CELLULAR REACTIONS TO WAX-LIKE MATERIALS FROM ACID-FAST BACTERIA

THE UNSAPONIFIABLE FRACTION FROM THE TUBERCLE BACILLUS, STRAIN H-37

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PLATES 32 AND 33

The Mycobacteria are discriminated as a group by their capacity for synthesizing large amounts and varied types of lipoids in the form of fatty acids, phosphatides, and wax-like substances. Since no sterols have been found in them (1), the materials which have the properties of waxes are not waxes in the chemical sense. Rather they have been found to be composed of higher solid alcohols (2), and hydroxy acids of high molecular weight combined with polysaccharides, or, in the case of the corresponding substances from the Bacillus leprae, glycerides (3, 4). Like waxes, however, these materials are not only completely insoluble in water, but they cannot even be wet with water, properties which offer difficulties to the study of their effects on cells. Nevertheless, it can be shown that they are powerful stimulants for the new growth of cells. These wax-like materials have the property of acid-fastness and contribute it to the bacilli. For convenience we shall speak of these compounds as waxes.

As is well known, the separation of mixtures of lipoids is only to be made by the appropriate use of solvents. In general, Anderson has found that with the first use of each solvent, some of all the different types of lipoids come out. When it is recalled that almost all of the early biological tests of tuberculo-lipoids were made upon original alcohol-soluble, or alcohol-ether-soluble, or chloroform-soluble extracts, it is easy to understand why the effects so induced were complex and brought out no single cell reactions. Dr. Anderson has separated these lipoids from each other and given us three types of material.

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The first, the so called acetone-soluble material, is a mixture of many fatty acids and is so irritating that it stimulates every type of connective tissue cell (5). The other two preparations, on the other hand, the phosphatides and the waxes, give practically a single cell reaction. Both of these materials stimulate monocytes, but their effect on the monocyte is different. With the phosphatide, monocytes become epithelioid cells and form tubercles in all their essential characteristics; while on the other hand, the waxes bring about a fusion of monocytes into foreign body giant cells. This paper is concerned primarily with the reactions to the unsaponifiable material or wax which will be considered in comparison with those to the phosphatide.

Materials and Methods[1]

The Unsaponifiable Wax from Human Tubercle Bacilli, H-37.—This wax was obtained by Anderson (2) from the bacterial residue remaining after the extraction with alcohol-ether. The residue was treated with chloroform and the extract, after evaporation of the chloroform, gave a large amount of crude wax. This was then dissolved in ether, to which either acetone or methyl alcohol was added. A precipitate formed which was then separated into a saponifiable and an unsaponifiable portion by boiling in alcoholic potassium hydroxide. It is the unsaponifiable portion which is acid-fast and which contributes the wax-like characteristics to the chloroform extract. Some of this unsaponifiable material was also obtained from the original ether-alcohol extract both in the process of purification of the phosphatide and from the acetone-soluble material. After purification it is a white, amorphous powder, soluble in ether or chloroform, insoluble in water, and extremely stable. It is an hydroxy acid with the formula $C_{94}H_{188}O_4$ (1).

In all of the earlier work in this laboratory with the waxes, including the crude wax as well as the more purified, the materials were introduced in solution in mineral oil (6). Since mineral oil itself, the most inert oil yet found, causes considerable reaction by inducing a new growth of fibroblasts, by inducing adhesions, and by being phagocytized by clasmatocytes (macrophages), we have now devised other methods of introducing the waxes. The intraperitoneal route has been used for the most part, but the material has also been introduced subcutaneously, intradermally, and intravenously.

The wax has been injected intraperitoneally in three ways: first, as a dry

powder through a cannula and either pushed in by trocar or blown in with air; second, as a dry powder through an incision under anesthesia; and third, in colloidal suspension. The first method proved the least instructive, because the wax-like materials frequently became packed into a single bolus within the needle. The second method is the most satisfactory for all of the different materials. It is possible, if aseptic precautions are used, to open the peritoneal cavity several times and dust the powdered waxes onto the omentum. The crude waxes, which were in large lumps, could be ground into fine powder if they were first chilled with dry ice until brittle. The wax often became charged electrically when scraped from the watch glass into the peritoneal incision. This, however, did not alter the cellular reactions.

The colloidal suspensions proved instructive on account of the opportunity they afforded to introduce these inert materials in fine particles. They were made by one of us as follows: A given weight of the unsaponifiable wax from H-37 was dissolved in chloroform and then an equal amount of hot alcohol was added. The material remained in solution. When an equal amount of distilled water was added drop by drop, the wax came out in a precipitate of particles so fine as to make a milky suspension. The flask was then placed in a water bath and kept at 100°C. until all of the chloroform and alcohol had been driven off. The water was then concentrated until 1 cc. contained 5 mg. of the wax. This suspension proved to be stable with no aggregation of the precipitate into clumps on standing.

