

CELLULAR REACTIONS IN THE MENINGES OF RABBITS  
TO TUBERCULO-LIPOID, PROTEIN, AND POLYSACCHA-  
RIDE, COMPARED WITH THE EFFECTS OF  
TUBERCLE BACILLI

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PLATE 1

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The experiments presented in this paper involve the study of meningitis produced in rabbits by the introduction of living and killed tubercle bacilli into the subarachnoid space, as compared with the effects of the injection of various extracts from the tubercle bacillus. Every extract tested has produced some symptomatic or pathological change. The tests have been made in tuberculous and in normal rabbits. The differences in their reactions have proved to be only quantitative.

Recent experimental studies of tuberculous meningitis have been made by Opie (1), Manwaring (2), Austrian (3), Foot (4), Kasahara (5), and Soper and Dworski (6, 7). In 1925, Soper and Dworski (6) injected tubercle bacilli into the meninges of tuberculous rabbits. They obtained striking results, showing that the phenomena attending superinfection manifest themselves in the meninges of the rabbit as well as in the other serous membranes and organs of various experimental animals. They found that with high superinfecting doses death was probably hastened by an allergic state; with medium doses the period of survival was double that of the controls; while with relatively small doses the superinfected animals often showed high resistance and survived a long period in comparison with the controls, which had received a single inoculation in the meninges. Examination of the spinal fluid indicated that in the superinfected animals an intense meningeal reaction occurred immediately, with increased red and white cell counts which persisted for a few days after inoculation but gradually diminished. In the controls the counts were relatively low at first and then followed an irregular course upward as the disease progressed. In 1930 they published further work (7) showing that by using very small doses for superinfection, the rabbits were able to survive the disease, while all the control animals which had received a single inoculation into the meninges died.

In 1929, Rich and McCordock (8) in their studies on the relation of allergy to immunity and to the lesions in tuberculosis gave evidence to show that, clinically, meningitis occurs when a tuberculous focus in the brain or meninges ruptures, liberating living bacilli into the subarachnoid space. They think that the bacilli do not pass directly from the blood stream to the spinal fluid, but that the involvement of the meninges is due to the fact that some focus which had developed in a blood vessel or in the periphery of the brain becomes caseous and ruptures into the subarachnoid space, thereby liberating organisms into it. This idea fits well with the observations made in experimental work, for intravenous inoculations with living organisms rarely result in meningitis, and, when it occurs, a caseous area can be located which has ruptured into the subarachnoid space.

The first of the present studies was made with living tubercle bacilli.

#### *Methods*

The organisms were introduced by the postorbital route, but this was soon given up since it was too difficult to obtain cerebrospinal fluid and to determine the exact position of the point of the needle. It is essential to withdraw as much fluid as is injected in order to avoid the phenomena due to increased intracranial pressure. Cisternal puncture through the atlantooccipital ligament was found feasible. Later it was discovered that this method had been described in 1919 by Wegeforth, Ayer, and Essick (9). Better results are obtained if, after entering the skin and muscles over the area, the stylet of the needle is withdrawn before piercing the ligament. This affords the advantage of obtaining a free flow of fluid as soon as the point of the needle enters the cisterna cerebellomedullaris, with avoidance of the possibility of entering the medulla before one is aware that the needle has passed through the ligament. After one has become familiar with the method there is no need for an anesthetic, since it causes little disturbance of the animal.

The bovine tubercle bacillus used was the Strain B-1, originally isolated by Dr. Theobald Smith and obtained from the Saranac Laboratory. It was known to be virulent for rabbits. The suspension was prepared from a weighed quantity of bacilli grown on Petroff's egg-gentian violet medium. Normal saline was added to make a suspension, the organisms were then counted in the Petroff-Hausser bacterial counting chamber, and a further dilution made until 0.2 cc. contained the desired number (usually 500,000). If any large clumps were present, the suspension was filtered and recounted.

