CELLULAR REACTIONS TO DEFATTED TUBERCLE BACILLI AND THEIR PRODUCTS*

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PLATES 45 AND 46

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Since 1926 we have been following the cellular reactions to various fractions from tubercle bacilli. The present study completes our general biological survey of the main lipoidal fractions of acid-fast organisms as analyzed by Dr. Anderson.

In Chart 1 is given in briefest possible outline the chemical procedures used by Dr. Anderson to obtain the materials we have studied. It represents only the end products of his analyses; for example, each of the solvents used originally, namely, alcohol-ether, acetone, and chloroform, extracted some of all the forms of lipids in the bacilli and only predominantly the type indicated on the chart. The separation of phosphatide, acetone-soluble fat, and the so called wax from each other after these primary mixed extractions has been described in Dr. Anderson's publications, of which a complete list to date is appended (1–52). The solvents used are shown on the chart in black face type; the residues and the extracts which are acid-fast are enclosed with double lines, while those which are non-acid-fast are enclosed with single lines.

The cellular reactions to the tuberculo-phosphatide, the acetone-soluble fat, and to the so called waxes have been published previously (53–77). The present study involves the biological reactions to the lipids and polysaccharides from the second residue, the so called defatted bacilli.

*All the members of the laboratory have taken part in these studies. The work has been a part of a cooperative study on tuberculosis initiated by the Research Committee of the National Tuberculosis Association, Dr. William Charles White, Chairman. The analyses of tubercle bacilli for lipids have been done by Dr. R. J. Anderson and his associates at Yale University, to whom we are indebted for the various lipids and polysaccharides from different acid-fast strains.

Materials and Methods

The defatted bacilli lack all the lipids that can be removed by neutral solvents such as alcohol-ether and chloroform, but they are in reality only partially defatted, for they are still strongly acid-fast. They still contain what Dr. Anderson

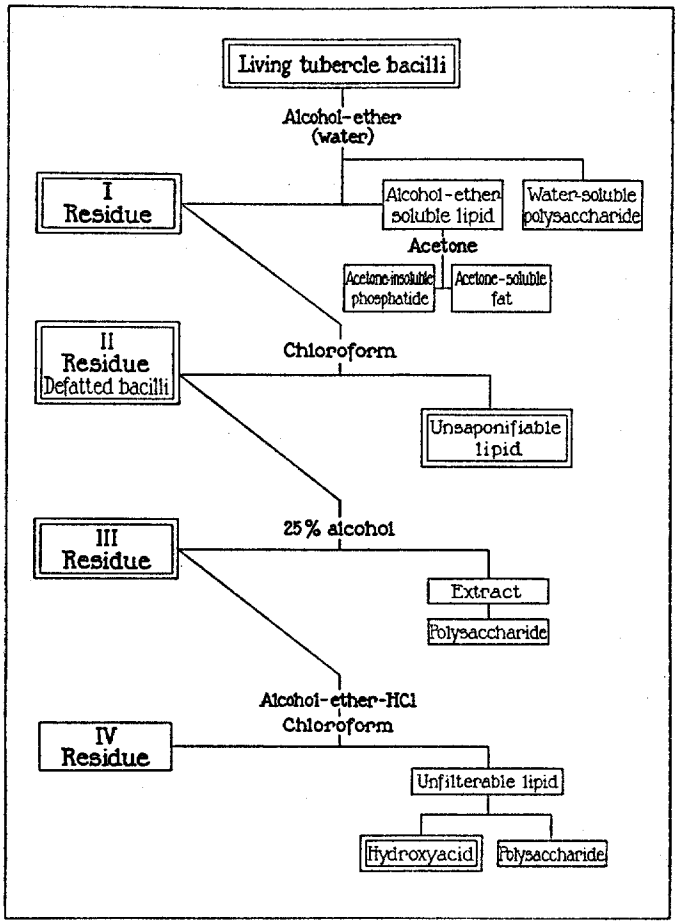

CHART 1

has called the "firmly bound lipid." They were first treated with 25 per cent alcohol by him, which gave an extract which proved to be predominantly of polysaccharide. The residue, the third on the chart, was also of strongly acid-fast organisms. This third residue was then treated with alcohol-ether-HCl after

which it was possible to remove the firmly bound lipid with ether and chloroform; only the chloroform is recorded on the chart. This lipid he found to be unfilterable; it could be split into about equal parts of an hydroxy acid and a polysaccharide. The remaining residue was non-acid-fast.

