STUDIES ON ANTIBODY PRODUCTION

IV. The Role of a Wax Fraction of Mycobacterium tuberculosis in Adjuvant Emulsions on the Production of Antibody to Egg Albumin*

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It is the purpose of this paper to draw attention to the very active adjuvant properties of tubercle bacillary wax in potentiating the serum antibody responses to ovalbumin, administered as a water-in-oil emulsion, subcutaneously into guinea pigs. Also the morphological effects of the injection of this antigenic mixture, with or without added tubercle bacillary wax, are described.

The usefulness of the Freund-type adjuvants in which the antigen is incorporated into the watery phase of a water-in-oil emulsion, the oily phase of which contains added killed mycobacteria, is now firmly established. The knowledge concerning their action and the realization of their usefulness has accumulated from many sources, some of which are quite unrelated to one another. Lewis and Loomis (1, 2) observed that living, virulent tubercle bacilli, injected into the peritoneal cavity of guinea pigs, enhanced antibody formation to various antigens subsequently introduced by the same route. This report led to many attempts to substitute killed mycobacteria for the living organisms, owing to the dangers and inconvenience of the latter. But killed bacteria are ineffective in this respect if administered in a watery medium. Coulaud (3) later demonstrated that persistent and intense skin sensitization could be produced in rabbits providing the killed mycobacteria were incorporated into melted paraffin. Many subsequent publications were devoted to various aspects of the relationship of paraffin oil and tubercle bacilli to the allergy of disease and to the mechanism of delayed skin sensitivity. In recent years this problem has received clarification from the work of Raffel and his associates. Raffel (4) showed that defatted tubercle bacilli were incapable of establishing tuberculin sensitivity, even if employed in very large doses and over long periods of time. But, if the defatted bacilli were mixed with a

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chloroform-soluble substance, a wax-like lipid, the ability of the bacilli to induce tuberculin sensitivity was restored. It was also shown that isolated tuberculoprotein could be employed with the wax to produce tuberculin sensitivity. Later, it was demonstrated (5) with the antigen egg albumin, that the simultaneous presence of tubercle bacillary wax in the tissues injected, will cause hypersensitivity of the delayed type to this antigen. Although this work did not concern itself primarily with the simple adjuvant action of such wax in enhancing the level of humoral antibody, a pronounced adjuvant effect was demonstrated. At the same time it was shown that wax in a watery medium was ineffective as an ordinary immunologic adjuvant.

The use of killed tubercle bacilli as simple adjuvants followed the observation of Freund, Casals, and Hosmer (6) that guinea pigs, injected with these organisms in paraffin oil, produced complement-fixing antibodies in high titer for at least twenty months. Subsequent work demonstrated the general effectiveness of killed tubercle bacilli incorporated into water-in-oil adjuvant mixtures in augmenting the antibody response to a wide variety of antigens, *e.g.* horse serum (7), influenza PR8 virus (8, 9), and poliomyelitis virus (10). Also, an increased ability to confer protection against infection with *Plasmodium knowlesi* (11, 12), and *Plasmodium lophurae* and *Plasmodium cathemerium* (13) was demonstrated following the use of killed tubercle bacilli in water-in-oil emulsion vaccines of these parasites. Certain results exist in which this potentiation of antibody response by tubercle bacilli was not demonstrated. Halbert, Mudd, and Smolens (14) recorded that the addition of tubercle bacilli to an emulsion of *Shigella paradysenteriae* (Flexner) in aquaphor, paraffin oil, and water had little or no effect on the antibody response. Also some of the results of Freund (15) attributed only a slight potentiating effect to the tubercle bacilli within the mixture; *e.g.*, in the case of *Salmonella typhi.*

Recently, Fischel *et al.* (16) determined by quantitative methods the influence of tubercle bacilli incorporated into oil-in-water emulsions of ovalbumin in potentiating the antibody response to the latter, and found that antigen emulsions with tubercle bacilli resulted in antibody nitrogen levels several times as high as those without this component.

Materials and Methods

Antigen and Mode of Administration.—The antigen used throughout this study was crystalline egg albumin as supplied by Armour & Company. Except when stated, a dose of 10 mg. per injection was used. Antigen was administered as a water-in-oil emulsion into the footpad of the guinea pig. This procedure was adopted in order to study the reactions in the popliteal and other regional lymphatic glands. The volume injected was 0.2 ml. The antigen was composed of saline solution of antigen, arlacel (batch 3375, Atlas Powder Company, Wilmington, Delaware) and bayol F (Esso Standard Oil Company), in the proportions (by volume) of 1:1:3 respectively. To prepare a batch of antigen-emulsion mixture (10 mg. crystalline egg albumin per dose) 100 mg. of egg albumin was dissolved in 0.4 ml. saline, 0.4 ml. of arlacel was added, and emulsification was achieved by drawing the mixture up into a 2 cc. syringe with an 18 gauge needle and expelling repeatedly into a test tube. If the needle point is kept below the surface it is possible to achieve good emulsification without the frothing which would tend to denature the antigen. 1.2 ml. of bayol F was then added and emulsification of the whole accomplished as before. This mixture was injected through a 26 gauge needle. When the tubercle bacillary wax was added to the mixture it was dissolved in the bayol F before emulsification.

Tubercle Bacillary Wax.—The wax used in this study was kindly supplied by Dr. Rudolf J. Anderson and consisted of "purified wax" (Anderson) (17). The initial stages in its preparation involved extraction of bacilli with alcohol-ether followed by chloroform extraction of the bacillary residues. The chloroform extract was then filtered and reduced to dryness in a current of carbon dioxide. The "crude" wax obtained at the end of these operations was separated (18, 19) into two fractions by precipitation thrice from ether solution by cold methyl alcohol and twice from toluene by methyl alcohol and acetone. The principal product is "purified wax" while "soft wax" remains in solution.

Animals.—Guinea pigs of weight 350 to 500 gm. were purchased from local dealers. They were fed on a pellet diet with supplementary cabbage *ad lib.*

Tests for the Presence of Delayed Sensitivity.—

Skin tests: 1 mg. of crystalline egg albumin in 0.1 ml. of physiological saline solution was injected intracutaneously into the skin of the flank, previously carefully shaved. Readings of ensuing reactions were made at 30 minutes, 1 hour, 24 and 48 hours.

Corneal tests: After preliminary anesthetization of the conjunctiva by instillation of a drop of a 0.5 per cent solution of tetracaine, intracorneal injections were made using a 30 gauge needle attached to a 0.25 ml. syringe. A solution of crystalline egg albumin, 20 mg. per ml. was used. An amount of antigen sufficient to cause a disc of opacity in the cornea, 2 mm. in diameter, was injected.

Tuberculin tests: Intracutaneous injections of 1:1000 dilution of Old Tuberculin (supplied by the Massachusetts Biologic Laboratories, Boston) were performed on a few animals.

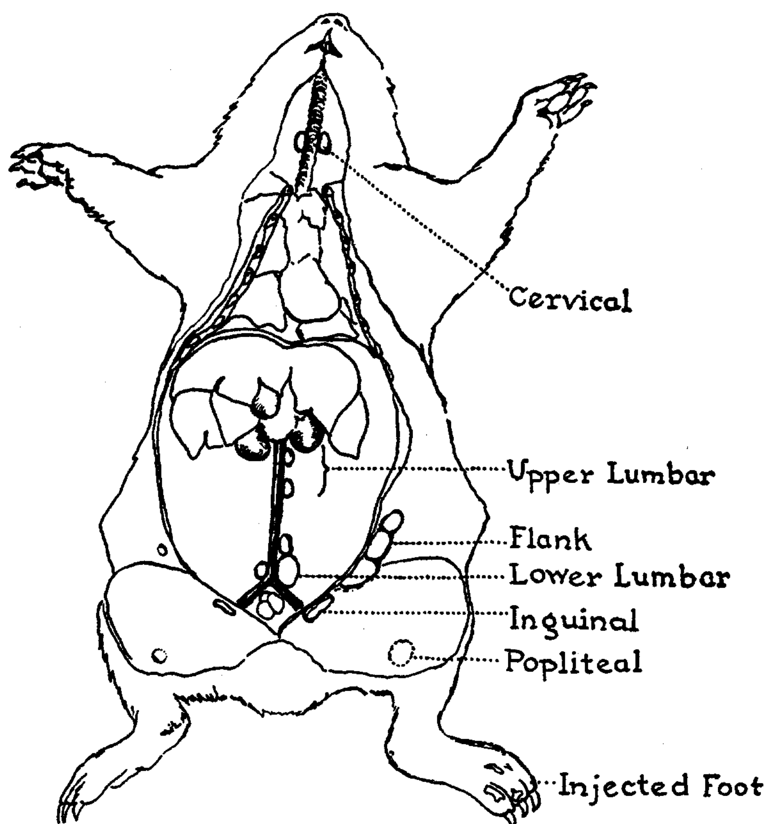
Estimation of Antibody Levels.—Sera were collected aseptically by cardiac puncture at the time of sacrifice, frozen, and stored at −15°C. until the time when all samples could be estimated together by a quantitative precipitin method modified from Kabat and Mayer (20). Analyses for anti–egg albumin nitrogen were carried out in duplicate. All sera were decomplemented by the addition to each milliliter of 100 μg. of rabbit anti-pneumococcal antibody nitrogen and an equivalent amount of SSS II, followed by refrigeration at 4°C. for 24 hours. The supernatant serum was recovered by centrifugation.