Heat-killed organisms were prepared from a subculture of the B-1 strain as above. 100 mg. (moist weight) of organisms were ground in 10 cc. of freshly prepared normal saline. The suspension was heated in a water bath at 70°C. for 1 hour. Dilutions were made so that 0.2 cc. of the final suspension contained the desired number of organisms.

Formalin-killed organisms were also prepared from a subculture of B-1 described above. 400 mg. (moist weight) of organisms were suspended in 20 cc. of normal

saline. Sufficient formalin was added to make a concentration of 0.4 per cent. This was incubated at 37°C. for 10 days. The suspension was then centrifuged and the fluid decanted. The remaining formalin was removed by washing three times with freshly prepared normal saline solution, and the organisms were suspended in normal saline solution with 0.5 per cent phenol added as a preservative. Dilutions of this suspension were made so that the amount used for inoculation contained the desired number of organisms.

The lipid used for the subarachnoid injections was the phosphatide A-3, isolated from the bovine tubercle bacillus by Dr. R. J. Anderson (10) at the Sterling Chemistry Laboratory of Yale University. This extract has proved to have the specific biological property of producing tubercular tissue, that is, epithelioid cells, when introduced subcutaneously and intraperitoneally in rabbits and guinea pigs (11).

The tuberculo-protein was received from the H. K. Mulford Biological Laboratories and was designated as MA-100. This was prepared from the filtrate of cultures of the human tubercle bacillus, Strain H-37.

Fresh tissue from the dura, pia, and brain was examined immediately after removal at autopsy, using supravital staining with neutral red and Janus green (12). The entire brain and upper portion of the cord were fixed in 10 per cent formalin for 2 days. After this, they were cut into blocks, washed, dehydrated, and embedded in paraffin. The sections were stained with hematoxylin and eosin. The other organs were fixed in Helly's mixture (Zenker base with 5 per cent formalin), and treated as above. Needless to say, all the animals were kept under the same conditions as to air, sunlight, feeding, and general care.

The occurrence of spontaneous encephalitis in apparently normal rabbits is well known. It was first described and related to a protozoan parasite by Wright and Craighead (13) in 1922 and has since been repeatedly studied (14-19). The lesions consist of round cell infiltrations of the meninges, perivascular infiltrations in the brain tissue, necrotic areas and cysts in which the parasite may be seen. They may closely resemble tubercles with caseous centers and were frequently encountered in this study.

#### *Tuberculous Meningitis in Rabbits*

Fifteen normal, healthy, adult rabbits were inoculated intrameningeally with 500,000 (0.0000125 gm.) undissociated tubercle bacilli, Strain B-1, from a 3 weeks' growth of Subculture 223. All the animals appeared well after operation. There were no symptoms of meningitis or of paralysis. At short intervals after inoculation they were killed to determine the cellular changes.

The cellular reactions, both to tubercle bacilli themselves and to the phosphatide extracted from them, were characterized by a type of cell which merits especial description.

The cell was predominant in the reaction in the pia in the early stages of the disease, and later was found in the cerebrospinal fluid. It is shown in a photograph of living cells from the pia in Fig. 1. Its size, as can be judged from the red corpuscle in the lower right hand corner of the figure, was about that of an actively phagocytic clasmatocyte or a large epithelioid cell. The cytoplasm was so completely filled with vacuoles that the nucleus was entirely obscured. The vacuoles were of even size, uniformly stained, and reacted with the neutral tint of vital neutral red. The reason for the variations in the photograph is that the content of the vacuoles was highly refractive, suggesting lipoidal material, and, as is well known, highly refractive globules appear black when the focus is not in the equatorial plane.

It is difficult to classify this cell. It differs from the actively phagocytic clasmatocyte usually found in the tissues, the latter having vacuoles of different size which show the complete range of color of the dye at different pH values, namely, from red to yellow. Likewise, it has none of the characteristics of the typical monocyte.

