MECHANISM OF THE AUGMENTING ACTION OF MINERAL OIL ON ANTIBODY PRODUCTION

Tissue Reactions and Antibody Response to Dysentery Vaccine in Saline, and in Saline–Lanolin–Mineral Oil Emulsion*

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Of the numerous methods which have been used to intensify and prolong antibody production,[1] the technique of Le Moignic and Pinoy (1) with mineral oil has been found to be the most effective. It has been shown that in the presence of mineral oil emulsified in lanolin-like substances with or without the addition of killed acid-fast bacteria, there may ensue marked augmentation in the production of complement-fixing antibodies to tubercle bacilli (Freund and coworkers (2–4)), of precipitins to horse serum (5, 6) and influenza virus (Friedewald (7, 8), Henle and Henle (9)), of agglutinins to typhoid bacilli and of antitoxins to diphtheria toxoid (Freund and Bonanto (10)). Recently mineral oil has been used to enhance the production of antibodies to dysentery antigen (Halbert, Mudd, and Smolens (11)).

Several hypotheses have been postulated as to the mechanism of the augmenting action of mineral oil. Rist (12) who studied the tissue response to killed tubercle bacilli and mineral oil, found that bacilli alone produced merely a local reaction, mineral oil alone affected also the regional lymph node, while the combination of killed tubercle bacilli and mineral oil produced tubercle-like lesions in the regional lymph node and also in the lungs. On the basis of these findings, Rist came to the conclusion that the mineral oil had a twofold function: it maintained tubercle bacilli at the site of injection in the subcutaneous tissue, and at the same time promoted their dispersion through the draining lymph node.

Freund and his coworkers observed that "the lesions at the site of injections of killed tubercle bacilli and paraffin oil consisted of large mononuclear cells, lymphocytes and numerous polymorphonuclear cells," that "phagocytosis was abundant and giant cells were present" (3), and that a remarkable parallelism existed between the amount of adjuvant injected, the size of the local lesion, and the antibody response (4). Freund and his coworkers concluded that the mineral oil acted by retarding the distribution of the antigen and protecting it against elimination. However, they believed that it was also likely that promotion of antibody formation was due to the reaction of the monocytes produced by the adjuvant.

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[1] In a recent unpublished dissertation on "non-specific stimulants to antibody formation" R. P. Mosch recorded 65 publications on various adjuvants including unrelated bacteria (notably *Staphylococcus aureus*), foreign proteins, metal salts, adsorbents (especially alum and and aluminum hydroxide), and various lipoids and oils. Adjuvants were first used by Pasteur and Joubert in 1877 (*Compt. rend. Acad. sc.*, 1877, **85**, 107).

Friedewald, recognizing the complexity of the mechanism involved, expressed the view (8) that under ordinary conditions the antigenic stimulus of a given virus was apparently short-lived, for the antigen was absorbed and probably destroyed within a short period. He believed that adjuvants functioned probably by setting up a reactive tissue wall about the inoculum which localized and maintained the antigenic material at the inoculation site and through slow continuous absorption produced "hyperimmunization." It was also considered that the monocytes called forth by this material were directly involved in the enhanced antibody production.

As the augmenting action of mineral oil appears to be of considerable theoretical interest as well as of great practical importance, it seemed to be desirable to study its mechanism in greater detail. Since the only histological study available (12) is not only incomplete, but the results are equivocal, as we shall see later, a comparative analysis of the tissue reactions at the place of injection, in the regional lymph node, and elsewhere in the body, and a comparison with the antibody response as it developed in the blood serum seemed to be indicated. Our investigation includes a comparative study of the severity of the tissue reactions locally and systemically, as well as a determination of the duration of persistence of the oil at the site of injection.

Materials and Methods

All experiments were performed on Chinchilla rabbits weighing about 2000 gm. each. Alcohol-killed cells of *Shigella paradysenteriae* Flexner Z911A[2] were used as the antigen. The saline-in-mineral oil[3] emulsions, stabilized with the lanolin-like substance, Falba,[4] were prepared according to the procedure described by Freund (10). The ratios of the volumes of the constituents in the final emulsions were: saline (with or without vaccine) 1 volume, Falba 1 volume, and Mineral oil 4 volumes. All injections were into the pads of the hind feet of the rabbits.

A first group of 10 rabbits received 0.15 ml. of saline in oil emulsion (without antigen) into each foot. A second group were injected with the antigen suspended in saline. Fifteen of these rabbits received 0.25 mg. of antigen into the right foot, and 1.25 mg. into the left, each in a volume of 0.15 ml. Ten additional rabbits received 4.0 mg. of antigen into the right foot, and 8.0 mg. into the left, each in a volume of 0.3 ml. A third group received the antigen suspended in a saline-in-Falba and mineral oil emulsion. The doses of antigen, and the final volumes administered were exactly the same as in the second group. Fifteen rabbits were injected with the smaller doses, and 12 with the larger.

The days on which the rabbits were sacrificed are recorded in Tables I and II. The tissues studied microscopically were: the site of injection, the regional popliteal lymph nodes, lungs, liver, adrenals, spleen, heart, and gastrointestinal tract. The fixatives used were 4 per cent formalin solution and Helly's fluid. The strains employed were hematoxylin and eosin, Azur II and eosin, and Scharlach R.

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[2] This strain was obtained from the Puerto Rican Department of Laboratories.

[3] The brand of mineral oil used was Atreol No. 9, a light mineral oil, U.S.P. prepared by the Atlantic Refining Company.

[4] Falba is the trade name of a lanolin-like substance prepared by Pfaltz and Bauer, Inc., New York.

TABLE I

Antibody Titers and the Weight of the Lymph Nodes Following the Injection of Moderate Doses of Saline in Oil, Vaccine in Saline, and Vaccine in Oil

Experimental period	Saline in oil					Vaccine in saline					Vaccine in oil				
	Rabbit No.	Antibody titer	Weight of nodes			Rabbit No.	Antibody titer	Weight of nodes			Rabbit No.	Antibody titer	Weight of nodes		
			Right	Left	Both			Right	Left	Both			Right	Left	Both
days			*gm.*	*gm.*	*gm.*			*gm.*	*gm.*	*gm.*			*gm.*	*gm.*	*gm.*
1	8-5					6-1					7-2				
2	8-4					6-0	200				7-1	200			
4	8-3		0.22	0.21	0.43	5-8	12,800	0.25	0.49	0.74	7-0	12,800	0.345	0.45	0.80
						8-9	12,800	0.39	0.365	0.755	9-8	6400	0.355	0.44	0.795
6	8-2		0.20	0.24	0.44	5-7	25,600	0.26	0.22	0.48	6-9	25,600	0.28	0.40	0.68
						9-0	25,600	0.30	0.60	0.90	1-00	25,600	1.12	1.995	3.11
9	8-1		0.075	0.185	0.26	5-6	12,800	0.16	0.205	0.36	6-8	25,600	0.37	0.50	0.87
						9-1	25,600	0.37	0.51	0.88	3-29	25,600	0.555	0.815	1.37
14	8-0	200	0.34	0.27	0.61	5-5	12,800	0.37	0.44	0.82	6-5	12,800	0.35	0.45	0.805
						9-2	12,800	0.355	0.525	0.88	3-33	25,600	0.465	0.675	1.14
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1	7-9	200	0.19	0.215	0.41	7-3	1600	0.325	0.34	0.665	6-7	6400	0.22	0.41	0.63
2	7-8	200	0.265	0.225	0.49	5-4	1600	0.165	0.12	0.285	6-6	6400	0.26	0.335	0.595
3	7-7	200	0.29	0.28	0.57	5-3	800	0.37	0.36	0.73	6-4	3200	1.02	0.77	1.79
6	7-6	200	0.43	0.36	0.79	8-8	200	0.43	0.33	0.76	6-3	1600	0.235	0.29	0.525
11						8-7	200	0.22	0.35	0.57	6-2	1600	0.31	0.375	0.685