RESULTS

Reactions to Unsaponifiable Wax from H-37 Given Intraperitoneally.—
The unsaponifiable material from tubercle bacilli, H-37, has been given intraperitoneally to seventeen rabbits in amounts shown in Table I. All of these animals showed also the reaction to the subcutaneous injection, since small amounts always lodged there in introducing the wax. In the table the animals are arranged according to the time which elapsed from the first injection to the date of killing the animal.

In the peritoneum the reaction to the wax is under the mesothelium of the parietal peritoneum, in the serosal coat of the cecum, in the capsule of liver and spleen, and in the omentum. The descriptions in the table are mainly of the omentum as representative of the entire reaction, because of the advantages in studying this structure. It can be studied as a film, while the cells are living, showing the supravital reaction to neutral red and Janus green. Then the same preparation can be fixed and stained in some manner, such as with the Ziehl-

TABLE I

Protocols of Rabbits Which Received Unsaponifiable Wax from Tubercle Bacillus, H-37, Intraperitoneally

Animal No.	No. and amount of injections	Time	Method of preparing material	Peritoneal exudate			Tissues
				Percentage of			
				PMN	Lymph.	Mono.	
		days					
R 3351*	1 24 mg.	1 K†	Colloidal suspension	41.5	2.6	55.8	Acid-fast material on milk spots of omentum and none in interspaces. 10% of monocytes in peritoneal exudate contain acid-fast material
R 2760	1 20 mg.	4 K	Dry powder through cannula	17.1	8.8	74.1	Milk spots of omentum show an increase in monocytes surrounding wax, but they have not started to fuse into foreign body giant cells. Infiltration with eosinophilic leucocytes
R 2793	1 20 mg.	4 K	Dry powder through incision under anesthesia	2.55	34.7	62.75	Wax became highly charged electrically and lodged in fat around the kidney, where foreign body giant cells developed. No infiltration with eosinophilic leucocytes
R 2840	2 20 mg.	4 2 D‡	" "	—	—	—	Died of pneumonia. Foreign body giant cells in omentum
R 2884	1 20 mg.	6 K	" "	—	—	—	Wax in small particles on milk spots, surrounded by newly formed monocytes which are starting to fuse into foreign body giant cells. Coccidiosis present
R 3301	2 20 mg.	4 3 K	Colloidal suspension	3.3	19.8	76.85	Milk spots show dense masses of monocytes around wax; some have fused into foreign body giant cells. Reaction to acid-fast stain brilliant; it is in part free on the milk spots and in part within the monocytes and giant cells. Infiltration with eosinophilic leucocytes. Small foci of monocytes in septa between air sacs in lungs

R 3322	1 20 mg.	8 K	" "	0	3.0	96.5	Milk spots increased in size by new monocytes around particles of wax; some fusion into foreign body giant cells. Only a little acid-fast material within the cells but much on the milk spots. Infiltration with eosinophilic leucocytes. Foreign body giant cells in retrosternal lymph nodes. Many young monocytes in peritoneal fluid
R 2802	2 20 mg.	4 4 K	First through cannula; second through incision under anesthesia	—	—	—	First injection lodged in a bolus. Second caused increase in monocytes around particles of wax on the milk spots. Some fusion into foreign body giant cells. In second incision there was a marked increase in monocytes and infiltration with eosinophilic leucocytes. Increase in eosinophilic myelocytes in the bone marrow
R 2841	2 20 mg.	4 4 D	Dry powder through incision under anesthesia	—	—	—	Died of pneumonia. Omentum, adherent to peritoneal wall at site of operation, contains foreign body giant cells and has infiltration with eosinophilic leucocytes
R 2814	2 20 mg.	3 14 K	" "	0	33.7	66.3	Marked increase in size of milk spots, each of which has from 2 to 10 foreign body giant cells. In sections some of the giant cells have a hollow center, from which the wax has been dissolved out, but show no vacuoles in the fused cytoplasm. In others wax had been phagocytized. Infiltration with eosinophilic leucocytes. Nodule in skin of first incision has foreign body giant cells with partially fused cytoplasm. In second incision masses of monocytes but relatively little fusion into giant cells. Many eosinophilic leucocytes in incisions. Retrosternal lymph nodes have many complex giant cells

PMN = polymorphonuclear neutrophilic leucocytes. Lymph. = lymphocytes. Mono. = monocytes.
* These are serial experiment numbers covering the entire work of the department.
† K = Killed.
‡ D = Died.