Until the 15th day after the intrameningeal injection of the living bovine tubercle bacilli, the animals showed no abnormal symptoms. Those allowed to live 15 days or longer had first a weakness of the hind quarters, became progressively worse, and finally developed partial paralysis by the 27th day.

Although the rabbits showed no symptoms of meningitis until some time after injection, there was a definite cellular response which occurred during this incubation period.

On the 1st day after the inoculation, supravital studies of the pia revealed the characteristic cell described above as having the vacuoles of even size and uniform staining reaction with the vital neutral red. There was an abundance of these cells throughout the pia and some in the dura. On the 2nd day the specimens could not be distinguished from those of the preceding day. By the 3rd day a definite increase in the number of lymphocytes and plasma cells in the specimen from the pia was observed. At the 4th day a few of the characteristic vacuolated cells just described showed certain changes; namely, an increase in the number of the vacuoles, with a corresponding decrease in their size. The vacuoles at this time were larger than those of the cell designated by Sabin *et al.* (11) as the coarse vacuole epithelioid cell. The predominance of lymphocytes and plasma cells was observed. The next day (the 5th), there did not seem to be any increase in the number of cells with the smaller vacuoles. The cells with larger vacuoles were present in far greater proportion than those with the smaller. A scraping from the medulla and midbrain contained sheets of monocytes, some of which very nearly approached typical epithelioid cells (Fig. 2). Otherwise there was no change in the findings. For the period from the 6th to the 15th day, it was difficult

to distinguish any daily change in the cellular picture other than a steady increase in the number of lymphocytes and plasma cells in the specimens from the pia. By the 15th day, however, a great number of the mononuclear cells containing vacuoles of even size and uniform staining reaction showed an increase in number and a decrease in size of the vacuoles, which was a reversal of the previous findings. At the end of 27 days, only an occasional cell with the large vacuoles was observed. The vacuoles in most of the cells of this type had diminished in size until they were the size found in the coarse vacuole epithelioid cell. These cells correspond to those seen in Fig. 4.

Histological sections from these animals showed the visceral organs to be normal during the entire course of the experiments. The sections from the brain and cord during the first 13 days revealed a progressive lymphocytic infiltration of the meninges extending into the sulci. By the 13th day the vessels around the periphery of the cord for a short depth showed marked perivascular lymphocytic infiltration. On the 19th day the infiltration had greatly increased and there was beginning tubercle formation in some areas of the meninges. Sections on the 27th day showed extensive tuberculous processes in the meninges and invading the brain substance.

Ten rabbits were inoculated intrathecally with 800 organisms. Some of these animals are still living so that the data are incomplete but it is clear that both the period before the onset of symptoms, and the survival, are longer than in animals inoculated with the greater number of organisms.

#### *Subarachnoid Inoculation of Heat-Killed and Formalin-Killed Tubercle Bacilli*

Varying doses of killed organisms were injected in a small series of rabbits to determine whether there was any distinguishable difference in the reactions to bacilli killed by heat, and to those killed with formalin. In Table I are presented the findings in the animals inoculated intrathecally with heat-killed tubercle bacilli.

In the animals receiving the heat-killed organisms, the immediate reaction was similar to that with the living organisms. The predominant cell in the pia, as seen in fresh preparations, was the cell with even sized vacuoles taking the uniform stain with the vital neutral red. The vacuoles were of the coarse variety. This preparation could not be distinguished from that obtained in the first 6 days after

the injection of living bacilli. Acid-fast stains of the pia in one animal 2 days after the last injection demonstrated acid-fast debris, but no whole organisms. Phagocytosis of this debris could not be detected.