It has long been known that it is difficult to obtain materials from tubercle bacilli which are free from acid-fast organisms. In the case of the polysaccharides this offers no obstacle because, the material being soluble in water, the organisms can be readily removed by filtration through candles. With the lipids, on the other hand, which must be filtered in lipoidal solvents, one cannot be sure that every filtration will remove the last traces of bacilli, since a lipoidal solvent alters the conditions of filtration. We have described in another paper (74) the methods by which it was possible to free the tuberculo-phosphatide from the last traces of bacilli. Dr. Anderson filtered the material through Chamberland candles but not every preparation was entirely free of organisms even after such treatment. We have found that last traces of the organisms can be removed by centrifugation if the lipid is dissolved in a mixture of solvents the specific gravity of which allows the sedimentation of tubercle bacilli. For this purpose we have used chloroform three parts and ether seven parts. After centrifuging at 3600 R.P.M. for 3 hours in this mixture the bacilli have been found in the sediment and not in the supernatant fluid. As a further check on the presence of a few bacilli in the materials we have stained the tissues of the animals which have received lipids for the acid-fast reaction; when bacilli were present in the materials they were usually to be found in giant cells during the first week after the injections. In the case of the phosphatide, we presented further evidence that the material we used lacked bacilli in any considerable numbers by showing that the phosphatide itself injected intradermally did not sensitize guinea pigs to the tuberculo-protein which is contained in the bacilli, while when minute traces of the protein were added to the phosphatide, sensitization did occur (74).

In the materials described in this report tubercle bacilli were found as follows: The second and third residues were predominantly of acid-fast organisms, markedly beaded and looking much like the bacilli in old cultures. The fourth residue from which the so called firmly bound lipid had been removed by ether-chloroform after the material had been treated with HCl, was predominantly of non-acid-fast material, but there were a few residual organisms which were found both in the material itself and in the tissues. This fourth residue consisted of two kinds of material—first, there was much granular material without any suggestion of bacillary form, which took the green counterstain in the Ziehl-Neelsen technique; and secondly, larger masses of granular material which became a dull purple color in the same technique. These larger masses seemed to have shadows of the bacillary form. The acid-fast organisms found were only faintly acid-fast and were described as "suggestive of tubercle bacilli."

The original preparation of the unfilterable lipid was itself not acid-fast but contained a considerable number of acid-fast bacilli; these were separated out

from the material by centrifugation, as described above, until none was found either in the material itself or in the tissues.

The unfilterable lipid and the hydroxy acid obtained from it offer interesting materials for the study of acid fastness, because the unfilterable lipid is non-acid-fast and the hydroxy acid is acid-fast. We had difficulties in applying the acid-fast technique to the chemical fractions to obtain comparable results until we found that preparations made with the standard technique varied markedly in accordance with the melting point of the materials tested. Specifically, those materials which are highly acid-fast, the unsaponifiable wax and the hydroxy acid of these experiments, have so low a melting point, 54–56°, that the heating involved in the standard technique melts the material and makes it roll into droplets or spheres much too dense to analyze any structure in them. The method we have found best is to obtain a dilute solution of the material in its appropriate solvent, which is chloroform for the so called waxes, and spread this solution evenly on a clean slide. The slide is then thoroughly dried in an incubator at 37° and then covered with fuchsin and allowed to stand at 37° for from 12 to 16 hours. The decoloring, washing, and counterstaining are done by letting the fluids flow gently onto the end of the slide and run over the surface of the films. By this method it can be seen that the unfilterable lipid spreads in an exceedingly thin homogeneous film on the slide and that it not only does not retain the fuchsin but it reacts readily to the counterstain. The hydroxy acid, on the other hand, retains the fuchsin and rejects the counterstain. Under oil immersion lenses it could be seen that the hydroxy acid was made up of finely but slightly irregularly granular material which is acid-fast; it contained a very few clumps of acid-fast bacilli.