TABLE I—*Concluded*

Animal No.	No. and amount of injections	Time	Method of preparing material	Peritoneal exudate			Tissues
				Percentage of			
				PMN	Lymph.	Mono.	
		days					
R 2806	2 20 mg.	4 23 K	Dry powder through incision under anesthesia	0	46.5	53.0	Milk spots increased in size with monocytes and foreign body giant cells. 8 to 10 giant cells in each milk spot. Moderate infiltration with eosinophilic leucocytes. Sections show tubercles of giant cells
R 3323	1 20 mg.	30 K	Colloidal suspension	0	5.1	94.9	Milk spots of omentum not increased in size but show foreign body giant cells which, as well as the monocytes, show small acid-fast granules surrounded by a pink zone in Ziehl-Neelsen stain. Most of the reaction was in the retrosternal lymph nodes, where the sinuses were filled with small giant cells having a uniformly vacuolated cytoplasm
R 3325	1 20 mg.	32 K	" "	0.5	17.35	82.15	Milk spots of omentum massive with foreign body giant cells. Only the giant cells show any acid-fast material and this consists of tiny granules stained red and surrounded by a pink zone in Ziehl-Neelsen
R 3326	1 20 mg.	46 K	" "	1.03	2.06	96.90	Milk spots of omentum dense with foreign body giant cells, some of which have irregular masses and finely divided particles that stain faintly acid-fast
R 2839	1 20 mg.	55 K	Dry powder through incision under anesthesia	0	39.2	60.8	Milk spots of omentum massive with tubercles of foreign body giant cells; tissues infiltrated with lymphocytes and with some eosinophilic leucocytes. Cytoplasm of the giant cells is vacuolated. There is some calcification but nothing that simulates caseation

R 2845	2 20 mg.	4 133 K	"	"	0	8.0	91.0	Milk spots of omentum massive with foreign body giant cells. Similar masses in the body wall. All of the tissues infiltrated with some eosinophilic leucocytes; some calcification. Nodules of monocytes and eosinophilic leucocytes in the lung
R 2844	2 20 mg.	6 222 K	"	"	0	56.65	43.35	Milk spots of omentum massive with tubercles of foreign body giant cells. Some masses of giant cells raised on pedicles. Supravital reaction to neutral red showed that the wax had become much more finely divided in the cells than in R 2845. Some plasma cells and an occasional eosinophilic leucocyte

Neelsen technique.   Other preparations of the omentum can be fixed for sections.   Besides occurring in these locations, some of the wax, occasionally much of it, floods through the lymphatics of the diaphragm into the retrosternal lymph nodes.   In most instances, small particles also reach the lung and are there represented by small foci of monocytes usually infiltrated with eosinophilic leucocytes.

The immediate reaction to the material injected in the form of the colloidal suspension is instructive.   The material is identified by its property of acid-fastness.

The colloidal suspension itself shows only a diffuse, pink reaction to the fuchsin of the Ziehl-Neelsen technique, for the wax has to be in aggregates of a given size before the typical red color of the reaction can be obtained.   Aggregates of the material are formed when the colloidal suspension is introduced intraperitoneally and the acid-fast reaction is shown in Fig. 1, from the omentum of Rabbit R 3351 which was killed 24 hours after one injection of 24 mg.   This film was fixed in the vapor of formalin and stained with the Ziehl-Neelsen method.   The wax, stained red with the fuchsin, appears black in the photograph and is almost limited to the milk spots.

It was clear on studying the fresh films of the omentum of this animal, R 3351, that the wax was merely adherent to the cells of the milk spots and had not been phagocytized by them.   The cells of the peritoneal exudate were stained for acid-fast material and only 10 per cent of the monocytes contained any of this material and they only in small amounts.

In the fifth column of Table I is shown the differential counts of the cells of the peritoneal exudate expressed in percentages of the three strains of cells which are significant.   In some of the counts there was an occasional basophilic leucocyte, never more than 2 per cent; but in none was there even a single eosinophilic leucocyte; in some counts there were a few clasmatocytes with phagocytized leucocytes and occasionally desquamated serosal cells.   These cell types may be considered as accidental and hence have been omitted in the table.   Only the first two animals listed in Table I showed any rise in neutrophilic leucocytes in the peritoneal exudate.   In the case of Rabbit R 3351, the blood cells were counted hourly after the injection and by 4 hours the neutrophilic leucocytes had risen from 4,860 to 11,023 per c.mm.

Thus there is an immediate draining of neutrophilic leucocytes into the peritoneal cavity but it is not lasting unless infection supervenes. Rabbit R 3351 did not show any infiltration of the tissues with eosinophiles, which subsequently becomes a constant finding.

By 4 days, all of the animals receiving the wax have shown a multiplication of monocytes around the particles of the wax, but we have not found them fusing into giant cells until the 6th day. The omentum of Rabbit R 3301, which was studied 7 days after the first injection, showed the milk spots increased in size and dense with monocytes; most of the milk spots had giant cells, some as many as thirty. All the giant cells were small because the material was introduced in a fine suspension, the size of the foreign body giant cell being proportional to the size of the mass it has engulfed. The omentum stained for acid-fast material gave a brilliant result. The acid-fast material was almost all on the milk spots in masses from about 5 to 20μ in diameter. The largest masses were surrounded by monocytes not yet fused into giant cells. All of the giant cells were filled with the acid-fast material. In sections of the omentum the giant cells showed a vacuolated cytoplasm indicating the zones from which the wax had been dissolved out in the processes of embedding. This explains why it is impossible to obtain positive acid-fast stains in sections of this material, because the free waxes are so easily removed by lipoidal solvents. The milk spots of the omentum were infiltrated with considerable numbers of eosinophilic leucocytes, but practically no neutrophilic leucocytes. In spite of this, there were no eosinophiles found free in the peritoneal exudate.