TABLE I  
*Results of Intrameningeal Injections of Heat-Killed Tubercle Bacilli into Normal Rabbits*

No. of rabbit	Amount of bacilli	Interval between injection and autopsy	Course	Autopsy findings
R 1787	8 million	1 day	Operative trauma. Killed	Brain showed neither edema nor hemorrhage. Viscera normal. Supravital studies of the pia showed great numbers of the meningeal type of early epithelioid cell and a few lymphocytes and plasma cells
R 1786	8 million. 2 days later 8 million	4 days	Operative trauma at 2nd injection. Killed	Brain covered with a blood clot. Viscera normal. Supravital studies of the pia showed great numbers of the meningeal epithelioid cells. Lymphocytes were pronounced in both pia and dura
R 1791	8 million. 2 days later 8 million. 2 days later 8 million	4 days	Killed on day of last injection	Brain slightly injected; no edema and no hemorrhage. Viscera normal. Supravital studies of the pia showed the same cellular reactions as in R 1786
R 1782	8 million	28 days	Animal normal. Killed	Brain appeared normal; no edema and no hemorrhage. Viscera normal. Supravital studies of the pia showed enormous numbers of the meningeal type of early epithelioid cell. No diminution in the size of the vacuoles of these cells
R 1756	1 million	33 days	Animal normal. Killed	Same as in R 1782

TABLE I—*Concluded*

No. of rabbit	Amount of bacilli	Interval between injection and autopsy	Course	Autopsy findings
R 1757	1 million. 5 weeks later 8 million	78 days (41 days after last injection)	Normal until 65th day. Killed after 13 days illness, with weakness and loss of coordination	Brain appeared normal. Viscera normal. Cerebrospinal fluid from the 4th ventricle contained sheets of epithelioid cells and stimulated monocytes. Supravital studies of the pia revealed epithelioid cells with small, uniform, even sized vacuoles. Increase in lymphocytes. Sections revealed tubercles in the meninges and an invasion of the brain with lymphocytes from the pia
R 1783	8 million. 1 month later 8 million	93 days (65 days after last injection)	Normal until 68th day. Killed <i>in extremis</i> after 25 days illness, with weakness and loss of coordination	Same as in R 1757 except a more extensive tuberculous process in the meninges and the beginning of an invasion of the brain with tubercles

At the end of 1 month the animals appeared normal. There was no loss of weight, nor other signs of illness. Fresh preparations of the pia at this time revealed a predominance of the same cells as described above with the equal sized and uniformly staining vacuoles. The vacuoles did not seem to have diminished appreciably in size. The number of lymphocytes and plasma cells was far in excess of that found in earlier studies. Again, it was difficult to distinguish this reaction at the end of a month from that obtained during the middle of the incubation period after inoculation with living bacilli. Histological studies showed that the visceral organs were normal. The sections of the brain showed lymphocytic infiltration of the meninges of a mild character. There was no involvement of the brain substance and no perivascular reaction.

The two remaining rabbits, R 1757 and R 1783,<sup>1</sup> continued in good health until the 65th day after the first inoculation when R 1757 suddenly began to lose weight, became weak in the hind quarters and progressively worse. 13 days after this acute exacerbation, on the 78th day after injection, the animal was killed. R 1783 had a similar history. This animal became ill on the 68th day and, being *in extremis* after progressive weakness for 25 days, was killed on the 93rd day after injection. In these two animals no gross tubercles were observed in the brain or meninges. Scrapings from the ventral surface of the medulla, studied supravitaly, showed numerous stimulated monocytes and typical epithelioid cells. Cerebrospinal fluid removed directly from the fourth ventricle as soon as the cranial cavity was opened showed these same cells. Fig. 3 shows a sheet of these cells in spinal fluid. Preparations of the pia, using the same technique, revealed the coarse vacuole cell described previously. Plasma cells and lymphocytes were present in unusually large numbers. The dura was thickened but contained no abnormal cellular elements. Histologically, the brain condition closely resembled that of the last 2 animals (R 2029, R 2030) of the experiments with living bacilli.