RESULTS

Defatted Bacilli and Third Residue.—The cellular reactions to the defatted bacilli and to the next residue in series were so similar that we have included the records of both substances in Table I. The bacilli were from the human strain, A-14. The only difference between the two materials was that the reactions to the third residue, while exactly the same in kind, were consistently a little greater in amount. There were more cells, and more bacilli were found in the giant cells. The defatted residue was in the form of a white granular material moderately clumped; the third residue was slightly yellowish and was in larger clumps. Both were from human tubercle bacilli, strain A-14. Both were rubbed into suspensions, which settled so quickly that it was better to weigh the material for each injection separately; some of the suspension was unavoidably lost by sticking to

TABLE I

Cellular Reactions to Defatted Tubercle Bacilli (Human Strain, A-14) and to the Third Residue

Rabbit No.	Material	Number, amount, and site of injections	Time interval after intraperitoneal injection	Results
			days	
R 5407, 5408 *	Defatted tubercle bacilli	1 of 1 mg. in 1 cc. saline i.p. and 6 of 0.1 mg. in 0.1 cc. saline i.d.	10	The last intradermal injection was like a + or ++ tuberculin test. The dermal lesions showed neutrophiles, monocytes, and giant cells, the latter containing acid-fast bacilli. The peritoneal exudates were of monocytes and lymphocytes in about equal numbers. The omenta showed foci of monocytes and epithelioid cells surrounded by lymphocytes, and some giant cells
5409, 5410	Third residue			
5542, 5543	Defatted tubercle bacilli	6 of 1 mg. in 1 cc. saline i.p.	12	4 days after the last injection animals tested with 1 mg. of MA-100 i.d. and gave +++ and ++++ reactions. The peritoneal exudates were of monocytes and lymphocytes in about equal numbers. The omenta showed abscesses; tubercles of epithelioid cells with infiltration with lymphocytes; giant cells containing acid-fast bacilli
5540, 5541	Third residue			
6044, 6045	Defatted tubercle bacilli	1 of 20 mg. in 5 cc. dist. H_2O i.p.	8	Peritoneal exudates of the two groups showed: *PMN* 43% *Lymphocytes* 4% *Monocytes* 52%
6046, 6047	Third residue			35% 2% 62%
				About 10% of the monocytes had phagocytized neutrophiles. The omenta and body walls had massive and complex reactions; many abscesses, monocytes, and epithelioid cells both scattered and in tubercles; free and phagocytized neutrophiles; edema; giant cells with acid-fast bacilli

i.p. = intraperitoneal; i.d. = intradermal.

* These are serial numbers covering the work of the laboratory for a term of years.

the wall of the syringe. There were three experiments with approximate dosages and intervals, as shown in Table I. The material had power to sensitize to tuberculo-protein, shown both in the increasing size of the reactions to intradermal injections of the material itself in the first experiment, and in the reactions to tuberculo-protein MA-100 in the second experiment. The cellular reactions were exceedingly complex and are shown in Figs. 1, 2, 5, and 6. There were small abscesses of neutrophilic[1] leucocytes; more often there were tubercles of epithelioid cells and giant cells infiltrated with neutrophiles, as in Fig. 1. This tubercle was very small; others measured 2 to 3 mm. in diameter. Some of the tubercles showed new fibers; other areas showed a diffuse infiltration with monocytes, as in Fig. 2. There were many giant cells, some in the tubercles, as in Fig. 1, some quite free in the tissues. One of these giant cells is shown in Fig. 5; the stain was hematoxylin and eosin and the deeply eosinophilic cytoplasm indicated that the cell was badly damaged or dead. This cell was so large that it appeared in several serial sections, and so a part of it is also shown in Fig. 6, stained for tubercle bacilli. It contained only a few bacilli as compared with many of the giant cells, for in some of them there were such dense masses that no photograph could resolve them into separate organisms.

The Products of Extraction with 25 Per Cent Alcohol.—The extract obtained by treating the defatted bacilli with 25 per cent alcohol was a brown, salve-like material from which Dr. Anderson obtained a polysaccharide. The cellular reactions to the original extract and to the purified material obtained from it are shown in Table II.