A later stage of the process is shown in Figs. 2, 3, and 4, representing 17, 27, and 32 days after the first injection. All the figures show giant cells of the omentum, photographed while the cells were living. All of the black color in the photographs represents the neutral red in the vacuoles that contain the wax. An acid-fast stain shows the wax itself; the supravital neutral red shows the fluid that the cell has secreted around the wax. It is clear that with the longer time, Fig. 3 at 27 days, much more wax has been phagocytized than was present at 17 days, Fig. 2. The reaction shown in Fig. 3 is of small tubercles of giant cells.

Three of the rabbits, R 3323, 3325, and 3326, which received the material in colloidal suspension gave suggestion of the fate of the wax within the cells.

These animals were killed 30, 32, and 46 days respectively after a single injection of the material in colloidal suspension. When the fresh omenta of these three animals were stained in the Ziehl-Neelsen technique, it was noted that instead of the massive red staining of the giant cells seen in Rabbit R 3301, killed 7 days after the injection, the reaction had now been modified. The giant cells of the first two animals, 30 and 32 days after the injection, all had diffuse, pink-stained zones in the center of which were tiny, bright red granules, while in the cells of the third animal, killed at 46 days, only the diffuse pink reaction remained. The type of giant cell seen in the omentum of Rabbit R 3325 is shown in Fig. 4. This cell is stained in neutral red; it is the type that showed the tiny, red granules surrounded by a diffuse, pink zone when stained for acid-fastness. It will be noted that the vacuoles of the cell, dark in the photograph, are irregular in size, but many of them, especially in the lower part of the cell, are small. In the cells of Rabbit R 3326, which had lost all of the material staining typically acid-fast, the cytoplasm of the living cells showed that the wax had become much more finely divided. Indeed, the cytoplasm reminded one of masses of platelets showing the same clumping of tiny granules.

These observations indicate that the wax becomes broken up in the cytoplasm of the giant cells until it is too finely divided to give an acid-fast stain, just as occurs when the wax is put into colloidal suspension.

In the case of Rabbit R 3323, most of the wax had flooded into the retrosternal lymph nodes, where the sinuses were distended with foreign body giant cells and the follicles had been much encroached upon by them. In these giant cells, the vacuoles were all small, giving evidence that the cells had been breaking the wax into small particles. Around these giant cells were many eosinophilic leucocytes. The omentum had so little of the reaction that the milk spots, though they showed a few giant cells, were not increased in size by them. In Rabbit R 3325, on the other hand, the milk spots were massive with giant cells.

The last three animals in the group, Rabbits R 2839, R 2845, and R 2844, killed 55, 137, and 228 days after the first injection of the wax, all had massive tubercles of foreign body giant cells in the omentum. Some of the milk spots were so thick that they were markedly raised from the surface of the omentum and some were even on pedicles. Within the giant cells there had been a progressive breaking up of the wax into smaller particles.

The type of giant cells is shown in Figs. 5 and 6 from the omentum of Rabbit R 2844. Fig. 5 is a foreign body giant cell of enormous size; the photograph is at low magnification, namely, 400 diameters. The cell has over a hundred nuclei. Smaller giant cells with from three to six nuclei are seen in the neighborhood. The cytoplasm is rather uniformly mottled, showing places from which the wax has been dissolved out in embedding. This is not as marked in sections as in the living cell on account of the shrinkage suffered during embedding.

Fig. 6 is a part of a tubercle of giant cells; again there is a great range in the size of these cells. One of the giant cells shows a central cavity from which the wax has been dissolved out. In the fresh preparations a few of the giant cells still showed unchanged wax in the center and this is evidently one of them. It will be noted in Figs. 5 and 6 that there are no signs of necrosis in any of the cells. All of the nuclei appear normal. This is characteristic of the sections throughout. In the sections of this animal there are many zones, such as the one marked with an arrow in Fig. 6, in which there is the suggestion that this type of giant cell may split into its component monocytes when the wax has become sufficiently finely divided. The history of the view that certain giant cells in tuberculous tissue do not regress by necrosis but rather break up into smaller cells was reviewed by Hektoen (7) in 1898, who brought evidence in support of this view from the study of a case of tuberculous peritonitis.

Reactions to Unsaponifiable Wax from H-37 Given Intravenously.—In Table II are given the protocols of eleven rabbits which received the unsaponifiable wax intravenously, five of which were subsequently inoculated with bovine tubercle bacilli.

The first five animals received the wax in doses of 3 mg. suspended in mineral oil and all showed foci of oil droplets in the septa between air sacs. In these zones there was an increase in monocytes which had phagocytized the oil.