Two rabbits were inoculated with formalin-killed organisms. Neither showed abnormal signs. One was killed at the end of 1 month and exhibited pathological features similar to those of the heat-killed group at this period. The other rabbit was finally killed on the 129th day to terminate the experiment and at autopsy was found to be normal except for an occasional cell with equal sized vacuoles of both large and small type in the pia.

The number of animals in these two groups is too small to warrant conclusions. One can only say that the evidence points to the fact that heat-killed organisms are not innocuous when introduced into the meninges of the rabbit. They produce lesions similar to those obtained with living organisms, but the time required for development is greatly prolonged. Further studies of these reactions are being made.

*Reaction in Normal and Tuberculous Rabbits to Subarachnoid Introduction of Bovine Phosphatide A-3*

Sabin and her associates (11) have injected as much as 80 mg. of phosphatide intraperitoneally in rabbits. It was plain that so much could not be injected into the subarachnoid space.

<sup>1</sup> These are serial numbers of the work of the department covering a term of years.

A dose of 7.5 mg. of the phosphatide A-3 suspended in 1.5 cc. of freshly distilled water was introduced into one animal (R 1591). The rabbit died in 21 hours with signs of increased intracranial pressure. The autopsy findings in the brain were chiefly those of a non-specific reaction to a foreign body. It was found that the optimum amount of fluid for each subarachnoid injection was 0.2 cc. Since the phosphatide was sparingly soluble in water, the maximum dose was limited to 2.0 mg. in 0.2 cc. of fluid.

Nine normal (Table II) and seven tuberculous rabbits (Table III), were given phosphatide A-3 into the subarachnoid space.

The total amount introduced in each animal varied from 0.1 mg. (R 1605 and R 1606) to 12.0 mg. (R 1882); and the amount of phosphatide in the smallest dose (0.1 mg.) represents that in 1.6 mg. of bacilli, which is 160 times the amount of living bacilli which we used in these experiments. The largest dose represents nearly 20,000 times this amount. It was found necessary to grind the phosphatide in a mortar while adding water drop by drop. In this manner a uniform suspension was obtained, which did not precipitate on standing. Unfortunately in the tuberculous group two of the rabbits (R 1741 and R 1743) were traumatized at later operations as the needle punctured the medulla and caused hemorrhage. These were autopsied immediately afterward, and the fresh blood in the cisterna did not obscure the cellular response in the fresh tissues. None of the animals suffered from secondary infections.

In the studies with the phosphatide there have been no complications from the presence of a diluting menstruum; the sterile water used was itself inert to the connective tissues (11). The amount of reaction from the mechanical effects of the operation and the injection of 0.2 cc. of fluid in the cisterna was controlled by injecting one animal, R 1607, with a weak (non-toxic) solution of neutral red (0.0025 per cent). There was no rise in temperature after this operation and the organs were normal at autopsy. Five rabbits were also given 0.2 cc. of a filtrate obtained by grinding a normal rabbit's brain in normal saline and filtering through double layers of Whatman's No. 5 filter paper. Culture of the filtrate was sterile. In these rabbits there was no rise in temperature and they remained normal.

The phosphatide itself could not be sterilized, but other precautions for sterility were taken, and there were no signs of a contaminating infection after its use. In the animals receiving the phosphatide A-3, the early reaction was characterized by the cells with even sized and uniformly staining vacuoles. These cells were identical with those

TABLE II

*Results of Intrameningeal Injections of Tuberculo-Phosphatide into Normal Rabbits*