TABLE II

Antibody Titers and Weight of the Lymph Nodes Following the Injection of Large Doses of Vaccine in Saline, and Vaccine in Oil

Experimental period	Vaccine in saline					Vaccine in oil				
	Rabbit No.	Antibody titer	Weight of nodes			Rabbit No.	Antibody titer	Weight of nodes		
			Right	Left	Both			Right	Left	Both
days			*gm.*	*gm.*	*gm.*			*gm.*	*gm.*	*gm.*
1/8	2-6		0.215	0.205	0.42	2-9		0.22	0.215	0.435
1/4	2-7		0.135	0.13	0.165	3-0		0.36	0.42	0.78
1/2	2-8		0.33	0.41	0.74	3-1		0.20	0.20	0.40
1	3-45	400	0.30	0.475	0.77	9-3	400	0.27	0.35	0.62
2	3-47	400	0.395	0.415	0.81	3-33	400	0.295	0.47	0.765
4						3-32	12,800	0.98	0.905	1.885
6	3-48	25,600	0.485	0.36	0.84	3-36	25,600	0.935	1.28	2.215
9						3-40	25,600	0.72	0.88	1.60
14	3-50	25,600	0.41	0.50	0.91	3-44	25,600	0.975	1.065	2.04

RESULTS

The antibody titers which developed in the blood serum of our animals are presented in Tables I and II. It can be seen that in all experiments the highest titers were encountered as early as 6 days after injection of the antigen, and, taking all experiments, the greatest titers amounted to 1:25,600. But while with antigen alone the titers began to fall 9 days after injection and disappeared between the 5th and 6th months, with antigen and mineral oil they did not begin to drop until 14 days after injection and the titer was still 1:1,600 in an animal sacrificed after 11 months.

Turning to the tissue reactions, let us first study the changes at the site of injection, then the reactions in the regional popliteal lymph nodes, and finally the systemic alterations which were observed.

Changes at the Site of Injection

1. Saline in Oil Emulsion.

Gross Changes.—During the first day of the experiment, we found inflammatory edema of the pads of the hind feet; beginning with the 4th day we observed a small amount of a grayish exudate surrounded by granulation tissue. After 3 months a small amount of a brown granulation tissue was seen in the pads of one rabbit, while two other rabbits sacrificed after 2 and 6 months showed no gross changes.

Microscopic Findings.—After 1 day, the injected tissue was diffusely infiltrated with granulocytes.

After 2 days, the oil emulsion showed early segregation; there were large oil drops surrounded by macrophages (Fig. 1); many macrophages contained Sudan-positive vacuoles. The granulocytes were less numerous; many showed signs of disintegration or had been phagocytosed by macrophages. There was conspicuous activation of the endothelial and adventitial cells of the small blood vessels.

After 4 days, we found large oil drops surrounded by macrophage layers which varied in thickness, but mostly were not prominent. Between the oil drops we observed proliferation of macrophages and of cells resembling fibroblasts. There were many mitotic figures in places. Many macrophages contained large Sudan-positive droplets. Granulocytes had greatly diminished.

Between 6 and 14 days, the production of granulation tissue appeared to be at its peak. The macrophage layer now often showed two distinct zones, namely an inner zone of large pale cells with large pale nuclei and abundant vacuolated cytoplasm, and an outer zone of macrophages and fibroblast-like cells resembling the cells of the earlier preparations. The cells of the inner zone were partially filled with Sudan-positive droplets. But even at this stage, the granulation tissue was not conspicuous (Fig. 2).

After 1 month, we still found many large oil drops, often surrounded by two distinct zones of granulation tissue; but proliferation now was greatly diminished.

After 2, 3, and 6 months finally, oil drops were still present, but the cellularity of the granulation tissue showed progressive diminution (Fig. 3).

2. Antigen in Saline (right 0.25 mg., left 1.25 mg.).

Gross Changes.—During the first few days of the experiment, inflammatory edema was found in the pads of both hind feet, the left being more severely affected than the right. In one animal the edema was hemorrhagic. Oh the 6th day granulation tissue was seen, especially

in the pad of the left foot. At later dates all foot pads appeared to be normal, with the exception of one animal which on the 14th day showed a little infiltration of the left foot.

Microscopic Findings.—After 1 day, the injected tissue was edematous and diffusely infiltrated with granulocytes. The left foot was more severely inflamed than the right.

After 2 days, an abscess was found in the left foot. There was activation of endothelial and adventitial cells as well as of other mesenchymal elements, while macrophages were not conspicuous.

After 4 and 6 days, the injected tissue showed multiple granulomata consisting of granulocytes, lymphocytes, plasma cells, macrophages, and fibroblast-like cells. There were abscesses in the left foot. There was marked activation of the endothelial and adventitial cells of the small blood vessels.

After 9 and 14 days, small abscesses were present in the left foot of some animals, while others showed merely granulomata. The granulation tissue now consisted largely of lymphocytes and macrophages, but the latter were never very numerous (Fig. 4).

After 1 month and later, in spite of the presence of abundant muscle tissue in all slides, no trace of granulation tissue was found.

3. Antigen in Saline (right 4.0 mg., left 8.0 mg.).

Gross Changes.—During the first 6 hours no gross changes were noted. Twelve hours to 2 days after injection, hemorrhagic inflammation was found in both foot pads. After 6 days, little exudate was recovered from the site of injection; after 14 days a small amount of granulation tissue was found in the right foot pad, hardly any in the left.

Microscopic Findings.—Three hours after injection, large quantities of antigen were found at the site of the injection. There were numerous bacteria staining blue with Azur II-eosin. Leukocytes were conspicuously absent.

After 12 hours, the tissue was diffusely infiltrated with polymorphonuclear leukocytes. Bacteria were still visible, though now they stained red with Azur II-eosin.

After 1 day, the tissue was heavily infiltrated with granulocytes. Bacteria were no longer detectable.

After 2 days, the granulocytes showed marked disintegration. There was conspicuous abscess formation. There was activation of endothelial and adventitial cells as well as of other mesenchymal elements. The right and left pads were equally severely affected.

After 6 days, we found abscesses filled with disintegrating granulocytes surrounded by a granulation tissue consisting of blood vessels, fibroblasts, macrophages, and other chronic inflammatory elements. The granulation tissue was not very cellular. Macrophages were not very conspicuous.

After 14 days, we found granulomata consisting chiefly of macrophages. The granulocytes had largely disappeared.

4. Antigen in Oil (right 0.25 mg., left 1.25 mg.).

Gross Changes.—On the first day of the experiment, inflammatory edema was found in the pads of both hind feet On the 2nd day, the edema was replaced by a grayish creamy exudate which on the 4th and 6th days became surrounded by granulation tissue forming a cake measuring up to $1.5 \times 1.0 \times 0.5$ cm. The left was more severely involved than the right. After 2 months the cake began to shrink and turn brown in color; after 6 months we still found a good deal of granulation tissue, but after 10 months no definite lesion was detected in either pad.

Microscopic Findings.—After 1 day, the injected tissue was edematous and diffusely infiltrated with granulocytes. This infiltration was more severe in the left than in the right foot.