Rabbit R 3350 received six intravenous injections of the wax in colloidal suspension in water and was killed 32 days after the first injection. In this manner all of the complicating factors from the menstruum were removed and the reaction to the wax came out. Throughout the liver there were many giant cells in the sinuses, two to four in every oil immersion field. There was no reaction around them; no eosinophilic leucocytes were found. Some of these giant cells had only two or three nuclei; they were simple in type, with the nuclei in the periphery and the cytoplasm vacuolated in the center. Others had as many as thirty nuclei; these were complex, having two or three cytoplasmic centers surrounded by nuclei in rings or with clumps of them along the border. In almost all of these giant cells the cytoplasm was vacuolated. In the spleen there were many small giant cells, both in the sinuses and in the follicles; many of the follicles had small, tubercle-like bodies of monocytes. Also many of the follicles had small patches of early amyloid degeneration; occasionally a follicle was almost replaced by this type of degeneration. There were no more eosinophilic leucocytes in the spleen

TABLE II

Protocols of Rabbits Receiving Unsaponifiable Wax from Tubercle Bacillus, H-37, Intravenously

Animal No.	No. and amount of injections	Time	Method of preparing material	Inoculation with bovine tubercle bacilli	Tissues
R 2001	2 3 mg.	1 wk. apart. K 24 hrs. after second injection	Dissolved in 1 cc. mineral oil	—	Many foci of oil droplets, monocytes, and neutrophilic leucocytes in septa between air sacs in lungs. Tracheal lymph nodes were filled with leucocytes and the spleen had many clasmatocytes engorged with them
R 2002	3 3 mg.	1 wk. apart. K 24 hrs. after third injection	" "	—	Foci of monocytes and leucocytes, with occasional highly vacuolated mononuclear cells in septa between air sacs in lungs. Some of these foci large enough to obliterate a few air sacs. Spleen shows much destruction of leucocytes
R 2003	4 3 mg.	1 wk. apart. K 24 hrs. after fourth injection	" "	—	Many foci of monocytes and leucocytes with some highly vacuolated mononuclear cells and a few foreign body giant cells in lungs. Some large enough to obliterate 20 air sacs. Spleen shows a few small, tubercle-like masses of monocytes
R 2004	4 3 mg.	1 wk. apart. D 7 days after fourth injection	" "	— .	Larger foci of highly vacuolated mononuclear cells in lungs. These foci are vascularized and contain a few free leucocytes. Liver shows extreme involvement with coccidiosis and spleen has foci of necrosis
R 2006	6 3 mg.	1 wk. apart. K 2 days after sixth injection	" "	—	Foci of cells in lungs, 2 to 3 mm. in diameter, consisting mainly of highly vacuolated mononuclear types. A few dead leucocytes and many eosinophilic leucocytes. No destruction of leucocytes in the spleen but some foci of monocytes

| R 3350 | 6
12 mg.
24 "
12 "
10 "
8 "
8 "
——
74 " | Daily except last
interval was 2
days. K 25
days after
sixth injection | Colloidal suspen-
sion 12 mg. of
wax per 1 cc.
water | — | Many small, foreign body giant cells in sinuses throughout
the liver. Many foreign body giant cells in spleen
both in sinuses and in follicles. Few giant cells in septa
between air sacs in lungs and in glomeruli of kidney. No
infiltration with eosinophiles |
| R 2006
R 2007
R 2008
R 2009
R 2010 | 6
3 mg. | 1 wk. apart | Dissolved in 1 cc.
mineral oil | 0.1 mg. Strain
B-1, intrave-
nously, 2 days
after sixth in-
jection | Survived an average of 176 days, with a range of 57 to 306
days, as compared with a survival of 162 days, range of
30 to 287 days for 15 controls (R 2073–R 2087). There
were no differences in the type of disease in the rabbits
which had received the wax in oil, but they all had foci
of highly vacuolated mononuclear cells in lungs; these
foci did not become invaded with the tuberculous process |

than normally. An occasional small giant cell was found in the lung and also in the glomeruli of the kidney. The blood cells of this animal were studied, and from the time of the fourth injection until the animal was killed, the eosinophilic leucocytes were above 500 per c.mm., and twice they were above 1,000. The average number of eosinophiles in the normal rabbit is 110 per c.mm.

The last five animals on Chart 2 received six injections each of 3 mg. of the wax in mineral oil at intervals of 1 week and, 2 days after the last injection, were inoculated intravenously with 0.1 mg. of bovine tubercle bacilli, Strain B-1. At the same time, fifteen rabbits were inoculated with the same dose from the same suspension for controls. There were no significant differences in longevity between the injected animals and their controls, nor in the range of survival. All of the animals which had received the wax had small foci of vacuolated cells in the septa between air sacs, but these foci did not become invaded with the tubercle bacilli, resulting in setting up tubercles in them. Occasionally two or three of these foci became surrounded by tuberculous masses, but even then they were not invaded by epithelioid cells. Thus there was no sign that these abnormal foci of cells in the lungs had any effect whatever on the tuberculous infection.

DISCUSSION

The most interesting biological property of these solid alcohols and hydroxy acids which make the unsaponifiable material of Mycobacteria and contribute the property of acid-fastness to them is that, though they are stable and seemingly inert substances chemically, they are remarkable stimulants for the new growth of cells. Their essential property is that they stimulate the formation of monocytes wherever they lodge in the tissues. These monocytes then fuse around the wax and become foreign body giant cells.