No. of rabbit	Amount of phosphatide in distilled water	Interval between injection and autopsy	Course and autopsy findings
R 1591	7.5 mg. in 1.5 cc.	Died in 21 hrs.	No symptoms after injection. Appeared ill following morning; head retracted to left; later clonic generalized convulsions, vertical nystagmus, coma, and death. Viscera normal. Brain edematous and hyperemic. Supravital studies of the pia and dura showed clasmatocytes
R 1605	0.1 mg. in 0.2 cc.	Killed 4th day	No symptoms after injection. Following day seemed lethargic and had hyperpnea. Recovered on the 2nd day and remained normal until killed on the 4th day. Viscera normal. Brain showed no edema and no hyperemia. Supravital studies of the pia revealed a number of meningeal epithelioid cells with coarse vacuoles. There were moderate numbers of lymphocytes
R 1606	0.1 mg. in 0.2 cc.	Killed 10th day	No symptoms after injection. Animal remained well until the 10th day, when it was killed. Viscera normal. Brain hyperemic but no edema. Supravital studies of the pia showed many epithelioid cells, both those with coarse and those with fine vacuoles. Moderate increase in lymphocytes
R 1638	1 mg. in 0.2 cc.	Killed on 24th day	No symptoms after injection. Animal remained well until the 24th day, when it was killed. Viscera normal. Brain normal in the gross. Supravital studies of the pia showed monocytes, epithelioid cells, and many giant cells with fine vacuoles. Increase in number of lymphocytes
R 1723	1 mg. in 0.2 cc.	Killed on 24th day	No symptoms after injection. Animal remained well until the 24th day, when killed. Brain normal in the gross. Supravital studies of the pia showed an increase in the meningeal type of epithelioid cells. Many clasmatocytes and moderate numbers of lymphocytes

TABLE II—*Concluded*

No. of rabbit	Amount of phosphatide in distilled water	Interval between injection and autopsy	Course and autopsy findings
R 1724	2 mg. in 0.2 cc.	Killed on 24th day	Same as R 1723
R 1725	2 mg. in 0.2 cc.	Killed on 24th day	Reactions same as in R 1723 and R 1724
R 1607	5 injections of 1 mg. each in 0.2 cc.	Killed on 32nd day	Animal remained well for 32 days. Killed 32 days after the 1st injection and 7 days after the last. Peritoneal cavity contained 30 cc. of clear fluid free of cells. No evidence of peritonitis. Brain normal in the gross. Supravital studies of the pia same as in preceding three animals

found after the introduction of heat-killed organisms both in the early reaction and after 1 month. Fig. 1 illustrates these cells well, although this particular photograph was from the pia of a rabbit killed 1 month after inoculation with heat-killed tubercle bacilli.

At the end of 1 month (24 to 32 days) the findings in the normal animals injected with phosphatide remained unchanged. The predominant cell was that with the coarse vacuoles. There was no diminution in the size of these vacuoles. This observation indicates that the meningeal cells break down the phosphatide much more slowly than the corresponding cells elsewhere.

In the tuberculous group at 16 days the picture also was unaltered.

One animal (R 1739) was finally killed to terminate the experiment, 99 days after the first injection of phosphatide. At that time the rabbit appeared in excellent health, had gained weight, and showed no signs of infection in spite of the fact that it had had tuberculosis for 4 months. At autopsy the brain appeared normal in the gross. A thick brown exudate covered the medulla and midbrain. A scraping from this contained the typical cells as described before, but the vacuoles were now much smaller in size (Fig. 4). It is interesting to note that some of these cells appeared to contain a lipoid showing myelin-like figures, which has been found to characterize the phosphatide itself (20, 21). The pia contained some of these cells but showed no marked stimulation. The other organs had extensive generalized tuberculosis.

TABLE III

*Results of Intrameningeal Injections of Tuberculo-Phosphatide into Tuberculous Rabbits*