Based on preliminary precipitin tests (20), analyses were carried out on 0.2 to 2.0 ml. samples of serum, such as would yield approximately 100 μg. of nitrogen in the final precipitate. The amount of antigen used was calculated from the preliminary precipitin test and was such as to give maximum precipitation of antibody and a slight excess of antigen in the supernate. The precipitation was allowed to proceed for 30 minutes in the 37°C. bath and for 3 days in the refrigerator at 4°C. The nitrogen values of the twice washed precipitates were estimated by the method of Koch and McMeekin (21). Tests of the supernatants from this precipitation were made and the results were accepted when a small excess of antigen was indicated. Sera are recorded as containing no antibody when 5 μg. of egg albumin–nitrogen added to the sample of 2 ml. of serum showed no evidence of precipitation after 3 days in the refrigerator. In a few instances the results of sera containing larger amounts of antibody were checked by using 2 ml. amounts of serum in the macroprecipitin technique of Heidelberger and Kendall (22). Comparisons of the results showed that the method given above gives low values since an appreciable loss occurred during the washing of these small quantities of precipitate.

Morphological Techniques.—The tissues subjected to histological study were quick-frozen, stored, and sectioned by methods described previously (23, 24). Staining for ovalbumin and for anti-ovalbumin were carried out by the methods reported in references 25–27 and the controls of specificity of the reactions observed were identical with those reported there.

In order to localize the staining by the above techniques to structures stained by more conventional methods, adjacent sections were stained by Giemsa's method after fixation in

Bouin's solution. This method yields good staining of cytoplasmic basophilia and the cytoplasmic granules of eosinophil myelocytes and leucocytes. Nuclear detail is, however, lacking; nucleoli are obscured and nuclear chromatin pattern is difficult to determine. On this account, further tissue samples were fixed in Bouin's fluid and stained with hematoxylin and eosin. For the demonstration of cytoplasmic basophilia, the use of this staining method must be carried out so that adequate hematoxylin staining of cytoplasm remains after decolorization



TEXT-FIG. 1. Diagram of lymph gland system of guinea pig (10 mg. ovalbumin in water-in-oil emulsion, with 5 mg. wax from the tubercle bacillus.

and this should be followed with minimum eosin counterstaining. Accordingly, sections were stained for 15 minutes in Ehrlich's hematoxylin, washed in tap water, given adequate controlled decolorization in 2 per cent acid alcohol (1/2, 2 minutes) and washed thoroughly in running tap water for 10 minutes for adequate blueing of the basophilic structures. Staining with eosin lasted about 5 seconds only.

The nomenclature adopted in the descriptions of histological material follows the definitions of cells described in Marshall and White (28).

The nomenclature adopted for description of the gross morphological changes involving the lymphatic glands is illustrated in Text-Fig. 1, which shows the glandular involvement

following the injection of antigen (10 mg. ovalbumin) in water-in-oil emulsion with added wax (5 mg.). The flank glands were situated along the course of the inferior epigastric vessels which descend on the anterolateral aspect of the abdomen to join the femoral vessels in the inguinal region. The abdominal glands included the upper and lower lumbar groups on the posterior abdominal wall on either side of the inferior vena cava. The lower lumbar glands were constantly observed in normal animals on either side of the inferior vena cava just above the junction of the two iliac veins. The "cervical glands" were two glands constantly present on either side of the trachea at a point just above the middle of a line joining point of the lower jaw and xiphisternum.

RESULTS

The following descriptions refer to animals which received a single injection of 10 mg. of egg albumin antigen, administered as a water-in-oil emulsion, with the addition of 5 mg. of tubercle bacillary "purified" wax in animals referred to as "wax" animals, and without this addition in animals referred to as "controls." They were sacrificed 3 weeks after a single injection.

Local Lesion

The antigen mixtures were injected in a total volume of 0.2 ml. into the left foot-pad of guinea pigs. It was apparent that all mixtures, with and without added wax, acted as local tissue irritants. At 24 hours following injection the feet were obviously red and swollen. In the control group this swelling did not increase markedly beyond 48 hours and by the 7th day had decreased, although leaving the foot slightly larger than the opposite uninjected one. The animals injected with wax, inspected on the 7th day, showed a foot lesion which was obviously increasing in size, with a shiny bright blue cyanotic surface. There was a patchy denudation of hair from the dorsum, desquamation, and often small superficial ulcers on the lateral margin and on the dorsum of the toes near the base of the nail. The injected foot of the wax animals was at 10 to 12 days several times the size of its opposite member and had multiple superficial ulcers of the surface.

At autopsy (3 weeks after injection) dissection of the "wax" animals revealed that the swelling within the foot-pad was composed of firm, translucent, pale yellow tissue enveloping the plantar tendons and other normal anatomical structures. Occasionally small lacunae were encountered containing oily thick yellow exudate, and sometimes these presented at the surface to form sinuses. By contrast the lesion in the control animals was a just perceptible enlargement of the foot with a slight increase in spongy subcutaneous tissue.

Sections of the control animals revealed a small area of macrophage proliferation (Fig. 1) with increases in fibroblasts and young collagen fibres. The macrophages were small in size with a small extent of weakly basophilic cytoplasm. Throughout the area were "oil spaces." In the paraffin-embedded tissues these appeared as empty spaces, 25 to 50 μ in diameter, surrounded

by macrophages. Giant cells of foreign body type were present. Scattered areas containing polymorphonuclear neutrophil leucocytes and nuclear debris were encountered. The foot lesion in the "wax" animals was voluminous by comparison, and consisted of an extensive proliferation of macrophages (Fig. 2). Many of these had the so called epithelioid appearance. The cytoplasm was bulky, with spindle or polygonal outline, and possessed a slightly eosinophilic, finely granular, or opaque appearance. The nuclei were oval or reniform with undulating clear cut nuclear membranes and an "empty" interior with prominent nucleoli. Giant cells of the Langhans type were numerous. Oil spaces were present surrounded by typical epithelioid cells, as well as irregular areas of necrosis containing polymorphonuclear leucocytes in varying stages of disintegration. The areas which were composed almost exclusively of macrophages were enclosed by strands of cellular fibrous tissue with spindle fibroblasts and scanty groups and columns of plasma cells and occasional lymphocytes. Tissue of the same composition extended from the foot among the tendons and muscles of the lower leg. White, thickened cords of lymphatic vessels were a prominent feature on macroscopic inspection.

Sections which were stained for *ovalbumin* and for *anti-ovalbumin* antibody gave the following results. The local lesion from the wax-treated animals showed evidence of the presence of antigen in small areas related to the oil spaces. The sections stained for antibody demonstrated that the main mass of cells—the macrophages—was completely devoid of antibody staining. There was a very bright staining of collagen fibres and of the adventitia of blood vessels in the area. This staining corresponded with a surface-staining of the collagen fibres sharply outlining the individual fibres. The appearances were similar to those illustrated in Figs. 6 and 7. Occasionally the foot-pad contained numerous non-specifically stained eosinophil leucocytes. Antibody-containing cells were rare and a prolonged search was often necessary to find them. When they were found, they were present as small groups or rows of 5 to 10 cells, often alongside small arterioles or in the strands of young granulation tissue enclosing the masses of macrophages. Adjacent sections stained with Giemsa revealed these cells as mature and immature plasma cells. In lesions of control animals receiving egg albumin in water-in-oil emulsion without wax, the sections stained for antigen revealed a similar persistence of antigen in foci related to the oil droplets. In sections stained for antibody, as above, the most striking feature was staining of collagen fibres but this was much less intense than in the case of the wax-treated animals. The number of antibody-containing cells was rather greater than in the case of the wax-treated animals. As in these, they were situated commonly as small groups in strands of young fibrous granulation tissue, or to one side of small arterioles and corresponded with immature and mature type plasma cells (Fig. 3). It should also be noted that the young fibroblasts with basophilic cytoplasm did

not show evidence of staining for antibody in either wax or control groups of animals.