It is clear that whenever lipoids are introduced parenterally, they are dealt with by the phagocytic mononuclear cells, that is, by monocytes. For this study the omentum gives the most valuable data; after intraperitoneal injection, it is easy to see that lipoids lodge on the milk spots of the omentum and not in the interspaces between them. This has been illustrated in Fig. 1 by means of the acid-fast property of the tuberculo-wax. When the lipoid is introduced in the form of a uniform colloidal suspension, we must assume that the fluid passes both through milk spots and interspaces, but only the part of the wax which floods through the milk spots becomes fixed (Fig. 1). This must be either because of the greater density of the primitive cells and monocytes which make up the milk spots, or on account of

the characteristics of their surface films.   Tiny foci of young, connective tissue cells, so readily identified as milk spots in the omentum, exist throughout the connective tissues, and their property of fixing lipoids to their surface and then phagocytizing them may well be the source of the monocytes in the local formation of tubercles.

We have previously studied the effect of another lipoid especially characteristic of tubercle bacilli, namely, a phosphatide (6, 8–10), on the cells of the connective tissues.   The phosphatide, in contrast to the waxes, is readily dispersed in water, making stable suspensions which are suitable for parenteral injection.   In water these phosphatides form myelin-like figures (10) which can be readily identified within cells.   It is thus possible to show that these phosphatides are phagocytized by monocytes and that they are acted upon within the cell in a specific manner.   The material is at first irregular in size, making a cell that appears highly vacuolated in fixed material, since the processes of treating tissues for sectioning result in a complete solution of the lipoid.   However, it is soon broken up into small and then smaller particles within the cytoplasm.   This process seems to go on uniformly so that the particles are all about the same size at any stage, except immediately after phagocytosis.   When they have become finely divided, the cell is the typical epithelioid type, indistinguishable from the form seen in the disease tuberculosis.   The time necessary for the formation of epithelioid cells ranges from 4 days to 2 weeks.   Some of the monocytes which have phagocytized the phosphatide become multinuclear, making Langhans' giant cells.

The only constituent of the phosphatide capable of bringing about this reaction is a fatty acid, discovered by Anderson and named by him phthioic acid.   This acid is optically active and has the formula of $C_{26}H_{52}O_2$.   It has not been found in nature before and is highly characteristic of tubercle bacilli.   It occurs in all of the three major lipoid fractions as first obtained through solvents, namely, in the alcohol-ether-soluble phosphatide, in the acetone-soluble mixture of fatty acids, and in the chloroform-soluble material (1, 11–13).   When this fatty acid is given either in the form of salts (5), or with its burning properties cut down by suspension in nujol, it forms typical epithelioid cells, singly and in tubercles (8–10).

The fate of the epithelioid cell is an essential point in judging its

meaning; after the introduction of the lipoid either as phosphatide or as fatty acid, it is broken up into fine particles, after which no further change can be detected in the cell, except that refractive bodies may appear in the periphery. The end-result, though it may be long delayed, is the death of the cell. Thus the monocyte can readily phagocytize this material and disperse it to a certain state, but it seems not physiologically adapted to a disintegration of this fatty acid into its simpler molecular groups. Epithelioid cells then regress, either through the death of the individual cells, or *en masse*, in which case the phenomenon is called caseation (10).

The giant cells produced in response to the waxes must be compared with the epithelioid types. The unsaponifiable material from H-37, which is a higher hydroxy acid with the formula $C_{94}H_{188}O_4$, can be identified within the cells by the property of acid-fastness. Only the finest particles of this material, as when introduced in colloidal suspension, are phagocytized by the individual monocyte; rather the masses of the wax become surrounded by many monocytes which fuse into a giant cell which is at first a hollow sphere. The large inner surface of this fused cytoplasm is then able to engulf the material.

When the wax is sufficiently finely divided, as in the colloidal suspension, it gives only a diffuse pink reaction with the Ziehl-Neelsen technique, but when the small particles again become aggregated, typical acid-fastness is restored. This property gives an opportunity to follow the action of the giant cells upon the wax. Whether the material has been introduced in colloidal suspension or in powder form, it appears within a period of 2 to 3 weeks as massive aggregates which are acid-fast. The material remains for a long time within the giant cell in the form of irregular masses, with no immediate tendency toward the formation of particles of equal size, so characteristic of the epithelioid type. But in the period of months, typical acid-fastness gradually grows less, until only a diffuse, pink reaction like that of the colloidal suspension remains. This indicates that the cell is able to disperse this material into finer particles, such as are in the colloidal suspensions. In sections the giant cells which were originally coarsely vacuolated become much more finely vacuolated, as is shown in Figs. 5 and 6. If such a giant cell is small and has peripheral nuclei, as is shown in Fig. 5, it is indistinguishable from the typical Langhans' type

Thus the method of dealing with the wax within the giant cells is different from that of dealing with the phosphatide. It is a slower process and there is nothing that might be compared to a process of emulsification as with the phosphatide, but there is a gradual disintegration of the material until it is too finely divided to give an acid-fast stain.