After 2 days, the granulocytes had increased in number; there were large abscesses filled with well preserved granulocytes. The infiltration was more severe in the left than in the right

foot. There was distinct segregation of oil drops. Macrophages were not yet conspicuous, but there was marked activation of the endothelial and adventitial cells of the small blood vessels.

After 4 days, large segregated oil drops were found in the right foot, the drops being surrounded by disintegrating granulocytes and proliferating macrophages. The left foot showed large abscesses filled with necrotic granulocytes; these were surrounded by a moderately cellular granulation tissue consisting of blood vessels, fibroblast-like cells, and some macrophages.

After 6 days, the changes in the right foot resembled those after 4 days. The left foot continued to show large abscesses filled with disintegrating granulocytes; but now there was marked proliferation of macrophages and fibroblast-like cells (Fig. 5). There were many mitotic figures present. In the periphery of the granulomata we found early infiltration with lymphocytes.

After 9 days, we still saw scattered abscesses of various sizes, often in or around oil drops, particularly in the left foot, but now macrophages were prominent (Fig. 6). The right foot showed much thicker macrophage layers than the left, while the left foot showed more granulocytic infiltration than the right. Now we also found marked lymphocytic infiltration.

After 14 days, the granulocytes were greatly reduced in number, while the macrophages were very conspicuous; they were especially numerous next the oil drops (Fig. 7). Now we found many giant cells as well as many lymphocytes. The latter were found particularly at the periphery of the granulomata. There were still mitotic figures present. The only difference between right and left was the presence of small foci of granulocytes in the granulomata of the left foot.

After 1 month, the macrophages were found to be large, and there were many foreign body giant cells present. We also observed lymph follicles now.

After 2 months, we still found some abscesses as well as large aggregations of macrophages, though the total amount of granulation tissue was smaller than after 1 month.

After 3, 6, and 10 months, we still found oil drops and granulation tissue (Fig. 8), but the latter showed gradual regression with the passing months.

5. Antigen in Oil (right 4.0 mg., left 8.0 mg.).

Gross Changes—Three hours after injection, no gross changes were detected at the site of inoculation. After 6 hours, we found edema of the foot tissue, after 12 hours marked inflammation. After 1 to 4 days, the pads were acutely inflamed, more on the left than on the right. After 6 days, we found large granulomata measuring 2.0 × 1.5 × 0.7 cm., the centers showing creamy suppuration. Similar granulomata were found after 9 and 14 days.

Microscopic Findings.—After 6 hours, the foot pads were diffusely infiltrated with polymorphonuclear leukocytes. There were large numbers of bacteria staining blue with Azur II-eosin.

After 12 hours, oil drops began to segregate, and there was diffuse infiltration with polymorphonuclear leukocytes. There were numerous bacteria in the oil drops.

After 1 day, acute suppuration was observed, and there were large numbers of bacteria in both foot pads (Fig. 9).

After 2 days, the granulocytes had further increased in number; large abscesses filled with disintegrating granulocytes were found in both feet. The oil drops were crowded with bacteria. The tissues surrounding the abscesses showed early granulation tissue.

After 4 and 6 days, we found large abscesses filled with disintegrating granulocytes and segregating oil drops. The latter contained some bacteria staining blue with Azur II-eosin. The abscesses were surrounded by granulation tissue consisting of fibroblast-like cells and macrophages. The latter were not yet very prominent.

After 9 and 14 days, we found enormous abscesses surrounded by thick layers of macrophages; a good many lymphocytes and plasma cells were also found. The oil drops still contained some bacteria after 9 days (Fig. 10, arrow), but now they stained pink with Azur II-eosin. After 14 days bacteria were hard to recognize.

Summary of the Changes at the Site of Injection.—The changes at the site of injection differed greatly in our various experiments. With saline in oil emulsion, the local lesion was chiefly one of granulomata consisting of oil drops surrounded by macrophages. During the first 2 days, there were numerous polymorphonuclear leuckocytes present; after 4 days, these cells had greatly diminished. The granulomata reached the peak of their development between the 6th and 14th days. After 1 month they were less conspicuous, but even after 6 months oil drops surrounded by macrophages were still detectable. The size of the granulomata was never large. After 1 month they were not recognizable in the gross in most animals.

With antigen in saline the local reaction was chiefly one of acute inflammation. From 2 to 6 days after injection small abscesses were found microscopically. They were largest in the pads which received the largest dose of antigen. After 14 days, the granulocytes had largely disappeared and had been replaced by granulation tissue consisting of lymphocytes and macrophages. After 1 month or more no granulation tissue was found in the sections.

After large doses of antigen in saline, numerous bacteria staining blue with Azur II-eosin were noted at the site of injection. Twelve hours after injection they were still visible, though now they stained red with Azur II-eosin. After 24 hours bacteria were no longer detectable.

The injection of antigen in oil resulted in grossly visible suppuration and abscess formation which reached its peak during the 2nd week of the experiment.[5] This was followed by the formation of large granulomata which reached their greatest size during the second half of the first month. They began to regress after the 2nd month, but even after 6 months a good deal of granulation tissue could be detected in the gross, and after 10 months microscopically. The character of the granulomata was essentially the same as that of those which followed the injection of saline in oil emulsion. However, with antigen in oil we found many more macrophages, and numerous giant cells; and many lymphocytes were also observed.

After large doses of antigen in oil, large numbers of bacteria staining blue with Azur II-eosin were found at the site of injection. After 12 to 24 hours they became concentrated within the segregating oil drops where they remained visible for more than a week. During the 2nd week they changed from blue to pink with Azur II-eosin. After 14 days they had completely disappeared.

[5] It may be mentioned here that no perforation of the skin was observed at the site of injection in any of our experimental animals.

Reactions in the Regional Popliteal Lymph Nodes

1. Saline in Oil Emulsion.

Gross Changes.—The gross appearance of the lymph nodes was essentially normal throughout the experiment. The weight was possibly slightly increased on the 4th and 6th days; but our data were not numerous enough to establish this observation (Table I).

Microscopic Findings.—After 1 day, the sinus endothelial cells contained a good many Sudan-positive droplets. There were some granulocytes in both the marginal and intermediary sinuses.

After 2 days, a mild lymphatic hyperplasia was observed. There were large tertiary nodules containing a fair number of lymphoblasts. There were a good many lymphocytes within the sinuses. The sinus endothelial cells continued to contain Sudan-positive material.

After 4 days, the picture resembled that after 2 days, but in the animal studied the macrophages were more prominent. There were a good many of them in the sinuses and the tertiary nodules. Polymorphonuclear leukocytes were inconspicuous.

After 6 to 9 days, we found fewer tertiary nodules, but more and larger secondary nodules showing a good many medium-sized and large lymphocytes and macrophages containing tingible bodies.[6] After 9 days, one of the slides showed many macrophages in the sinuses and tertiary nodules. For the rest these cells were not very conspicuous. There was still a good deal of Sudan-positive material in the sinus endothelial cells.

After 14 days and more, we found various degrees of normal lymphocytopoiesis. There were well developed tertiary and secondary nodules of varying appearance. The only remainder of the reaction described above was the presence of nests of epithelioid cells containing abundant Sudan-positive material, particularly in the tertiary nodules. These were present in all rabbits sacrificed from 1 to 10 months after injection.

2. Antigen in Saline (right 0.25 mg., left 1.25 mg.).

Gross Changes.—Following the injection of antigen into the foot pad, the weight of the right node rose from an average of 0.2 gm. to one of 0.3 gm. during the first 4 days, that of the left node from 0.2 gm. to 0.45 gm. (Table I). Similar weights were found from the 6th to the 14th days of the experiment. Thereafter the weights became smaller.