The reactions in these two experiments were typical of the effect of polysaccharides. The tissues were studied 24 hours after the injections were made. There was a high percentage of neutrophiles in the peritoneal exudates and many of these extravasated neutrophiles had been engulfed by the monocytes in the milk spots of the omentum.

Fourth Residue and Its Derivatives.—In Table III are shown the cellular reactions to the fourth residue and to the unfilterable lipid and its two derivatives, namely, the hydroxy acid and the polysac-

[1] The term neutrophile is used for the pseudoeosinophilic leucocyte of the rabbit.

charide. These materials were from the human strain, A-10. The fourth residue was a fine, granular material which was non-acid-fast but contained a few tubercle bacilli. The four rabbits which received the fourth residue showed a moderate increase in monocytes in the milk spots of the omentum, and some infiltration of these milk spots with neutrophiles. The number of the neutrophiles varied in the different animals. There were some giant cells containing acid-fast bacilli.

TABLE II

Cellular Reactions to a 25 Per Cent Alcoholic Extract and a Polysaccharide

Rabbit No.	Material	Number, amount, and site of injections	Time interval after intraperitoneal injection	Results
			hrs.	
R 5704, 5705	25% alcoholic extract from human tubercle bacilli, strain A-14	20 mg. in 1 cc. saline i.p.	24	Increase in neutrophiles in the peritoneal exudates, averaging 82%; monocytes, 15%; lymphocytes, 1%; serosal cells, 2%. Marked phagocytosis of neutrophiles in milk spots of omentum
5664, 5665, 6118	Polysaccharides from human tubercle bacilli, strain A-10	10 mg. in 1 cc. saline i.p.	24	Increase in neutrophiles in peritoneal exudates. Neutrophiles, 58%; lymphocytes, 3%; monocytes, 39%, of which 10% had phagocytized neutrophiles. Marked phagocytosis of neutrophiles in milk spots of omentum
6111, 6118, 6049		10 mg. in 5 cc. saline i.p.		

The unfilterable lipid gave a moderate reaction consisting of an increase in monocytes and some giant cells. Practically every milk spot contained a giant cell, as seen in films of the omentum. As shown in Fig. 4, some of the giant cells were surrounded by neutrophilic leucocytes. This is interesting because this lipid is made up of two substances, an hydroxy acid which itself gives rise to giant cells, and a polysaccharide which attracts neutrophiles from the

TABLE III

Cellular Reactions to the Fourth Residue, the Unfilterable Lipid and Its Derivatives, the Hydroxy Acid, and the Polysaccharide, Human Strain A-10

Rabbit No.	Material	Number, amount, and site of injections	Time interval after intraperitoneal injection	Results
			days	
R 5706 5707	Bacterial residue IV (contains a few acid-fast tubercle bacilli)	20 mg. in 1 cc. saline i.p.	7	Moderate increase in monocytes and some infiltration of tissues with neutrophiles; a few giant cells containing acid-fast bacilli
6335 6336		20 mg. in 5 cc. dist. H_2O i.p.		
5676 5677	Unfilterable lipid	20 mg. dry powder through operative incision under anesthesia, i.p.	7	Moderate reaction; peritoneal exudates contain neutrophiles, 33%; lymphocytes, 3%; monocytes, 63%. Omentum, diffuse increase in monocytes; some giant cells surrounded by neutrophiles
5702 5703	Hydroxy acid (contains very few acid-fast tubercle bacilli)	" "	7	Moderate reaction; peritoneal exudates contain neutrophiles, 3%; lymphocytes, 4%; monocytes, 92%. Omentum showed diffuse increase in monocytes; some giant cells surrounded by eosinophiles
6029 6030 6115 6116	Polysaccharide from unfilterable lipid	10 mg. in 5 cc. saline i.p.	24	The peritoneal exudates of the two groups differed in proportion of free neutrophiles: *PMN* *Lymphocytes* *Monocytes* 80% 1% 18%
6125 6126		10 mg. in 0.5 cc. dist. H_2O i.p.		24% 6% 68% The omenta showed neutrophilic leucocytes and their phagocytosis by monocytes

vessels. The reactions shown in Fig. 4 suggest that at this interval, 7 days after the injection, the giant cells had been able to break down the lipid enough to liberate some polysaccharide.