More important still is the difference in the fate of the cell which has taken in the wax. We have not detected any signs of damage to the cells; they do not disappear through caseation; there is no sign of cell death in the tissues. It is probable that the giant cells eventually separate into single monocytes, though our experiments have not been carried far enough to prove this point.

The first reaction to the introduction of the wax into the peritoneal cavity is the calling of neutrophilic leucocytes into the peritoneal cavity, but this follows the introduction of any foreign material whatever. It is a reaction which does not persist, and in about 2 weeks' time the neutrophilic leucocytes have disappeared from the tissues and eosinophilic leucocytes appear. This reaction is so marked that it can be detected by an increase in eosinophilic myelocytes in the bone marrow and, if the blood cells are followed, by an increase in their number in the blood stream. They do not seem to wander into the peritoneal exudate but rather remain around the giant cells. Their presence is so constant around these lesions that they suggest that the giant cells break some material from the wax which is chemotaxic to eosinophiles. Some of the foci of giant cells, especially those that form quite large tubercles, also become surrounded or infiltrated with lymphocytes. It is not a constant reaction, tubercles of giant cells in any one section varying as to whether there are few, many, or no lymphocytes around them.

The study of the effect of the unsaponifiable material injected intravenously on the course of a subsequent infection with tubercle bacilli indicates that the waxes have no effect on resistance. These materials are elaborated by the Mycobacteria and give them the property of acid-fastness by which they are classified, but their function is probably in connection with the life and the survival of the bacillus and they induce no reactions in the host that are associated with resistance.

CONCLUSIONS

1. The unsaponifiable fractions of the Mycobacteria, though insoluble in water and extremely stable chemical compounds, are nevertheless remarkable stimulants of cells.

2. They give rise to new monocytes which surround these waxes and then fuse into giant cells which engulf them.

3. The property of acid-fastness of the waxes makes it possible to identify them within the giant cells which have phagocytized them.

4. Within the foreign body giant cells the waxes are slowly disintegrated. They appear not to damage the cells which engulf them, and hence one may infer that they take no part in caseation.

5. They have no effect on the resistance of the host.

BIBLIOGRAPHY

1. Anderson, R. J., *Physiol. Rev.*, 1932, **12**, 166.
2. Anderson, R. J., *J. Biol. Chem.*, 1929–30, **85**, 339.
3. Uyei, N., and Anderson, R. J., *J. Biol. Chem.*, 1931–32, **94**, 653.
4. Anderson, R. J., and Uyei, N., *J. Biol. Chem.*, 1932, **97**, 617.
5. Smithburn, K. C., and Sabin, F. R., *J. Exp. Med.*, 1935, **61**, 771.
6. Sabin, F. R., Doan, C. A., and Forkner, C. E., *J. Exp. Med.*, 1930, **52**, suppl. 3, 1.
7. Hektoen, L., *J. Exp. Med.*, 1898, **3**, 21.
8. Sabin, F. R., and Doan, C. A., *J. Exp. Med.*, 1927, **46**, 645.
9. Sabin, F. R., *Physiol. Rev.*, 1932, **12**, 141.
10. Smithburn, K. C., and Sabin, F. R., *J. Exp. Med.*, 1932, **56**, 867.
11. Anderson, R. J., *J. Biol. Chem.*, 1929, **83**, 169.
12. Anderson, R. J., *J. Biol. Chem.*, 1929, **83**, 505.
13. Anderson, R. J., and Chargaff, E., *J. Biol. Chem.*, 1929, **84**, 703; **85**, 77.

EXPLANATION OF PLATES

PLATE 32

FIG. 1. Film of omentum of Rabbit R 3351, which had received one intraperitoneal injection of 24 mg. of the unsaponifiable material from human tubercle bacilli, Strain H-37, suspended in 2 cc. of water. Killed 24 hours after the injection. Film stained with the Ziehl-Neelsen technique to bring out acid-fast material which shows as black masses against the milk spots. × 10.

FIG. 2. Foreign body giant cell in the center of a milk spot of the omentum, photographed while the cells were living, of Rabbit R 2814, which had received two intraperitoneal injections of the unsaponifiable material from the human

tubercle bacillus, H-37, and was killed 14 days after the second injection. The material was introduced as a dry powder through an incision under anesthesia. The film is stained with neutral red and was photographed while the cells were still living. The material stained black represents the fluid around the particles of wax, which had been phagocytized; there is only a small amount of this reaction within the giant cell; there are well stained monocytes in the border. The refractive bodies seen around the giant cell are in the slightly stimulated serosal cells covering the milk spot. × 1040.

FIG. 3. Small tubercle of foreign body giant cells in a film of omentum of Rabbit R 2806, which had received two intraperitoneal injections of the unsaponifiable material from the human tubercle bacillus, H-37, and was killed 23 days after the second injection. The material was introduced as a dry powder through an incision under anesthesia. The film is stained with neutral red and was photographed while the cells were still living. The material stained black represents the fluid around the particles of wax, which had been phagocytized, and shows a marked increase from the stage of Fig. 2. The wax is in relatively large masses. × 693.