Microscopic Findings.—After 1 day, we found many granulocytes scattered through the lymph nodes. The secondary nodules contained a good many large and medium sized lymphocytes; there were a fair number of lymphoblasts in the lymphoid tissue. The sinus endothelial cells contained a good many erythrocytes. All these reactions were more severe in the left than in the right node.

After 2 days, the number of granulocytes was greatly diminished, their debris being demonstrable within macrophages. The lymphatic tissue showed many lymphoblasts everywhere. The hyperplasia was rather diffuse; the secondary nodules undergoing dissolution.

After 4 and 6 days, we found marked diffuse lymphoid hyperplasia with numerous lymphoblasts everywhere (Fig. 11). This was much more severe on the left than on the right. On the other hand, the sinus endothelial cells were hardly increased in number. There were only a few nests of epithelioid cells within the lymphoid tissue.

After 4 days lymphoblasts were still conspicuous; after 6 days small lymphocytes had come into prominence. In some places we now observed large secondary nodules filled with medium sized and large lymphocytes showing many mitotic figures and macrophages filled

[6] The term "tingible bodies" has been introduced to designate the often numerous pyknotic nuclei contained in the macrophages of the secondary nodules. The great majority of these nuclei are remnants of phagocytosed and disintegrating lymphocytes.

with tingible bodies. The right node was much less affected than the left. It showed chiefly large secondary nodules filled with medium sized and large lymphocytes.

After 9 and 14 days, diffuse lymphocytopoiesis had largely subsided. The lymphoid tissue was partially depleted of lymphocytes. We now found large secondary nodules (Fig. 12) with many mitotic figures and numerous tingible bodies everywhere in the right and left lymph nodes; we also found many prominent macrophages particularly in the tertiary nodules (Fig. 13).

After 1 month, the left node still contained large active secondary nodules, while the right approached normal. The left node still contained a fair number of macrophages in places.

After 2 months, the left node also approached normal, though it still contained some accumulations of macrophages in places.

After 3 and 6 months, lymphocytopoiesis was possibly still somewhat hyperactive in the left node.

Finally after 10 months the nodes appeared to be normal.

3. Antigen in Saline (right 4.0 mg., left 8.0 mg.).

Gross Changes.—From 12 to 24 hours after injection, the lymph nodes were markedly edematous. There were petechial hemorrhages in both nodes 1 and 2 days after injection of the antigen.

There was a marked increase in weight of both lymph nodes, the left more than the right (Table II). This increase was greater than that following the injection of 0.25 mg. but not much different from that following 1.25 mg. of antigen (see Table I).

Microscopic Findings.—Three hours after injection of the antigen into the foot pad, a few polymorphonuclear leukocytes were present in the sinuses. After 6 hours, the sinuses were distended and contained a larger number of leukocytes. After 12 hours the sinuses were filled with numerous polymorphonuclear leukocytes; there were many leukocytes everywhere in the lymph nodes.

The lymphatic tissue was well preserved in some places, while marked destruction of lymphocytes was apparent in other places (Fig. 14). These changes were more severe on the left than on the right.

After 1 day, the lymph nodes were heavily infiltrated with granulocytes in places. The lymphatic tissue contained rather few cells. There were large macrophages filled with numerous tingible bodies in both secondary and tertiary nodules. Some secondary nodules contained small abscesses.

After 2 days, lymphocytopoiesis was well under way. There were many large lymphoblasts everywhere. The secondary nodules still contained small abscesses. There were no lymphoblastic centers. Macrophages were inconspicuous.

After 6 days we found marked lymphoid hyperplasia with numerous lymphoblasts everywhere, the secondary nodules showing all stages of dissolution. The granulocytes had largely disappeared. Macrophages were inconspicuous.

After 14 days, we found many large secondary nodules with large and medium sized lymphocytes showing many mitotic figures as well as many tingible bodies. We also found prominent macrophages especially in the tertiary nodules and sinuses.

4. Antigen in Oil (right 0.25 mg., left 1.25 mg.).

Gross Changes.—The weight of the right node rose from an average of 0.2 gm. before injection to one of 0.35 gm. on the 4th day, and about 0.5 gm. on the 6th to 14th days after injection; that of the left from 0.2 gm. before injection to 0.45 gm. on the 4th day and about 0.8 gm. on the 6th to 14th days after injection. Thereafter the weights decreased again (Table I).

Microscopic Findings.—After 1 day, many granulocytes were diffusely scattered through the lymph nodes. The sinus endothelial cells contained many erythrocytes. In places, fat droplets were found in the node surrounded by granulocytes and macrophages.

After 2 days, many granulocytes were still present in the left node, while the right showed large sinus endothelial cells filled with erythrocytes. There were many lymphoblasts in the tertiary nodules and elsewhere in the lymphoid tissue. Lymphoblasts and proliferating reticuloendothelial cells differed markedly in morphologic appearance.

After 4 and 6 days, we found marked lymphocytopoiesis in the nodes with marked enlargement of the cortex, at the left more than at the right (Fig. 15). There were numerous dividing lymphoblasts. There were many lymphoblasts and small lymphocytes in the secondary nodules, most of which showed dissolution. The sinus endothelial cells were moderately hyperplastic.

After 9 and 14 days, lymphocytopoiesis was very active. There were many lymphocytes in the sinuses. The markedly enlarged lymphoid tissue still contained many lymphoblasts; but now many large secondary nodules had made their appearance (Fig. 16). Most of these were filled with numerous medium sized and large lymphocytes showing many mitotic figures and macrophages filled with tingible bodies. Nests of macrophages were found only in the left node of one rabbit sacrificed 14 days after injection (Fig. 17). Otherwise, the macrophages were not very conspicuous.

After 1 and 2 months, lymphocytopoiesis was still quite active. There were still many large secondary nodules filled with vigorously dividing medium sized and large lymphocytes. The tertiary nodules were large and still contained a fair number of lymphoblasts. We now found nests of epithelioid cells surrounding oil drops especially in the lymphoid tissues in between the secondary nodules. Lymphocytopoiesis was more active at the left than at the right.

After 6 and 10 months, lymphocytopoiesis was still slightly greater than normal, though the secondary nodules had come to rest. There were still some oil droplets (Fig. 18). The reticuloendothelial cells appeared to be normal again.

5. *Antigen in Oil* (right 4.0 mg., left 8.0 mg.).

Gross Changes.—In this experiment, both the right and the left lymph nodes were found to develop much greater weights than those following the injection of 0.25 and 1.25 mg. of vaccine (compare Tables I and II). The weight of the right node rose from an average of 0.2 gm. before injection to one of 0.9 gm. 4 to 14 days after injection, while the left node reached an average of over 1.0 gm. (Table II).

Microscopic Findings.—During the first day of the experiment, the changes closely resembled those after the injection of antigen in saline, though they were possibly slightly less severe. There appeared many polymorphonuclear leukocytes, and there was marked destruction of lymphocytes in places. After 12 hours, abscesses were found in some secondary nodules.

After 1 and 2 days, the nodes were heavily infiltrated with granulocytes, and a good many abscesses were found in the secondary nodules. However, there appeared to be less evidence of destruction than in the antigen in saline experiments. There were less macrophages filled with tingible bodies. Also, we found less lymphocytopoiesis.

After 4 and 6 days, we observed marked lymphocytopoiesis with marked enlargement of the cortex and numerous dividing lymphoblasts. The secondary nodules contained many large and medium sized lymphocytes and showed all stages of dissolution of them. There were still some abscesses in the secondary nodules. The reticuloendothelial cells were more prominent than on the preceding days.