The cellular reaction to the hydroxy acid is shown in Fig. 3. It consisted of the formation of foreign body giant cells, some with a few nuclei, some with many. There were a very few bacilli in this material but in our opinion there were far more giant cells than could be caused by them. No bacilli were found in the tissues. The foreign body giant cell is the characteristic reaction to the waxes from tubercle bacilli. At a certain stage the tissues around these giant cells become infiltrated, not with neutrophiles but rather with eosinophiles which are marked by arrows in Fig. 3. The polysaccharide from the unfilterable lipid gave rise to the same reactions as were described above for the polysaccharide from the defatted bacilli.

DISCUSSION

The defatted tubercle bacilli were of course rich in protein since this is the material from which the proteins of the organisms are obtained. No studies were made of the proteins from this material, however, since we have studied the cellular reactions to the bacillary proteins from material given to us by Dr. Michael Heidelberger (76).

The defatted tubercle bacilli were only partially defatted for they still contained enough lipid to be acid-fast. The defatted tubercle bacilli and the third residue gave cellular reactions which were much like those known to characterize the reaction to heat-killed tubercle bacilli. They were complex and consisted of abscesses; tubercles of monocytes and epithelioid cells; of monocytes diffusely scattered in the tissues; of giant cells, many of which contained masses of the tubercle bacilli. Some of the tubercles were infiltrated with neutrophiles. Most of the acid-fast organisms were to be found in the giant cells; occasionally one or two were found in a monocyte, and more rarely still, a few were free. The last residue, from which the firmly bound lipid had been removed, was relatively inert material. It was clear that the protein in this residue must have been much altered from its original state; this residue, however, still retained the property of stimulating a new formation of monocytes.

The unfilterable lipid and hydroxy acid gave interesting material for the study of cellular reactions because the unfilterable lipid was made up of the hydroxy acid plus a polysaccharide. The basic cellular reaction to the hydroxy acid was the formation of foreign body giant cells in greater numbers than could be induced by the small content of bacilli. The unfilterable lipid gave such giant cells, and after a week's time the tissues became infiltrated with neutrophiles, that is, reactions to this lipid included those of its constituents, the hydroxy acid and the polysaccharide.

SUMMARY

The cellular reactions to defatted tubercle bacilli are complex and like those to heat-killed whole tubercle bacilli.

The firmly bound lipid, when removed from these organisms, is non-acid-fast; it contains an hydroxy acid which is acid-fast and a polysaccharide, which is not.

This hydroxy acid gives rise to foreign body giant cells and the tissues eventually become infiltrated with eosinophiles.

The polysaccharides, both from the defatted bacilli and from the unfilterable lipid, call neutrophiles from the blood stream.

The reactions to the unfilterable lipid include those of both its constituents.