FIG. 4. Foreign body giant cell in a film of omentum of Rabbit R 3325, which had received one intraperitoneal injection of the unsaponifiable material from human tubercle bacillus, H-37, and had been killed 32 days later. The material was introduced in colloidal suspension in 3 cc. of water. The film was stained with neutral red and photographed while the cells were still living. The material which shows as black (neutral red) represents the fluid secreted by the cell around the particles of the wax and indicates that some of the wax is still in large aggregates while some is in fine particles. × 866.

PLATE 33

FIG. 5. Large and small foreign body giant cells in a section of the omentum of Rabbit R 2844, which had received two injections of the unsaponifiable material from the human tubercle bacillus, H-37, and had been killed 222 days after the second injection. Stained with hematoxylin and eosin. × 400.

FIG. 6. Tubercles of foreign body giant cells in a section of the omentum of the same animal as in Fig. 5. The arrow points to a zone in which a giant cell may be breaking up into its component monocytes. Stained with hematoxylin and eosin. × 400.
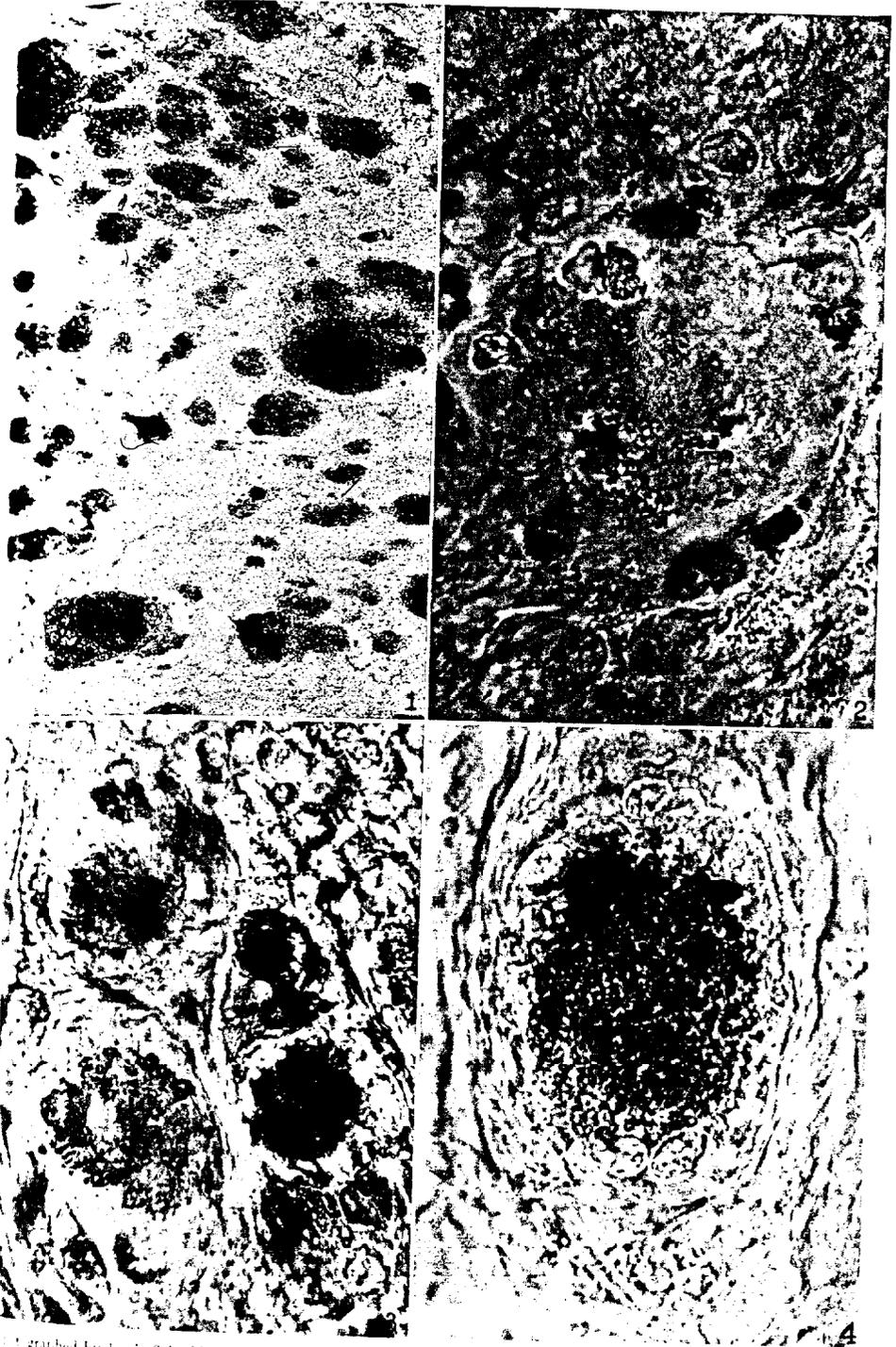
graphed by Louis Schmidt

(Sabin et al.: Wax-like materials from acid-fast bacteria)

Photographed by Louis Schmidt

(Sabin et al : Wax-like materials from acid-fast bacteria)