TABLE I

Weights of Lymphatic Glands in Experimental and Control Rabbits

Lymphatic gland	Wax, 5 mg.	Wax, 5 mg.	Control (no wax)	Control (no wax)
	gm.	*gm.*	*gm.*	*gm.*
Popliteal:				
Homolateral	0.23	0.16	0.07	0.020
Contralateral	0.055	0.045	0.055	0.015
Inguinal:				
Homolateral	0.12	0.14	0.06	0.035
Contralateral	0.06	0.05	0.05	0.020
Flank:				
Homolateral	0.35	0.16	0.095	0.060
	0.32	0.08		0.025
	0.18			
Contralateral	0.39	0.45	0.02	0.025
	0.15	0.15		0.025
Abdominal:				
Lower lumbar: homolateral	0.15	0.16	0.06	0.055
contralateral	0.08	0.07	0.06	0.040
Higher lumbar:	0.08	0.06	Nil	Nil
	0.05			
Bronchial:	—	0.03	0.04	0.03
Axillary:				
Homolateral	0.035	0.06	0.02	0.030
Cervical: (both glands)	0.14	0.11	0.12	0.160
Spleen:	1.04	0.85	0.77	0.76

Lymph Nodes

Table I sets out the detailed findings with regard to the autopsy findings of four animals; these findings are typical of the whole. Inspection of the results shows that in the control animals receiving egg albumin in water-in-oil emulsion, there was moderate enlargement of the homolateral popliteal, inguinal, flank, and lower lumbar glands. The animals which in addition received wax showed a much more striking enlargement of lymph glands over

a wide territory of lymphatic drainage. The homolateral, popliteal, and inguinal glands were 3 to 4 times normal mass and the homolateral flank glands were up 15 times normal mass. In addition, the lower lumbar glands, homolateral and contralateral, were both enlarged to several times the normal size and several glands were seen on the posterior abdominal wall above these. The contralateral flank glands were also obviously enlarged. The cut surfaces of the homolateral popliteal, flank, and lower lumbar glands were a firm, almost homogeneous, pale, yellow surrounded by a much thickened capsule of tough fibrous tissue. The cut surfaces of the remoter enlarged glands of the upper abdominal and contralateral flank glands were indistinguishable from normal gland tissue although areas of injection and circumscribed, more opaque, pale yellow areas could sometimes be seen.

Sections of Control Animals.—The popliteal, inguinal, homolateral, flank and lumbar glands showed isolated plasma cells and small collections of plasma cells in the medullary cords. The architecture of the lymphatic glands was well preserved, and the corticomedullary ratio varied little from normal. The capsule was not thickened. The lymphatic nodules contained germinal centers, and a normal cuff of small lymphocytes. The peripheral and medullary sinuses showed an increased content of rounded macrophages, polymorphonuclear neutrophil leucocytes, and eosinophils.

Sections of the popliteal glands stained for *anti-ovalbumin* antibody revealed numerous cells in the medullary cords with a cytoplasmic content of antibody. As illustrated in Fig. 4, they were uniformly scattered single cells for the main part, although small groups of cells occurred. The main cell type corresponded in size and cytoplasmic outline with immature plasma cells. A minority of the cells possessed more extensive cytoplasm of a brighter fluorescence, an eccentrically placed nucleus, (7 to 8 μ in diameter), and an oval space—5 $\times$ 3 μ approximately—which was devoid of fluorescence and abutted on the nucleus. These cells were considered to correspond to mature plasma cells, and the oval space corresponded with the prominent Golgi element of these cells. Also, larger cells occurred with a large round nucleus (12 to 14 μ in diameter) and a narrow rim of cytoplasm. The cytoplasmic fluorescence of these cells was less intense than that of the main type and somewhat patchy. This cell type was considered to correspond with the hemocytoblast (plasmablast) cell stage and to be more primitive than the others described previously. Usually the nucleus is represented by a completely blank oval or circle, but a proportion of all these cells showed irregular dots, rings or strands of fluorescent material in the nuclei. The shape of these nuclear fluorescent patches was variable. Sometimes they were multiple. A general impression was that they did not correspond with nucleoli. Many nuclei of cells with strongly fluorescent cytoplasm did not show any nuclear fluorescence, and the significance of the latter must await further experience.

Similar antibody-containing cells were scattered throughout the medullary cords of other glands in the region of lymph drainage and could be found in the homolateral flank gland, the homolateral inguinal gland, and the homolateral lower lumbar gland. They were very scanty or absent in the contralateral flank gland and absent in the contralateral popliteal gland.

These glands, also, occasionally contained non-specifically stained cells with the characteristics, established by comparison with Giemsa-stained adjacent sections, of eosinophil myelocytes and leucocytes.

Sections of Animals Receiving Wax.—The histological changes of the wax-treated animals varied with the situation of the gland in the pathway of lymphatic drainage. The gland nearest to the site of injection, the popliteal gland, showed a very intense macrophage proliferation (Fig. 5). The normal architecture was completely destroyed. The capsule of the gland was greatly thickened with fibrous tissue. Outside this and united to it by fibrous granulation tissue were dilated lymphatics with thickened fibrous walls and a content of packed macrophages and polymorphonuclears. Most of the gland was solidly filled with masses of macrophages enclosed by strands of fibrous tissue. Many of the macrophages had the appearance of epithelioid cells. There were numerous giant cells of the Langhans type. Oil spaces were present surrounded by epithelioid cells, as well as irregular areas of necrotic polymorphonuclear neutrophil and eosinophil leucocytes. The sole remaining recognizable lymphatic tissue comprised a few small islands of cortex isolated by strands of fibrous tissue. Plasma cells were scanty but a few single or small groups of immature and mature types of cells could be found in the strands of young fibrous tissue and under the capsule.

The inguinal and homolateral flank lymphatic glands also showed a marked disorganization of their architecture by macrophage proliferation, and the main part of a cross-section of a gland was occupied by masses of macrophages, but usually here the underlying architecture could be traced and the remaining normal lymphoid tissue was greater. The macrophage proliferation was mainly a sinus proliferation affecting both the cortical peripheral and the medullary sinuses. The capsule was thickened and the several glands of the flank were matted together by fibrous tissue containing thickened lymphatics with fibrous walls. In these glands the medullary strands showed a marked plasma cellular proliferation. Usually it was possible to see that this proliferation surrounded arterioles in the medulla. Often the reaction was so intense that over 90 per cent of the cells in such an area were plasma cells. The most common form was the immature plasma cell. Hemocytoblasts (plasmablasts) were also present as well as mature plasma cells. Mitotic figures were commonly seen.

The lymphatic glands more remote from the point of injection, the abdominal glands and the contralateral flank glands, demonstrated a reduced degree of disorganization by macrophage proliferation. The main feature here was an

intense plasma cellular proliferation of the medulla. Sometimes this resulted merely in broadening of medullary strands which are solidly composed of cells with a high proportion of plasma cells, but sometimes the proliferation became nodular in type with the development of rounded masses consisting almost entirely of plasma cells. The cortical tissue appeared reduced in amount, the lymphoid nodules were shrunken with prominent centers of large lymphocytes and macrophages containing phagocytosed material and a narrow rim of small lymphocytes.

Sections stained for *ovalbumin antibody* showed few antibody-containing cells in the homolateral popliteal gland. As stated above, the main feature of such a section is massive macrophage and epithelioid cell proliferation. These cells were devoid of staining for antibody. A similar statement holds for the homolateral flank glands. Here, too, were massive areas of macrophage proliferation and these were without antibody staining. A very interesting feature of these glands was, however, the strong antibody staining of the collagen of the thickened capsule and of the adventitia of vessels outside the capsule. This is shown in Fig. 7. As shown in Fig. 6 the larger vessels within these glands also showed striking antibody staining of the fibres of their adventitia. This staining of collagenous fibres was absent from the glands remote from the site of injection and also from the fibrous tissue of other parts of the body used in controls. An analysis of the factors contributing to this interesting localization of antibody to certain regions of fibrous tissue is too lengthy a subject for analysis here but it is hoped to make it a subject for a future presentation. Sections stained for *ovalbumin antigen* failed to demonstrate any antigen in the glands or the periglandular fibroses.