After 9 and 14 days, we found marked lymphatic hyperplasia with numerous large classical secondary nodules containing large and medium sized lymphocytes and large macrophages

filled with tingible bodies. In the nodes secured after 14 days we also found nests of epithelioid cells in the secondary and tertiary nodules. Otherwise, the macrophages were not very conspicuous.

Summary of the Reactions in the Regional Popliteal Lymph Nodes.—The changes in the regional popliteal lymph nodes appeared to be essentially the same in all experiments, though they differed greatly in intensity. With saline in oil emulsion, the weight of the nodes was possibly slightly increased on the 4th and 6th days. With antigen in saline the weight rose from an average of 0.2 gm. to one of 0.3 gm. following the injection of 0.25 mg. of antigen, while after 4.0 and 8.0 mg. of antigen, the weight rose to 0.45 gm. With antigen in oil, the weight rose to an average of 0.5 gm. after the injection of 0.25 mg. of antigen, to 0.8 gm. after 1.25 mg., to 0.9 gm. after 4.0 mg., and to over 1.0 gm. after 8.0 mg.

With saline in oil emulsion, we first found some granulocytes scattered through the lymph nodes. On the 2nd and 4th days, this was followed by a mild lymphatic hyperplasia mainly of tertiary nodules, and on the 6th and 9th days by a greater activity of the secondary nodules. Beginning on the 4th day, the reticuloendothelial cells became more conspicuous in some animals, but they were never very prominent. After 1 month and later only a few nests of epithelioid cells were found especially in the tertiary nodules.

With antigen in saline, we first observed many granulocytes in the lymph nodes. After 1 and 2 days small abscesses were found in the secondary nodules. This was followed by a marked lymphoid and lymphatic hyperplasia which was chiefly responsible for the weight increase of the nodes. On the 4th and 6th days the hyperplasia was largely diffuse, there were numerous lymphoblasts everywhere. On the 9th and 14th days, many large active secondary nodules were present and there were a good many macrophages filled with tingible bodies. Though the macrophages became more conspicuous after this time, they never gained prominence. One month after the injection of 0.25 and 1.25 mg. of antigen, the lymphatic reaction had greatly subsided, the right node approached its normal appearance. Thereafter, both nodes were essentially normal, though the left node continued to show somewhat hyperactive lymphocytopoiesis.

Necroses were not observed in the lymph nodes after 0.25 or 1.25 mg. of antigen. With larger doses, however, marked destruction of lymphocytes was apparent especially in the secondary nodules of the node draining the pad which received the largest dose of antigen.

With antigen in oil the reaction in the lymph nodes was essentially the same as that following the introduction of antigen in saline. However, it was somewhat slower, it reached a greater intensity, and it lasted longer. Thus, granulocytes were abundant still on the 2nd day, and lymphocytopoiesis was still quite active 1 and 2 months after the injection of 0.25 and 1.25 mg. of antigen.

The reticuloendothelial response was equally moderate in both the antigen in saline and the antigen in oil experiments.

Necroses were present in the lymph nodes that drained the pads which had received 4.0 and 8.0 mg. of antigen. The intensity of the necrosis was about the same as the intensity of the necrosis in the tests with the antigen-saline experiments, though perhaps slightly less severe.

The Systemic Alterations

1. Saline in Oil Emulsion.

No significant changes were detected in any of the internal organs studied microscopically.

2. Antigen in Saline.

No significant changes were found in heart, kidneys, or intestines. However, there were significant changes in lung and liver, and to a lesser extent in adrenals and spleen.

The *lungs* of these animals showed so called mesenchymal reactions consisting of lymphocyte and macrophage infiltration and proliferation in and around small blood vessels (Figs. 19 and 20). Of 19 animals sacrificed 1 day after injection or later, 7 revealed mild, and 5 marked mesenchymal reactions; while of 10 rabbits which received saline in oil emulsion none showed these changes.

The *livers* of the animals which were sacrificed 2 to 6 days after injection of 0.25 and 1.25 mg. of antigen, all revealed cloudy swelling of the parenchyma and swelling and proliferation of the Kupffer cells; the liver of one rabbit sacrificed after 4 days showed acute necroses. After 9 days and later no definite changes were found in 6 out of 9 cases, while in 3 cases old periportal scars were seen, representing possibly healed necroses.

After the injection of 4.0 and 8.0 mg. of antigen, necroses were present in the livers of 5 out of 7 animals. In some rabbits the necroses were severe enough to be recognized in the gross. In addition, we found many polymorphonuclear leukocytes in the sinuses during the first 24 hours of the experiment.

The *adrenals* appeared to be normal in most animals treated with 0.25 and 1.25 mg. of antigen. After 4.0 and 8.0 mg. many polymorphonuclear leukocytes were observed in the sinuses of these organs.

The *spleen* revealed some activation of the reticuloendothelial system of some of the rabbits during the first 6 days of the experiments. The Malpighian bodies were apparently not affected by the injections.

3. Antigen in Oil.

Of 21 rabbits sacrificed 1 day after injection of the antigen or later, 1 showed mild, and 4 marked mesenchymal reactions in the lungs. These might have been the result of our injections. However, as mesenchymal reactions are observed occasionally in untreated animals, this observation cannot be regarded as significant.

The liver was of normal appearance in all animals treated with 0.25 and 1.25 mg. of antigen, while in the animals sacrificed 3 to 12 hours after injection of 4.0 and 8.0 mg. of antigen, it showed an increased number of polymorphonuclear leukocytes in the sinuses. There was no cloudy swelling, however; there were no necroses or scars of necroses.

Adrenals and spleen appeared to be normal in all animals.

Summary of the Systemic Alterations.—Systemic alterations were observed in lungs, liver, adrenals, and spleen following injection of the bacterial cells in saline. There were mesenchymal reactions in the lungs; leuckocytosis,

reticuloendothelial activation, cloudy swelling and necrosis in the liver; leucocytosis in the adrenals; and reticuloendothelial activation in the spleen. With the exception of leuckocytosis in the liver 3 to 12 hours after the injection of 4.0 and 8.0 mg. antigen in oil, systemic alterations were observed *only* in animals injected with antigen in saline. They were more severe after 4.0 and 8.0 mg. of antigen, than after 0.25 and 1.25 mg. of antigen.

COMMENT

The experiments which have been reported here show that the production of antibodies against *Shigella paradysenteriae* Flexner is enhanced when the vaccine is injected as an emulsion in mineral oil and Falba. The antibody response was greatly prolonged: considerable antibody titers were found as late as 10 months after a single injection of the antigen. These observations confirm and extend the earlier findings of Freund and coworkers (2–6, 10), Friedewald (7, 8), and Henle and Henle (9).

Our experiments show further that the systemic effects of *Shigella* are greatly diminished when it is injected in oil. It was found that a total subcutaneous dose of 12.0 mg. of antigen in oil was essentially inactive systemically, while a subcutaneous dose of 1.5 mg. of antigen in saline produced mesenchymal reactions in the lungs, cloudy swelling and sometimes necrosis of the liver, and other systemic changes.

The mesenchymal reactions in the lungs were not unlike those which Rist (12) observed in experiments with killed tubercle bacilli in paraffin oil; but while Rist believed that they were tuberculous in nature, we find that they are nonspecific; they are identical with the mesenchymal reactions following the injection of killed staphylococci and other bacteria described earlier by Ehrich and coworkers (13–15). The nature of these reactions is not entirely clear. There is nothing to suggest that they are allergic in nature. The evidence available at present seems to show that they are due to direct action of bacterial material.