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EXPLANATION OF PLATES

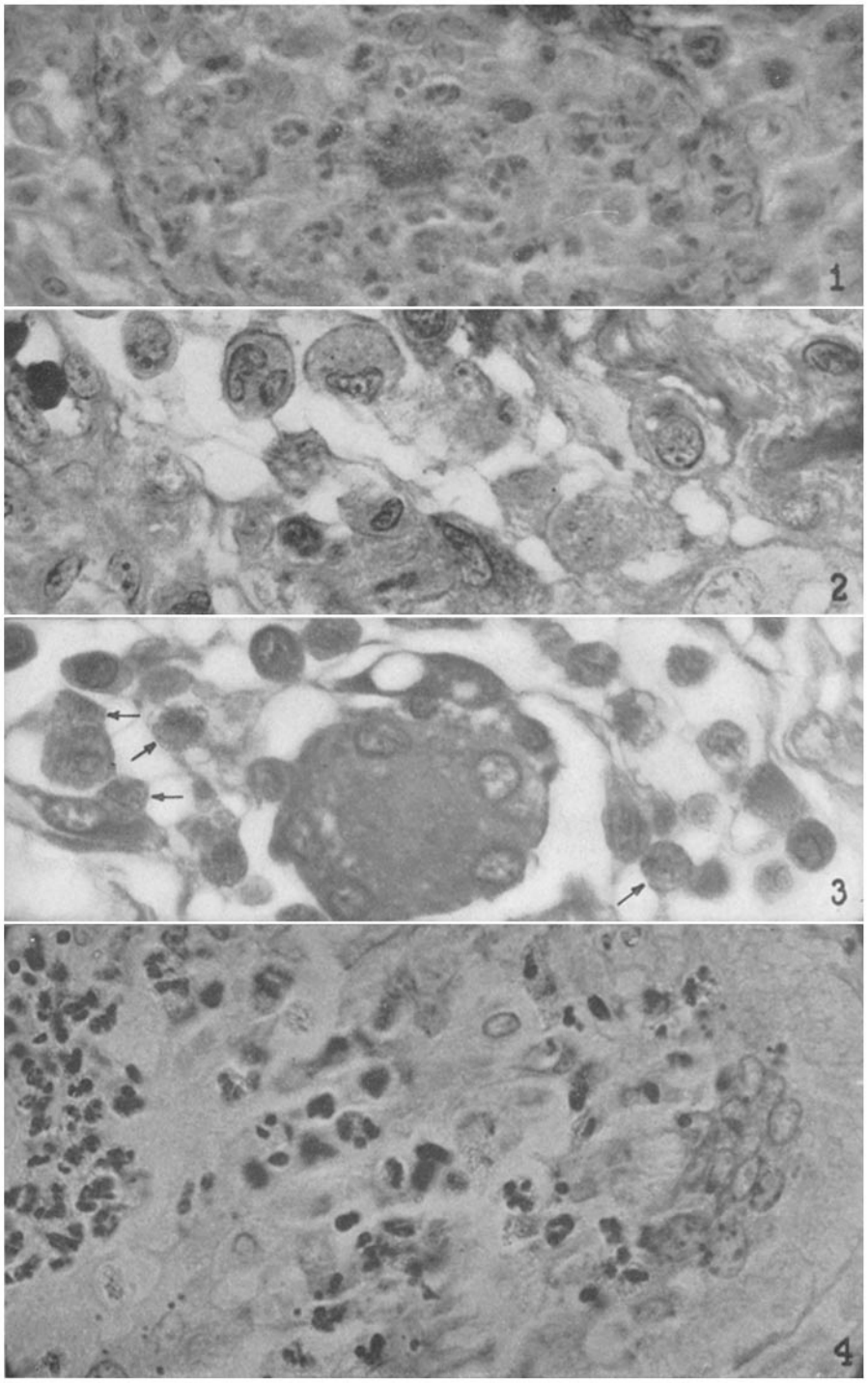
PLATE 45

FIG. 1. Section of the omentum of a rabbit, R 6046, which had received 20 mg. of the third residue, strain A-14, in 5 cc. distilled water, intraperitoneally. The tissues were studied 8 days later. It shows one of the smallest tubercles; it has a giant cell; it consists of epithelioids infiltrated by neutrophiles. It has a sharp border of fibers. Foot's modification of Masson stain. × 1000.

FIG. 2. Section of the omentum of a rabbit, R 6047, which had received the same material as in Fig. 1. The tissues were studied 8 days later. It shows an area with a diffuse infiltration of monocytes. Foot's modification of Masson stain. × 1000.

FIG. 3. Section of the omentum of a rabbit, R 5702, which had received 20 mg. of the hydroxy acid, intraperitoneally. It was introduced as a dry powder through an incision under anesthesia. The tissues were studied 7 days later. It shows a foreign body giant cell, some monocytes, and an infiltration of the tissues with eosinophiles, which are marked by arrows. Foot's modification of the Masson stain. × 1000.

FIG. 4. Section of the omentum of a rabbit, R 5677, which had received 20 mg. of the unfilterable lipid, intraperitoneally. It was introduced as a dry powder through an incision, under anesthesia. The material had had the bacteria removed by centrifugation. The tissues were studied 7 days later. It shows a foreign body giant cell and a marked infiltration of the tissues with neutrophiles. Stained with Giemsa. × 1000.