While few antibody-containing cells could be found in the homolateral popliteal gland, it was usual to find a few isolated strands and groups of antibody-containing cells in the homolateral glands of the flank. These were found often just beneath the capsule or as columns of cells in the cellular young fibrous tissue between the islands of macrophages. Careful inspection of adjacent sections of the same tissue showed that these areas corresponded to areas of plasma cells. In these glands it was possible by the same method to determine that the fibroblasts did not exhibit antibody staining.

The sections of those glands more peripherally situated in the region of lymphatic drainage, such as the contralateral flank glands, presented quite a different picture from the aforementioned glands. Here, the medullary region was the site of intense, almost confluent growth of antibody-containing plasma cells (Fig. 8). At higher magnification, the same gland, Fig. 9, showed a high proportion of the cells in the medulla of this gland to possess antibody. The regions of the lymphoid nodules are seen to be devoid of such cells and the boundaries of the nodules are sharply outlined. In some contralateral flank glands massive nodular proliferations of antibody-containing cells were seen.

Similar medullary proliferation of plasma cells was observed in the lower abdominal or lumbar glands. The contralateral popliteal glands did not show antibody-containing cells. Occasionally antibody-containing cells could be demonstrated in the medulla of cervical glands (Fig. 10).

Liver

While no abnormality could be detected in the control animals, sections of the liver from "wax" animals showed small granulomata in the portal spaces. The cells were macrophages together with plasma cells, lymphocytes, and scanty polymorphonuclear neutrophil leucocytes. These appearances resembled those obtained after multiple injection of antigens by the intravenous route (36). These small granulomata contained antibody-staining cells (up to 20 approximately) in the appropriately stained frozen sections. No antigen could be demonstrated in the liver.

Lungs

All animals demonstrated a very striking change which was visible to the naked eye on close inspection and which consisted in the presence of numerous grayish white areas 0.5 to 2 mm. diameter on the pleural surface of the pulmonary lobes. Sections of the lungs revealed that this appearance was due to numerous subpleural singular tiny nodules of firm gray translucent tissue, often surrounded by a narrow zone (0.5 mm. wide) of hyperemia. These areas were rendered more easily visible after the lungs had been immersed a short time in Bouin's fixative, then standing out clearly, pale gray against a yellow background. The main cellular component was the macrophage, often demonstrating epithelioid cellular characteristics together with variable numbers of polymorphonuclear leucocytes and eosinophils (Figs. 11 and 12). Giant cells of Langhans type were common. Lymphocytes and plasma cells did not make up more than a very small proportion of the cells. Sections of lungs stained for antibody content revealed few antibody-containing cells in these granulomata.

Spleen

The spleen of the "wax" animals was obviously enlarged beyond the size of that of the control animals. The thickness of the organ was increased and the upper and lower borders were rounded. The cut surface was a paler red than the controls. Malpighian corpuscles were still visible although somewhat obscured. The sections of the wax group showed a striking macrophage proliferation of the sinus endothelium, with the presence of numerous free macrophages in the sinuses which were packed and expanded. Also, a plasma cellular reaction was manifest throughout the red pulp, usually as scattered individual cells or small collections but in some animals large collections of cells numbering 50 to 100 were present. These were mainly composed of immature type plasma

cells with smaller proportions of hemocytoblasts (plasmablasts) and mature plasma cells. The sections stained to reveal antibody showed scattered antibody stained cells and collections of cells (Fig. 13). The correspondence between these areas of fluorescent antibody-stained cells and the areas of plasma cell aggregations as revealed by Giemsa staining of adjacent sections was established and an example of this is presented in Figs. 13 and 14.

No evidence of antibody-containing cells was found in the spleens of control animals, which received saline solutions of egg albumin antigen emulsified in paraffin oil.

TABLE II

Skin Sensitivity to Egg Albumin in Animals Sensitized with and without Wax

Sensitizing treatment (single doses in all cases)	Reactions*			
	½ hr.	1 hr.	24 hrs.	48 hrs.
10 mg. egg albumin in water-in-oil plus 5 mg. wax	0:6:1	0:12.5:2	20:35:6	20:30:4
	0:5:1	0:15:3	16:30:6	16:24:4
	0:5:1	0:11:3	15:28:5	14:25:4
	0:2:0	0:6:2	16:27:6	15:23:3
	0:6:1	0:15:3	19:34:6	20:30:4
10 mg. egg albumin in water-in-oil	0:5:1	0:14:3	0:8:2	0:1.5:0.5
	0:6:1	0:12:2	0:10:2	0:1.0:0.5
	0:7:1	0:14:2	0:8:2	0:2.0:0.5
	0:5:1	0:12:2	0:6:1	0:1.0:0.5
	0:6:1	0:10:2	0:5:1	0:1.0:0.5

* The first figure gives the diameter of necrosis of the skin, the second figure, the greatest diameter of the erythema and the third, the height above the surrounding skin approximately (measured in millimeters).

Cutaneous Reactivity to Egg Albumin in Variously Sensitized Guinea Pigs

Skin tests in the various groups of animals were carried out on the 18th day after injection of the antigen mixtures. Readings were made at constant time intervals thereafter up to 48 hours. In the tests, intracutaneous injection of 1 mg. of crystalline egg albumin dissolved in 0.1 ml. physiological saline was used. The results are presented in Table II for animals receiving a single dose of antigen in water-in-oil emulsion with 5 mg. added wax together with controls.

A further study of the effect of dosage of wax on cutaneous reactivity was carried out with animals receiving doses of wax varying from 0.040 mg. to 1 mg. In this case the test dose of antigen which was injected intracutaneously into the shaved flank was 0.1 mg. of crystalline egg albumin in 0.1 ml., since the reactions obtained in the above group of animals with 1 mg. of egg albumin were considered unnecessarily severe. The results are shown in Table III.

All animals used in this investigation were not skin-tested, owing to the possibility that an alteration of the cellular morphology would be caused thereby, especially within draining lymph nodes.

Corneal Reactivity to Egg Albumin in Various Sensitized Guinea Pigs

The corneal reaction, occurring as it does in an avascular tissue, has been accepted (5, 29, 30) as a means of revealing delayed types of hypersensitivity, on the theory that in the absence of blood vessels the Arthus response cannot occur.

TABLE III

Skin Sensitivity to Egg Albumin in Variously Sensitized Animals

Sensitizing treatment (all single doses as water-in-oil emulsion)	Reactions*		
	1 hr.	24 hrs.	48 hrs.
10 mg. egg albumin, 5 mg. wax	0:15:2	5:19:2	5:14:1
10 mg. egg albumin, 1 mg. wax	0:14:2	5:30:2	5:25:2
10 mg. egg albumin, 0.2 mg. wax	0:6:1	0:15:2	0:15:2
10 mg. egg albumin, 0.04 mg. wax	0:13:1	0:25:2	0:20:1
1 mg. egg albumin, 1 mg. wax	0:7:1	0:16:2	0:7:1
No antigen, 5 mg. wax	0:0:0	0:0:0	0:0:0
10 mg. egg albumin, no wax	0:4:0.5	0:1:0	0:1:0
1 mg. egg albumin, no wax	0:5:0.5	0:3:1	0:1:0.5

* The first figure gives the diameter of necrosis of the skin, the second figure, the greatest diameter of the erythema and the third, the height above the surrounding skin approximately (measured in millimeters).

Simultaneously with the skin tests, at 18 days following injection of the antigen emulsions, the animals were inoculated centrally into the cornea of one or both eyes with a droplet of albumin solution (20 mg. per ml.). The eyes were examined at 24 and 48 hours. The results are recorded in Table IV.

At 3 days following the cornea test the whole eyeball was dissected free at the autopsy and quick-frozen in a test tube which was plunged into an alcohol-CO_2 mixture. Frozen sections were prepared and the positive reactions confirmed by the histological appearances. In positive reactions the cornea appeared grossly thickened and there was an intense cellular exudation throughout the whole thickness. Individual corneal fibres appeared grossly swollen. In contrast the corneas from animals of the negative reactions showed either slight or no changes.