There can be no doubt that the cloudy swelling and necrosis of the liver occurring after injection of dysentery antigen in saline were toxic in nature. Their absence after large doses of antigen in oil confirms the recent observations of Halbert, Smolens, and Mudd (16) that the toxicity of various bacterial products may be considerably reduced by the addition of mineral oil.

As to the mechanisms of these various actions of our oil, it seems to be significant that the bacteria, when injected with oil, remained visible in the segregating oil drops for 1 week or more, while with saline they disappeared within 1 day. It seemed to be significant also that after the injection of antigen in oil, the oil drops remained detectable at the site of injection until the end of the experiment, *i.e.* as long as 10 months after inoculation, while with saline nothing, not even exudate or granulation tissue, was found after 1 month.

These facts show clearly, that antigen is actually retained and its destruction retarded when given in mineral oil. If we consider the various time elements recorded here, it seems certain that in the presence of oil antibody production was maintained for a much longer period than could be explained by the presence of microscopically visible bacteria. In mice antigen was demonstrable in the oil at the site of injection as late as 4 months after inoculation (unpublished data), so it seems that the retention of whole bacteria was succeeded by the presence of amorphous or dissolved antigen.

As to the site of antibody formation, it was found that at the place of injection the tissue reaction to antigen in oil was largely one of suppuration and production of mononuclear granulomata. The production of macrophages reached its peak during the second half of the 1st month; it began to regress only after the 2nd month. After the 1st week marked infiltration with lymphocytes was also observed.

After antigen in saline the local reaction was chiefly one of acute inflammation, macrophages not being very conspicuous at any time. After 1 month the inflammation had subsided.

Comparing these findings with the antibody response in these experiments, we noticed a discrepancy between the local tissue reactions and the antibody titers achieved. Whereas with antigen in oil we found an excellent antibody response together with an enormous proliferation of macrophages, with antigen in saline we also observed a good antibody production, but only few macrophages. We also noticed that in the antigen in oil experiments the local macrophage reaction followed on the heels of the antibody production; it reached its peak later and it remained there considerably longer than did the antibody response.

These observations do not eliminate the old reticuloendothelial theory of antibody formation; however, they do not strengthen it either. Nor is this theory supported by our observation of reticuloendothelial activation and proliferation in lungs, liver, and spleen after injection of antigen in saline, for in previous experiments with staphylococcus vaccine poor antibody response was observed whenever these reactions made their appearance (15). Monocytes, and also polymorphonuclear leukocytes, undoubtedly play an important rôle in antibody production whether by the killing of microorganisms, or the destruction of toxins, or through dissolving particulate antigens, or through retention of antigenic material which otherwise would be excreted or destroyed elsewhere in the body. For these reasons alone, macrophage production should aid in antibody formation.

In the lymph nodes, the tissue reactions closely resembled those observed during the formation of antibodies against typhoid vaccine or sheep erythrocytes (Ehrich and Harris (17). During the greatest rise in antibody titer in the blood serum, we found a diffuse lymphoid hyperplasia with numerous

dividing lymphoblasts, while later when the titers were at their peak, large secondary nodules made their appearance. The intensity and duration of these changes were directly correlated with the intensity and duration of antibody response, regardless of whether or not oil had been injected with the antigen.

A monocytic response was also observed in a good many lymph nodes, but as in the foot pad it followed on the heels of the antibody production, and it was never very conspicuous.

Thus a comparison of the various reactions at the site of injection, in the regional lymph nodes and elsewhere in the body, leaves us with the impression that the changes following the injection of antigen in oil fit better into the lymphocytic than the reticuloendothelial theory of antibody formation. According to the lymphocytic theory, antibody production is accomplished by the action of macrophages and polymorphonuclear leukocytes as well as of lymphocytes (Ehrich and Harris (18)); it may well be that the quantity of antibody produced is largely determined by the lymphocytes.

Finally, it may be emphasized that, although the local tissue reactions were prolonged when the vaccine was administered in oil, and the oil was slowly absorbed, the systemic toxic effects of the oil vaccine were significantly less than those of the saline vaccine. Preliminary accounts of the administration of dysentery vaccine in mineral oil to human subjects (16) and of influenza vaccine in mineral oil to human subjects (9) have been published. Further details will appear.

SUMMARY

A comparative study was made in rabbits of antibody production and tissue changes following the injection into the foot pads, of saline in Falba and mineral oil emulsion, of killed cells of *Shigella paradysenteriae* Flexner in saline, and of killed cells of *Shigella paradysenteriae* in saline in Falba-mineral oil emulsion.

It was found that antibody production was greatly prolonged by the emulsification in oil. While with antigen in saline the serum titers began to fall 9 days after injection and disappeared somewhere between the 3rd and 6th months, with antigen in paraffin oil they began to drop only after 14 days, and were still high after 10 months, when the experiment was ended.

The toxic effects of the antigen were greatly reduced by the emulsification in oil. A subcutaneous dose of 1.5 mg. of antigen in saline caused mesenchymal reactions in lung, liver, and spleen as well as toxic degeneration and sometimes necrosis of the liver whereas eight times as much of the antigen in oil produced no systemic lesions.

Oil drops remained detectable in the foot pad until the end of the experiment. Bacteria remained visible in the oil for 1 week or more, but with saline they disappeared within 1 day. The latter observation shows that retention of

antigen at the site of injection is at least one of the mechanisms of prolongation of antibody formation by paraffin oil.

The tissue reaction in the foot pad to antigen in oil was largely one of suppuration with the production of persisting mononuclear granulomata whereas after antigen in saline it was chiefly one of catarrhal inflammation, subsiding within a month. The changes in the regional lymph nodes were essentially those of lymphatic hyperplasia with the production of numerous lymphocytes and large active secondary nodules, the macrophages remaining subsidiary. The lymphocytic reaction in the lymph nodes closely paralleled the antibody response but the monocytic reaction at the site of injection was not correlated with this response; in fact, in the antigen in oil experiments the monocytic reaction reached its height after the peak of antibody production.

The tissue changes observed in the various experiments were consistent with the finding previously reported from this laboratory, that the lymphocyte is concerned in antibody formation.

BIBLIOGRAPHY

1. Le Moignic and Pinoy, *Compt. rend. Soc. biol.*, 1916, **79**, 201, 352.
2. Freund, J., Casals, J., and Hosmer, E. P., *Proc. Soc. Exp. Biol. and Med.*, 1937, **37**, 509.
3. Casals, J., and Freund, J., *J. Immunol.*, 1939, **36**, 399.
4. Freund, J., Casals, J., and Genghof, D. S., *J. Immunol.*, 1940, **38**, 67.
5. Freund, J., and McDermott, K., *Proc. Soc. Exp. Biol. and Med.*, 1942, **49**, 548.
6. Freund, J., and Walter, A. W., *Proc. Soc. Exp. Biol. and Med.*, 1944, **56**, 47.
7. Friedewald, W. F., *Science*, 1944, **99**, 453.
8. Friedewald, W. F., *J. Exp. Med.*, 1944, **80**, 477.
9. Henle, W., and Henle, G., *Proc. Soc. Exp. Biol. and Med.*, 1945, **59**, 179.
10. Freund, J., and Bonanto, M. V., *J. Immunol.*, 1944, **48**, 325.
11. Halbert, S. P., Mudd, S., and Smolens, J., *Proc. Soc. Exp. Biol. and Med.*, 1945, in press.
12. Rist, N., *Ann. Inst. Pasteur*, 1938, **61**, 121.
13. Ehrich, W., *J. Exp. Med.*, 1929, **49**, 361.
14. Ehrich, W., and Wohlrab, R., *Beitr. path. Anat. u. allg. Path.*, 1934, **93**, 321.
15. Ehrich, W., and Voigt, W., *Beitr. path. Anat. u. allg. Path.*, 1934, **93**, 348.
16. Halbert, S. P., Smolens, J., and Mudd, S., *J. Immunol.*, 1945, **51**, 39.
17. Ehrich, W., and Harris, T. N., *J. Exp. Med.*, 1942, **76**, 335.
18. Ehrich, W., and Harris, T. N., *Science*, 1945, **101**, 28.