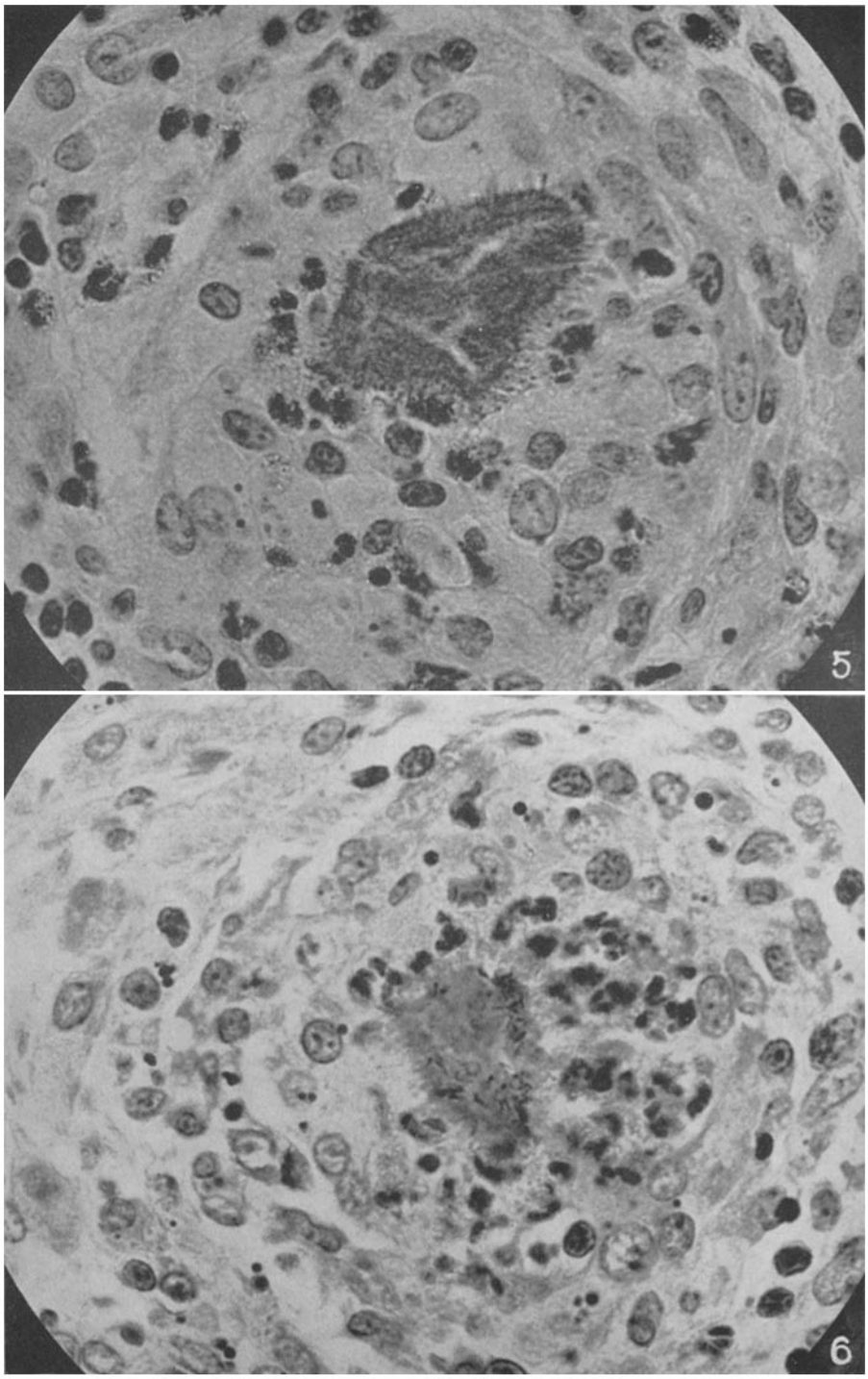
Photographed by Joseph B. Haulenbeek

(Sabin and Joyner: Cellular reactions to defatted tubercle bacilli)

PLATE 46

FIG. 5. Section of a nodule on the intestine of a rabbit, R 5410, which had received 1 mg. of the third residue, intraperitoneally. The tissues were studied 10 days after the injection. It shows a dead giant cell in the center of a tubercle of epithelioid cells. This tubercle contains a few neutrophiles. Stained with hematoxylin and eosin. × 1000.

FIG. 6. Section of a part of the same giant cell as shown in Fig. 5, stained to show the acid-fast bacilli within it. The technique used was that described by Fuller (77). × 1000.



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