As seen from the table definite positive reactions were obtained with all animals receiving egg albumin antigen and 0.2 mg. to 5 mg. wax in the sensitizing dose. Weak non-persistent reactions were obtained with 0.04 mg. wax.

Reactions in the control animals which received no wax were either completely absent or were slight and non-persistent.

TABLE IV

Corneal Reactivity to Egg Albumin in Variously Sensitized Animals

Sensitizing treatment (single dose of antigen in water-in-oil emulsion)	Result	
	24 hrs.	48 hrs.
10 mg. egg albumin, 5 mg. wax (3 animals)	+++	+++
10 mg. egg albumin, 5 mg. wax (2 animals)	+++ and C	+++ and C
10 mg. egg albumin, 1 mg. wax	+++	+++
10 mg. egg albumin, 0.2 mg. wax	+++	+++
10 mg. egg albumin, 0.04 mg. wax	+	−
1 mg. egg albumin, 1 mg. wax	+++	+++
1 mg. egg albumin, 0.04 mg. wax (2 animals)	−	−
No antigen, 5 mg. wax	−	−
10 mg. egg albumin, no wax		
2 animals	−	−
1 animal	+	−

+++, opacity affecting whole area of cornea.
+, small area of opacity about the site of needle penetration.
C, chemosis.

Histological Description of Skin Tests

Sections taken 72 hours following injection of 1 and 0.1 mg. of crystalline egg albumin intradermally demonstrated the following features. With the 1 mg. injection dose, necrosis occurred over a wide area of the epidermis and underlying dermis down to the muscle. The acellular eosinophilic debris enclosed necrotic, though recognizable, polymorphonuclear neutrophil leucocytes, and macrophages. The area of the dermis surrounding the necrosis demonstrated a massive infiltration with cells mainly macrophages. The superficial dermis was packed with sheets of elongated spindle-shaped macrophages lying between collagen fibres. Near the site of injection the collagen fibres were deeply eosinophilic and necrotic. The deeper layers of the dermis were packed with spherical and polygonal macrophages often massed in spherical nodules. This deeper zone also showed dilated vessels containing densely packed macrophages, many necrotic, and polymorphonuclear neutrophil leucocytes. The muscle bundles underlying the site of inoculation were necrotic. Irregular strands of macrophages separated the muscle bundles. Animals injected with a 0.1 ml. dose of egg albumin showed little or no necrosis and correspondingly less cellular infiltration. Plasma cells were not a prominent feature of the cellular extravasation. A few were sometimes observed in the superficial dermis around and between the hair follicles.

Frozen sections were also investigated by staining for the presence of antigen and antibody to egg albumin at 3 days after skin testing. The most striking feature was the brilliant fluorescent staining indicating the presence of antigen in the area of necrosis and surrounding dermis. The superficial layers of the dermis showed brilliant antigen staining in circumscribed globules or collections of globules resembling mulberries. The deeper layers of the dermis contained leucocytes packed with antigen, many appearing necrotic. The vessels in this zone were greatly dilated and crowded with antigen-containing microphages and macrophages (Figs. 17 to 19). No antibody-containing cells were found.

TABLE V

Intensity of Skin Reactions to 0.1 Mg. Old Tuberculin in Guinea Pigs 18 Days after Injection of an Antigenic Mixture (10 Mg. Ovalbumin in Water-in-Oil Emulsion) with and without Added Tubercle Bacillary Wax

Dose of purified wax	Results*	
	24 hrs.	48 hrs.
1 mg.	4:0:0	2:0:0
	10:2:0	7:2:0
	12:2:0	10:2:0
0.2 mg.	9:2:0	8:1:0
	12:2:0	7:1:0
	9:1:0	5:0:0
	8:?:0	3:1:0
No wax	5:0:0	2:0:0
	3:0:0	2:0:0
	2:0:0	1:0:0
	2:0:0	2:0:0

* The first figure represents the diameter of erythema; the second figure represents the diameter of blanching of surface; the third figure represents the diameter of obvious macroscopic necrosis.

Histological Findings in Animals Receiving Less Than 5 Mg. Wax

A few animals were observed following the injection of amounts of wax 1 mg., 0.2 mg. and 0.04 mg. per single injection dose of water-in-oil emulsion and subjected to autopsy at 3 weeks following injection. The morphological findings with animals receiving 5 mg., 1 mg., and 0.2 mg. of wax appeared very comparable. The smaller doses—1 mg. and 0.2 mg.—also caused the massive macrophage granulomatous swelling of the foot-pad, and the great enlargement of the popliteal and homolateral flank and intra-abdominal lymph glands. The same intense plasma cellular reaction was present in the more remote lymphatic glands. However, reactions to the 0.04 mg. dose produced an

obviously diminished response. The foot-pad of these animals, at 3 weeks, was only just appreciably larger than that of controls similarly injected minus wax. The reaction in the popliteal and homolateral flank lymphatic glands was a patchy macrophage reaction within the sinuses, and in these animals plasma cellular aggregations were present in the medulla of the popliteal (Fig. 15) and homolateral flank glands. The macrophage proliferation was thus much less extensive in the foot-pad and its draining lymphatic glands and the plasma cellular reaction occurred in glands closer to the site of injection. Moreover, little abnormality was present in the lungs of these animals. The reactions in the homolateral flank, inguinal, and abdominal (lower lumbar) lymphatic glands consisted of isolated scattered antibody-containing cells. The contralateral flank glands and the more remote glands usually showed no evidence of the presence of antibody-containing cells. The spleen usually, but not always, contained scattered single cells and small collections of antibody-containing plasma cells in the medulla (Fig. 16). None were found in the liver or lungs in these animals.

Certain animals injected with ovalbumin in water-in-oil emulsions, with and without added wax, were given intradermal injections of old tuberculin, 0.1 mg. to determine the presence of delayed type skin sensitization to this antigen. The results are shown in Table V.

DISCUSSION

Previous reports have demonstrated the remarkable effect of killed tubercle bacilli in augmenting the antibody response to an antigen when the bacilli and the antigen was incorporated in a water-in-oil emulsion (6–10, 15). Other mycobacteria (*M. butyricum*, *M. phlei*) are also active in this way (15), so that it seems likely that the adjuvant effect is dependent upon a chemical constituent common to these organisms. Raffel *et al.*, in their studies of a wax fraction of the tubercle bacillus, collected data indicating that this fraction had an adjuvant effect on antibody production to egg albumin when the injection mass was prepared in water-in-oil, but not when the wax was injected as a suspension in water (5).

The results of the studies reported in this paper shed some new light on some of the factors responsible for the adjuvant effect, but the complex effects of oil, wax, persistence of antigen, and cellular responses have by no means been unravelled.

Water-in-oil emulsions without added tubercle bacilli apparently have two effects, as pointed out by Freund (32, 33). In the first place, the destruction and elimination of the antigen are retarded. Halbert, Mudd, and Smolens found that *Shigella* antigen persisted in mice for 22 weeks after injection when incorporated in water-in-oil. Further, Freund (33) removed the tissue at the site of water-in-oil deposits of antigen at various intervals,

and found that at 14 days there was no diminution in antibody titer, compared with controls, for as long as 5 weeks after the antigen injection, and that surgical removal of the antigen mass ½ hour after injection lowered the titer but did not prevent antibody formation. In the work reported here, there was histological evidence of oil droplets associated with antibody in a cervical lymph gland 3 weeks after injection of the water-in-oil emulsion (Fig. 20). Similar droplets in adjacent sections stained with Sudan III. No antigen was demonstrable in these droplets, but we interpret this finding as indicating the persistence of antigen in the oil and the attraction to it of antibody from the circulation. Despite the effect of the oil in preserving the antigen at the site of inoculation, and in serving as a vehicle for its dissemination, the antigenic stimulus begins to wear off, at least to egg albumin, within 4 weeks as indicated by the fall in antibody nitrogen levels determined by Fischel *et al.* (16). This suggests the existence of a mechanism which prevents the antigen present locally from acting as an effective stimulus to antibody-producing cells. It is tempting to assume that this is accomplished by the macrophage granuloma and the fibrosis which develops at the injection site. The latter factor was suggested by Holt (38) to explain a similar occurrence in the case of the granuloma produced by alum-precipitated diphtheria toxoid. The accumulation of macrophages may lead to a more rapid destruction of antigen than would be the case if they were not present, or if their function were impaired.