PLATES

EXPLANATION OF PLATES

PLATE 21

All of the sections except that providing Fig. 19 were stained with Azur II and eosin. For Fig. 19 hematoxylin and eosin were used.

FIG. 1. Rabbit 8-4. Right foot pad; 2 days after injection of saline in oil. × 100.

FIG. 2. Rabbit 8-1. Right foot pad; 9 days after injection of saline in oil. × 100.

FIG. 3. Rabbit 7-7. Right foot pad; 3 months after injection of saline in oil. × 100.

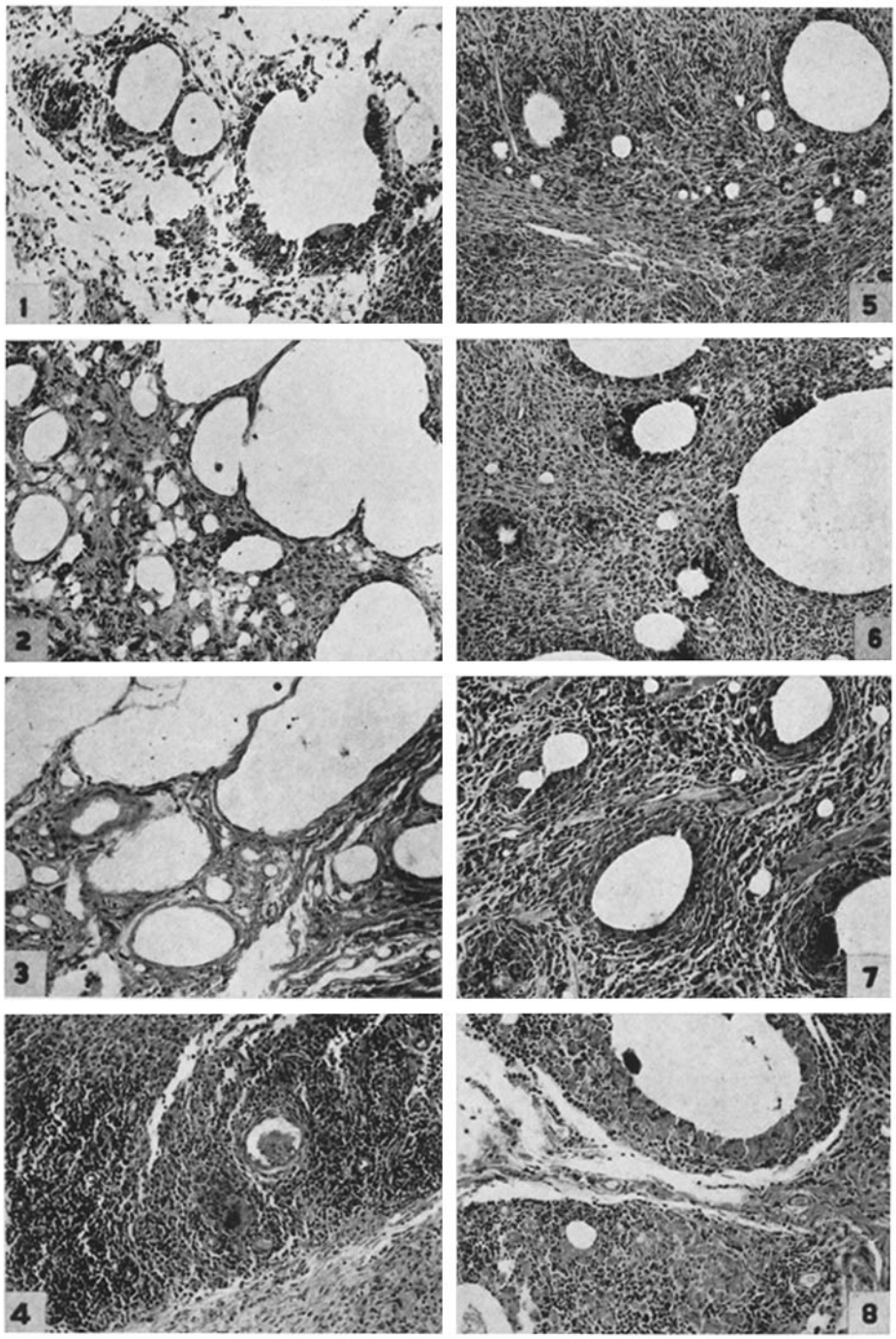
FIG. 4. Rabbit 9-2. Left foot pad; 14 days after injection of antigen in saline. × 100.

FIG. 5. Rabbit 1-00. Right foot pad; 6 days after injection of antigen in oil. × 100.

FIG. 6. Rabbit 6-8. Left foot pad; 9 days after injection of antigen in oil. × 100.

FIG. 7. Rabbit 6-5. Right foot pad; 14 days after injection of antigen in oil. × 100.

FIG. 8. Rabbit 6-4. Left foot pad; 3 months after injection of antigen in oil. × 100.



(Ehrich *et al.*: Action of mineral oil on antibody production)

PLATE 22

FIG. 9. Rabbit 9-3. Right foot pad; 1 day after injection of antigen in oil. Note the large number of dark staining bacteria in the oil drops. × 150.

FIG. 10. Rabbit 3-40. Right foot pad; 9 days after injection of antigen in oil. The bacteria in the oil drops (arrow) have largely disappeared. × 150.

FIG. 11. Rabbit 5-7. Diffuse lymphoid hyperplasia of left popliteal lymph node; 6 days after injection of antigen in saline. × 100.

FIG. 12. Rabbit 9-1. Large active secondary nodules in left popliteal lymph node; 9 days after injection of antigen in saline. × 90.

FIG. 13. Rabbit 9-2. Many macrophages in cortex of right popliteal lymph node; 14 days after injection of antigen in saline. × 140.

FIG. 14. Rabbit 2-8. Necrosis of secondary nodule of left popliteal lymph node; 12 hours after injection of antigen in saline. ×150.

