later, such structures were seen in cells which were identified as large, young, lymphocytes, and with increasing numbers of days after the injection of antigen, these elements were found in cells having progressively smaller and more dense nuclei. The cells showing these well stained cytoplasmic elements formed a continuous series from the transitional reticulum cell free in the cortex to the small, mature lymphocyte. These findings are consistent with observations on transitional forms between reticulum cells and lymphocytes as described by Sundberg in the case of lymphocytogenesis in human lymph nodes (29).

These histologic observations are of some interest in relation to the classical study of lymphocytogenesis by Downey and Weidenreich in 1912 (30). In those studies a complete series of transitional stages was traced in the lymph node of the guinea pig from the "reticulum cell just freed from the reticulum to the completely differentiated large lymphocyte." In the earlier work these stages were traced by transitions in the chromatin-parachromatin patterns in the nucleus. In the present study cytoplasmic structures have provided a

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means of demonstrating a development which is in agreement with, and may lend some corroboration to, those findings. Antigenic stimulation would, then, appear to offer a method of further study of this development since it constitutes a means of inducing heteroplastic lymphocytopoiesis in lymph nodes which can be controlled both in time and intensity.

SUMMARY

Following the injection of various antigenic and non-antigenic materials into the foot-pads of rabbits, the draining (popliteal) lymph nodes were removed on successive days and studied histologically, chemically, and serologically. On the 2nd day after injection of antigen, nucleoli and cytoplasmic granules and crescents stained with pyronine began to appear. They were found first in somewhat altered reticulum cells, later in transitional forms, then in young lymphocytes, and finally in more mature lymphocytes. The identity of this pyronine-stained material as ribonucleic acid was demonstrated by specific digestion with protease-free ribonuclease.

The concentration of ribonucleic acid was determined in aqueous extracts of the lymph nodes. It was observed that the concentration had risen to more than twice its normal value by the 2nd to 5th day following the injection of antigens into the foot, and then it declined. The peak of this change occurred at or slightly before the appearance of the maximal concentration of antibodies in the same node.

Non-antigenic materials, when injected into the foot, did not give rise to an increase in the ribonucleic acid content of the lymph node.

The concentration of desoxyribonucleic acid was constant in all lymph nodes, within the limits of experimental variation.

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EXPLANATION OF PLATE 8

All sections stained with methyl green-pyronine.

Fig. 1. Rabbit 3–6. Left. Popliteal lymph node of rabbit 3 days after injection of saline solution into foot-pad. × 460.

Fig. 2. Rabbit 3–6. Right. Popliteal lymph node 3 days after injection of dysentery bacilli into foot-pad. × 460.

Fig. 3. Rabbit 2–0. Left. Popliteal lymph node 5 days after injection of influenza virus, Type A. × 460.

Fig. 4. Rabbit 2–0. Left. Adjoining section from same block of tissue as Fig. 3. This section was treated with protease-free ribonuclease and then stained back to back with the slide shown in Fig. 3. × 460.

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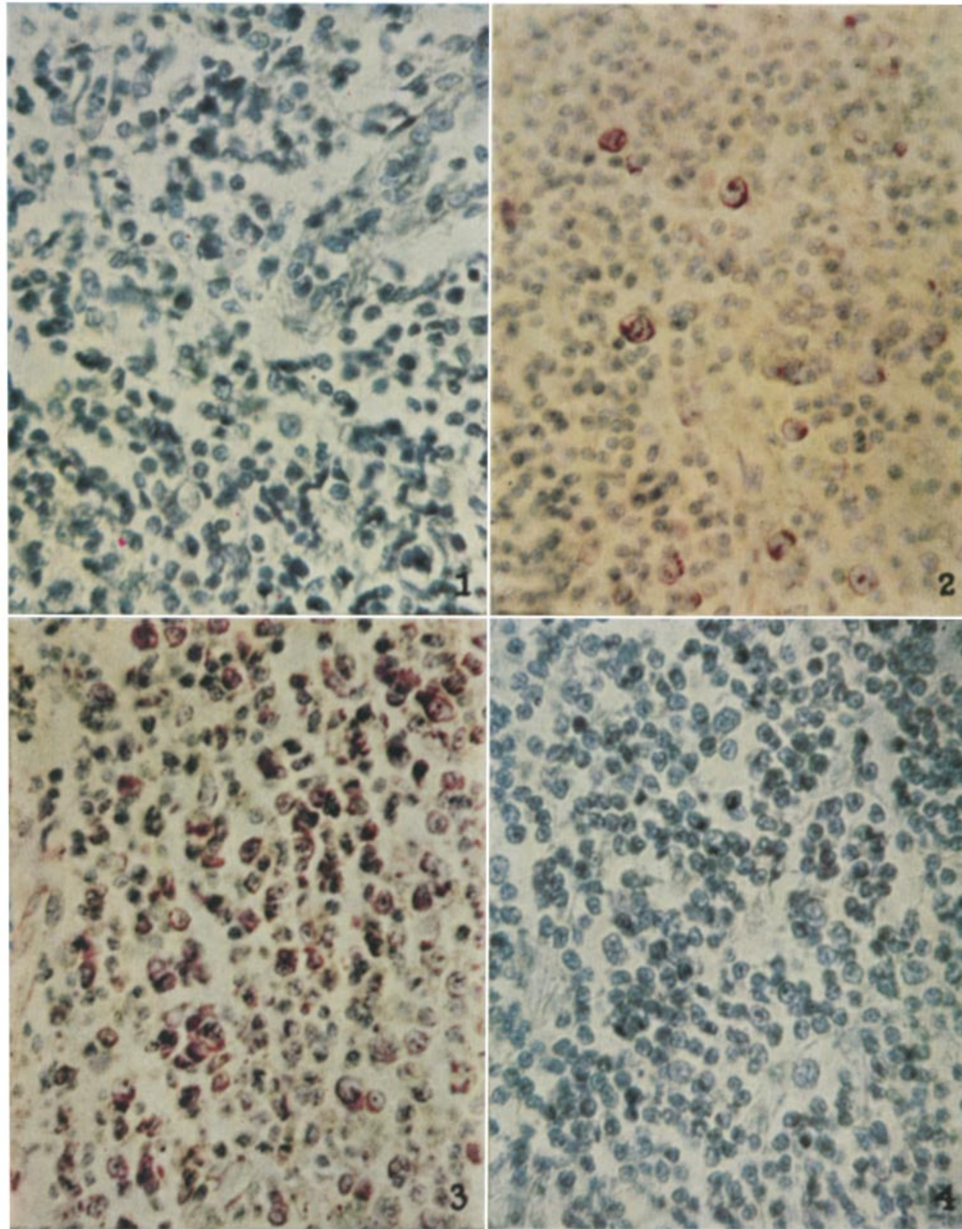

(Harris and Harris: Histochemical changes in lymphocytes)

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