Although the wax fraction is ineffective as an adjuvant in the absence of mineral oil (5), its addition to the water-in-oil emulsion greatly increases the antibody response. The data in Table VI show that an appreciable adjuvant effect is manifest when a dose of 40 μg. of wax is added to such an emulsion containing 10 mg. of ovalbumin. With this dose there is a 4 to 5-fold increase in the amount of antibody at the median over that in animals receiving the same mixture without the wax. When 5 mg. of wax was added, the antibody response was about 8-fold higher. The other results suggest that a dose of 200 μg. is but little less active than 5 mg., (although the results with the 1 mg. dose are surprisingly low).

A striking feature of the effect of the wax was a stimulation of macrophage proliferation. This was extraordinarily intense at the site of injection (the foot-pad), but also occurred in the regional lymphatic glands, and in more remote situations, for example, the lungs. These cellular changes are comparable to those reported by Rist (31) following the injection of whole killed tubercle bacilli in mineral oil injected into guinea pigs subcutaneously. The presence of the mineral oil is essential for the development of this systematized stimulation of the reticulo-endothelial system, as well as for the adjuvant effect, for when he injected the bacilli in aqueous suspension, a polymorphonuclear leucocyte abscess developed which quickly resolved.

Previous observation of the striking cellular characteristics of the local

lesion with oil and bacilli (32, 33) has led to speculation regarding the possibility that the cells assembled locally were responsible for the increased humoral antibody levels. Westwater (34) found complement-fixing antibody to antigenic components of the tubercle bacillus in the local granuloma before any was detectable in the blood. Freund *et al.* found higher titers of diphtheria antitoxin in fluid aspirated from local injection sites in the horse than in the serum (35). The findings reported here, however, do not support the conclusion that the local granuloma, composed mainly of macrophages and epithelioid cells, is important in the production of antibody. Both these cell types were devoid of

TABLE VI

Antibody Response (Serum) of Guinea Pigs Immunized to a Saline Solution of Crystalline Egg Albumin in Emulsion with Adjuvants Containing and Lacking Tubercle Bacillary Wax, 3 Weeks Following Injection

Group A: with tubercle bacillary wax				Group B: without tubercle bacillary wax
Dose 5 mg.	Dose 1 mg.	Dose 0.2 mg.	Dose 0.04 mg.	
µg. antibody N/ml.	*µg. antibody N/ml.*	*µg. antibody N/ml.*	*µg. antibody N/ml.*	*µg. antibody N/ml.*
				0
		280		0
216	191	300	106	0
282	195	311	198	21
310		380	220	22
368		476		36
495				39
				39
				90
				91
				105

detectable antibody. So also were the fibroblasts. Scanty cells of the plasma cell series were present in our material, and contained anti-ovalbumin, but the main mass of antibody detectable in such granulomata at 3 weeks was deposited upon collagen fibres, and apparently was formed elsewhere. A full analysis of the factors determining such a localization of antibody after its formation is beyond the scope of this paper. It is hoped that it may form the basis of a future communication.

The morphological studies described above demonstrate that the effective production of antibody is carried out mainly at sites remote from the point of injection. The lymphatic glands immediately receiving the drainage from the foot showed almost complete replacement by a massive proliferation of macrophages. Although areas of antibody-containing plasma cells could be

found in the homolateral popliteal and flank glands, the plasma cell reaction attained its greatest height in more remote glands; *e.g.*, the lower abdominal glands and the contralateral flank glands. Some antibody was also found in the red pulp of the spleen and in granulomata along the periportal spaces in the liver (*cf.* references 25, 36, 37). Whether the active chemical fraction of tubercle bacillary wax which is responsible for the adjuvant effect can directly stimulate the production of plasma cells by itself is unknown. Injections of the "purified wax" used in these experiments, in water-in-oil emulsions without added antigen resulted in an impressive plasma cell reaction in the local lymph nodes, and in remote situations like the spleen. However, our wax was not freed of tuberculoprotein (4) or other possible contaminating antigens.

The mode of action of the wax in causing macrophage and epithelioid cell proliferation, increased plasma cell response and humoral antibody levels, or the occurrence of delayed hypersensitivity is unknown. Several authors have stressed the prolongation of the antibody response, when tubercle bacilli are added to water-in-oil emulsions. This was noted in the original observations of Freund and his coworkers (6), and is clearly shown in the data of Fischel *et al.* (16). It is conceivable that this effect of tubercle bacilli and the wax derived from them may be dependent on interference with some mechanism which normally destroys antigen locally; for example, an interference with the enzymes of macrophages. Possibly the morphological changes in the nucleus and cytoplasm defined by the term "epithelioid cell" are a manifestation of such a process.

It is a pleasure to acknowledge the assistance of Mr. Philip L. Isenberg, student at the Harvard Medical School, Boston, in the estimation of levels of serum ovalbumin antibody.

SUMMARY

After injection of ovalbumin as a water-in-oil emulsion a pronounced adjuvant effect is demonstrable following the incorporation of tubercle bacillary wax into the oily phase of the mixture. With single doses of antigen (10 mg. ovalbumin) there is a 4- to 5-fold increase in the amount of antibody at the median of 3 week serum levels in animals receiving a small dose of wax (40 μg.). With a 5 mg. dose of wax there is an 8-fold increase in serum antibody levels at the median.

A striking feature of the action of wax is the stimulation of a macrophage proliferation locally at the site of injection, and the production of morphological abnormalities in these cells. As judged by staining techniques for antibody content, these locally assembled cells are not active in the formation of antibody. Wax injected in mineral oil results in a remarkable systematized stimulation of the reticulo-endothelial system.

The greatly increased serum antibody levels demonstrated after the use of

tubercle bacillary wax in antigen mixtures is attributed to a widespread proliferation of plasma cell elements in the lymphatic glands, spleen and liver.

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EXPLANATION OF PLATES

All photomicrographs are of organs from guinea pigs, inoculated into the hind foot-pad 3 weeks previously with 10 mg. ovalbumin in water-in-oil emulsion with or without various doses of added tubercle bacillary wax. In the fluorescence micrographs the lightest areas represent the yellow-green fluorescence of the deposited fluorescein antibody conjugate except when otherwise stated; the topography of the organs is made visible by the faint blue autofluorescence of the normal tissue. Sections were stained for anti-ovalbumin unless otherwise indicated. This procedure reveals antigen as well, and when the material stained is ovalbumin it will be so stated in the description.

PLATE 16

FIG. 1. Foot-pad, subcutaneous tissue at site of injection of antigen mixture (without wax). Hematoxylin and eosin. × 205.