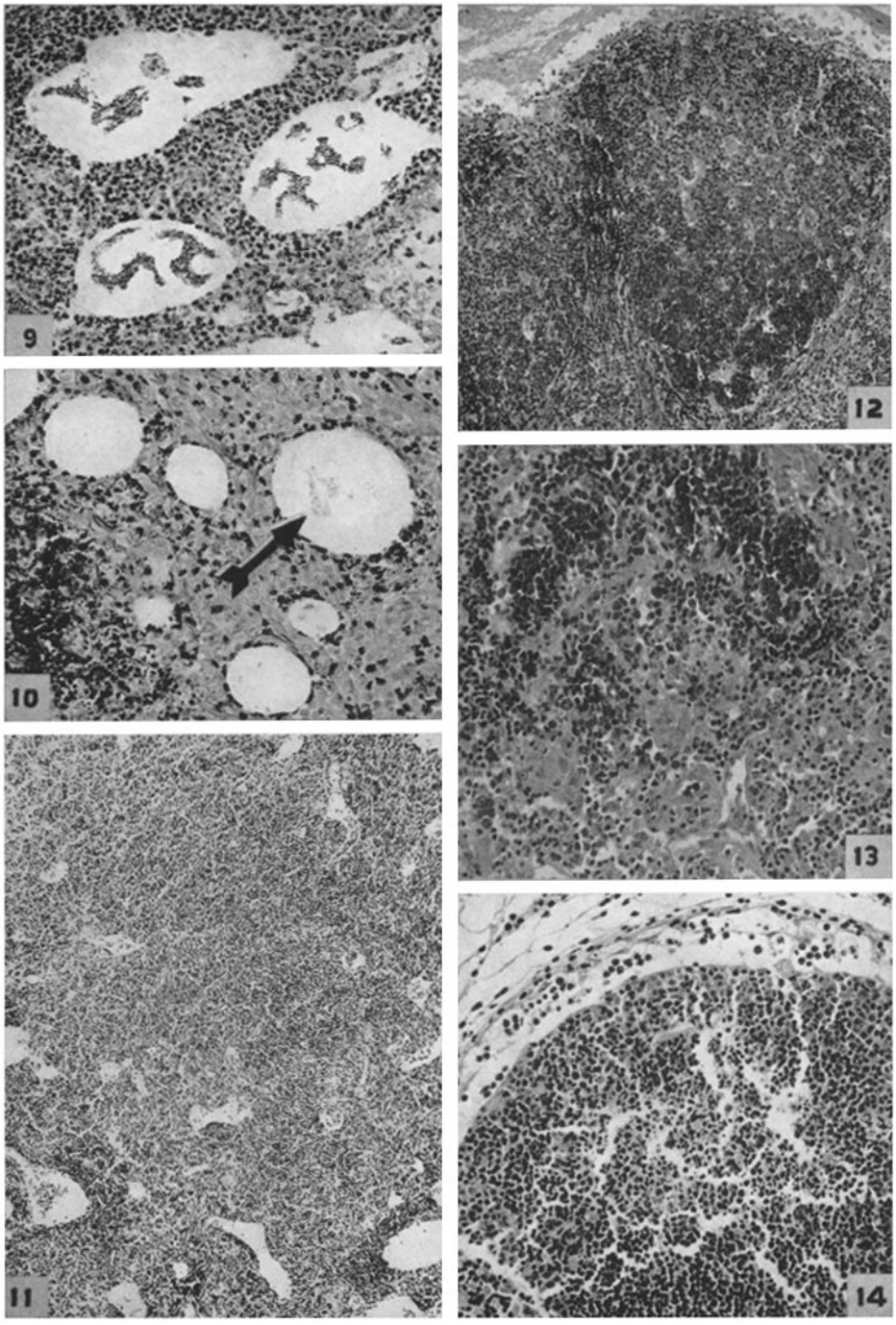


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PLATE 23

FIG. 15. Rabbit 1-00. Diffuse lymphoid hyperplasia of left popliteal lymph node; 6 days after injection of antigen in oil. × 100.

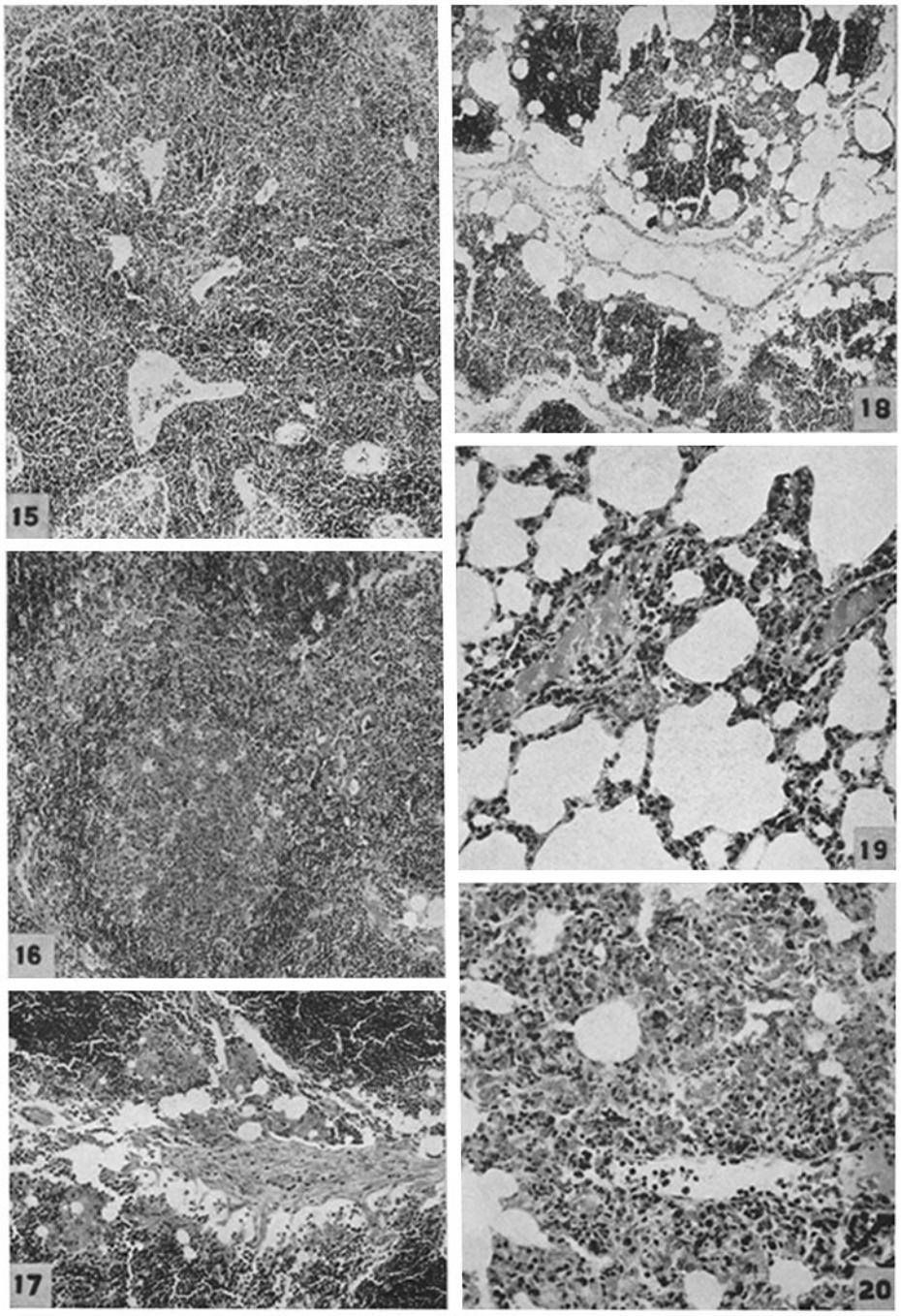
FIG. 16. Rabbit 3-29. Active secondary nodules in right popliteal lymph node; 9 days after injection of antigen in oil. × 90.

FIG. 17. Rabbit 3-33. Reticuloendothelial hyperplasia in left popliteal lymph node; 14 days after injection of antigen in oil. × 90.

FIG. 18. Rabbit 6-3. Oil drops in the sinuses and lymphatic tissue of the left popliteal lymph node; note absence of macrophages; 6 months after injection of antigen in oil. × 100.

FIG. 19. Rabbit 6-1. Early mesenchymal reaction in lung; 1 day after injection of antigen in saline. × 150.

FIG. 20. Rabbit 5-8. Marked mesenchymal reaction in lung; 4 days after injection of antigen in saline. × 150.



(Ehrich *et al.*: Action of mineral oil on antibody production)