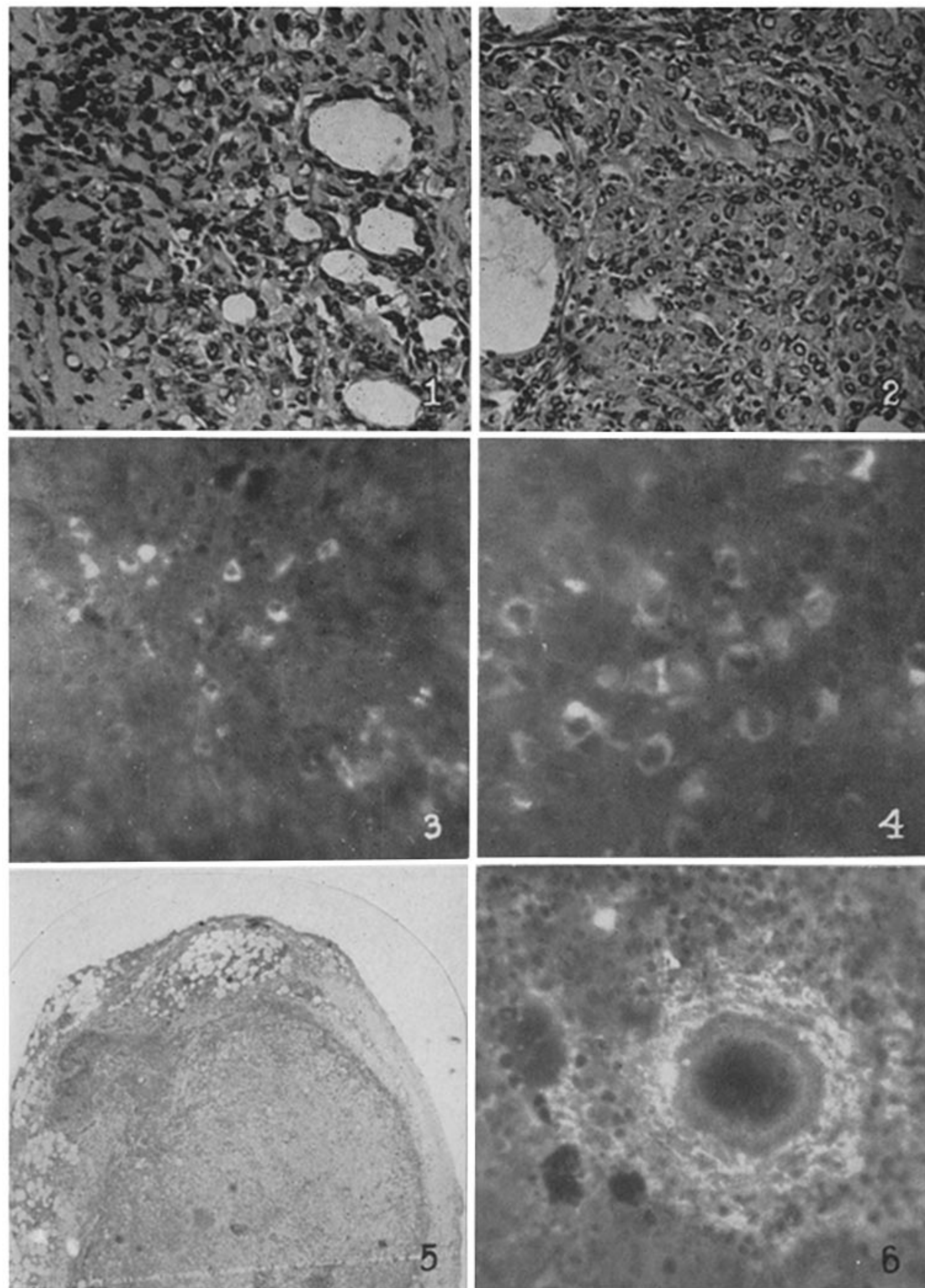
FIG. 2. Foot-pad, subcutaneous tissue at site of injection of antigen mixture with 5 mg. tubercle bacillary wax. Hematoxylin and eosin. × 205.

FIG. 3. Foot-pad, antibody-containing cells within lesion at site of inoculation of antigen mixture (without wax). Fluorescence micrograph. × 300.

FIG. 4. Homolateral popliteal lymphatic gland. Scattered, single, antibody containing cells. Antigen mixture (without wax). Fluorescence micrograph. × 600.

FIG. 5. Homolateral popliteal lymphatic gland. Antigen mixture with 5 mg. tubercle bacillary wax. Note gross thickening of capsule with fibrous tissue and almost complete replacement of the architecture of the gland with macrophage cellular proliferation. Hematoxylin and eosin. × 18.

FIG. 6. Artery of homolateral flank lymphatic gland. Antigen mixture containing 5 mg. tubercle bacillary wax. Note bright fluorescence denoting presence of antibody upon adventitial fibres. Fluorescence micrograph. × 300.



(White *et al.*: Studies on antibody production. IV)

Plate 17

Fig. 7. Homolateral flank lymphatic gland. Fibrous thickening of capsule. Antigen mixture with 5 mg. tubercle bacillary wax. Note very bright fluorescent staining of collagen fibres. Fluorescence micrograph. × 300.

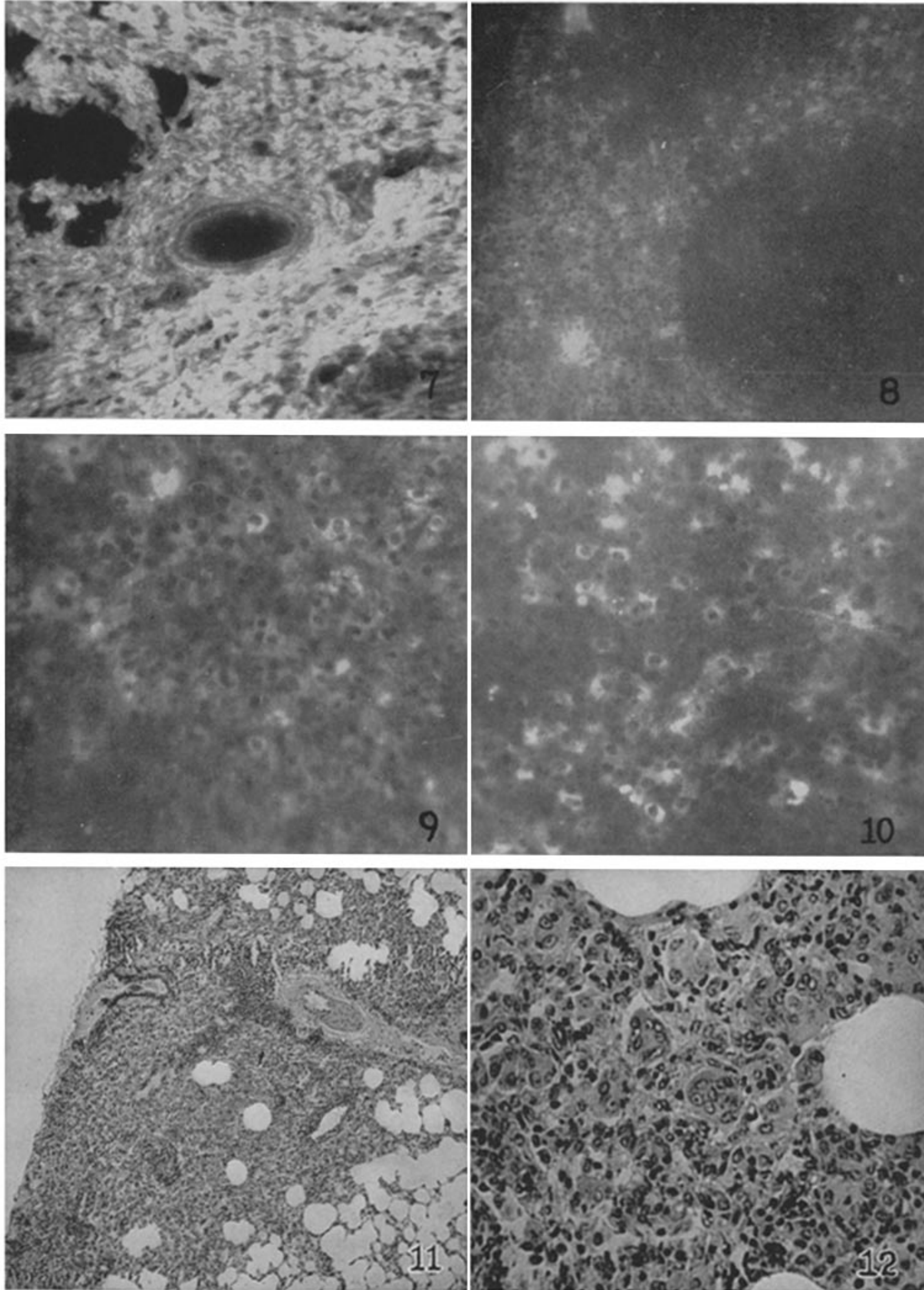
Fig. 8. Contralateral flank lymphatic gland. Antigen mixture with 5 mg. tubercle bacillary wax. Note intense medullary proliferation of cells with cytoplasmic antibody content, and the absence of these from lymphoid nodules. Fluorescence micrograph. × 150.

Fig. 9. Contralateral flank lymphatic gland. Higher magnification of medullary region from same gland as Fig. 8. Fluorescence micrograph. × 300.

Fig. 10. Cervical lymphatic gland. Antigen mixture with 5 mg. tubercle bacillary wax. Note numerous scattered antibody-containing cells in medulla of the gland. Fluorescence micrograph. × 300.

Fig. 11. Lung. Subpleural granuloma. Antigen mixture with 5 mg. tubercle bacillary wax. Hematoxylin and eosin. × 46.

Fig. 12. Lung. Subpleural granuloma. Higher magnification of Fig. 11. Note epithelial cells and Langhans giant cells. Hematoxylin and eosin. × 300.

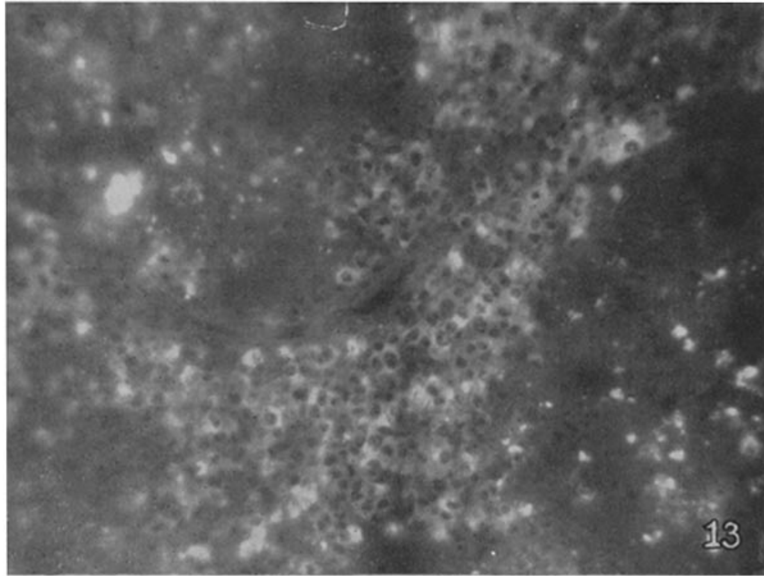


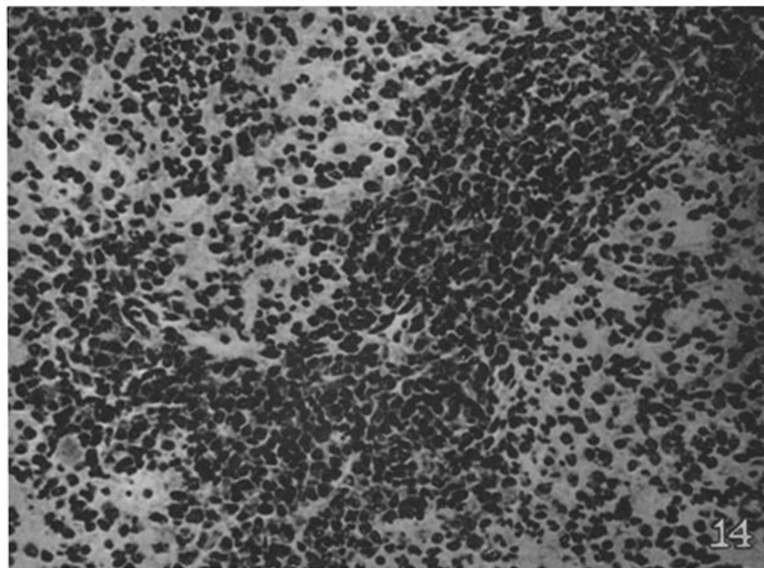
(White *et al.*: Studies on antibody production. IV)

Plate 18

Fig. 13. Spleen. Antigen mixture with 5 mg. tubercle bacillary wax. Note aggregation of antibody-containing cells around a vessel of the red pulp. Brightly fluorescent particles at top left and lower right were blue and part of natural fluorescence of the tissue. Fluorescence micrograph. × 300.

Fig. 14. Spleen. Corresponding area of adjacent frozen section stained with Giemsa. The aggregation of immature and mature plasma cells corresponds with the area of antibody-containing cells of Fig. 13. × 300.





(White *et al.*: Studies on antibody production. IV)

Plate 19

Fig. 15. Homolateral popliteal lymphatic gland. Antigen mixture with 40 μg. tubercle bacillary wax. Note intense proliferation of antibody-containing cells at edge of medulla. Fluorescence micrograph. × 300.

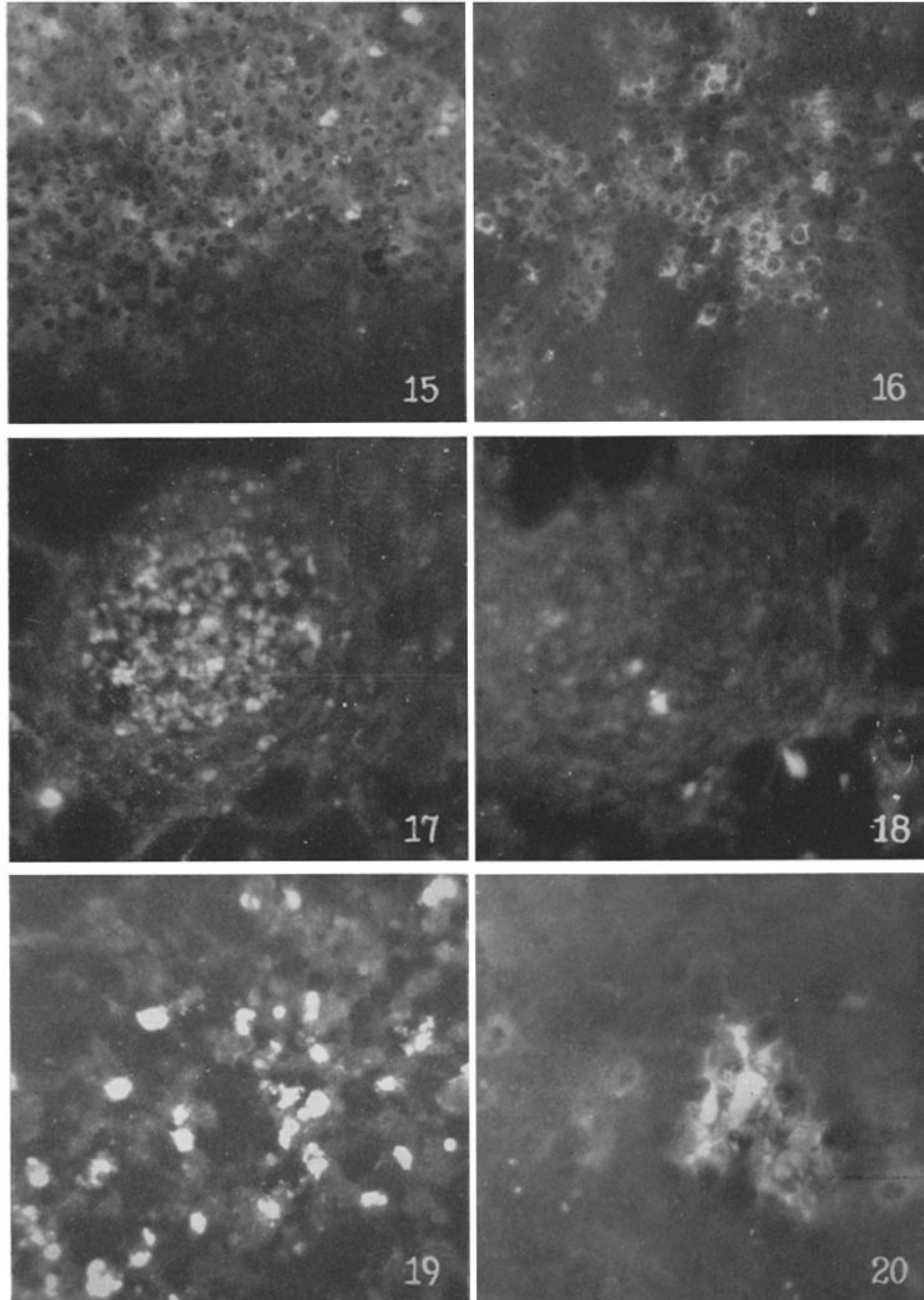
Fig. 16. Spleen. Antigen mixture with 40 μg. tubercle bacillary wax. Note proliferation of antibody-containing cells in red pulp. Fluorescence micrograph. × 300.

Fig. 17. Subcutaneous tissue at site of skin test, 3 days after intradermal injection of 0.1 mg. ovalbumin. Antigen mixture with 5 mg. tubercle bacillary wax. Note antigen content of phagocytic cells within blocked blood vessel. Stained for ovalbumin. Fluorescence micrograph. × 150.

Fig. 18. Subcutaneous tissue. Area corresponding with that of Fig. 17 in adjacent frozen section. Stained for ovalbumin following application of ovalbumin antiserum to inhibit specific antigen staining. (See morphological technique.) Fluorescence micrograph. × 150.

Fig. 19. Subcutaneous tissue at site of skin test, 3 days after intradermal injection of 0.1 mg. ovalbumin. Antigen mixture with 5 mg. tubercle bacillary wax. Intense fluorescent staining of precipitated antigen. × 300.

Fig. 20. Cervical lymphatic gland. Fluorescent oil droplet. Antigen mixture with 200 μg. tubercle bacillary wax. For interpretation see text. × 300.



(White *et al.*: Studies on antibody production. IV)