No. of rabbit	Interval between infection with tubercle bacilli and 1st injection of phosphatide	Amount of phosphatide in distilled water	Interval between injection of phosphatide and autopsy	Course and autopsy findings
R 1718	26 days. Skin test positive 16th day	2 mg. in 0.2 cc.	1 day	No symptoms immediately after injection and no rise in temperature. No paralysis or signs the evening after the injection but died during the night. Extensive pulmonary tuberculosis. Postmortem changes in the brain too marked to obtain any studies of the brain but no hemorrhages seen
R 1741	22 days. Skin test positive 18th day	1 mg. in 0.2 cc.	2 days. Killed at a 2nd operation	No symptoms immediately after injection but rise in temperature to 105.9°F. in 4 hrs. No paralysis. Killed 2 days later by trauma at a 2nd operation. Pulmonary tuberculosis. Moderate hyperemia of brain; no edema or old hemorrhages, but fresh clot in ventricles. Supravital studies of the pia showed masses of meningeal epithelioid cells with coarse vacuoles. Few lymphocytes
R 1743	22 days. Skin test positive 18th day	2 injections of 1 mg. each in 0.2 cc.	2 days. Killed by trauma of 2nd operation	No symptoms immediately after injection but temperature rose to 107.1°F. in 5 hrs. No paralysis. At 2nd injection 2 days later the cerebrospinal fluid removed had blood and animal showed loss of coordination and convulsions and died in 1 hour. Pulmonary tuberculosis. Brain showed hemorrhage in cisterna and trauma of the cord. Supravital studies of the pia showed masses of meningeal epithelioid cells with coarse vacuoles

TABLE III—*Continued*

No. of rabbit	Interval between infection with tubercle bacilli and 1st injection of phosphatide	Amount of phosphatide in distilled water	Interval between injection of phosphatide and autopsy	Course and autopsy findings
R 1705	52 days	2 mg. in 0.2 cc.	3 days. Killed <i>in extremis</i>	No symptoms immediately after injection but temperature rose to 107°F. in 5 hrs. Weakness and loss of coordination developed on the 3rd day and the animal was killed by intravenous injection of air. Pulmonary tuberculosis. Brain showed slight hyperemia; otherwise same as in R 1743
R 1737	110 days	2 mg. in 0.2 cc.	5 days. Died	Animal in very poor condition at time of injection. No symptoms immediately after but died on the 5th day. Extensive generalized tuberculosis elsewhere but postmortem changes too great for study of the brain
R 1740	22 days. Skin test positive 18 days	2 injections of 1 mg. each, 2 days apart	16 days. Killed	No symptoms immediately after injection but the temperature rose to 107° and 106.3°F. in 5 hours after the injections. Killed on the 16th day after the 1st and 14 days after the 2nd injection while still in good condition. Pulmonary tuberculosis. Brain slightly hyperemic. Supravital studies of the pia showed great numbers of meningeal epithelioid cells with the coarse vacuoles

TABLE III—*Concluded*

No. of rabbit	Interval between infection with tubercle bacilli and 1st injection of phosphatide	Amount of phosphatide in distilled water	Interval between injection of phosphatide and autopsy	Course and autopsy findings
R 1739	22 days. Skin test positive 18th day	2 injections of 1 mg. each, 2 days apart 1 injection of 2 mg. 16 days after 1st injection, 14 days after 2nd	99 days. Killed	No symptoms immediately after injection but subsequent rise in temperature. Animal remained in good condition and gained 800 gm. in weight. Killed on 99th day after the first and the 83rd day after the last injection. Pulmonary tuberculosis. Brain normal in the gross but a thin, brown exudate over the medulla. Supravital studies of the pia over the midbrain showed epithelioid cells whose vacuoles were much smaller than in any of the previous specimens after the phosphatide. These cells were like the typical coarse vacuole epithelioid cells of other tissues. Pia over the brain showed a few of the same cells

The striking result of the intrameningeal injection of the tuberculo-phosphatide was the production of the cells with vacuoles of even size stained uniformly with vital neutral red. This response was more pronounced than after the introduction of living and heat-killed bacilli. These cells remained unchanged at the end of 1 month, but, finally at the end of 3 months, the vacuoles of the cells had diminished in size until they were the size found in the coarse vacuole epithelioid cell, and corresponded exactly to the type observed in the terminal stages after the introduction of living bacilli. It is interesting to note the relatively great length of time after phosphatide that the cells in the meninges take to break down the large vacuoles into small ones.

Studies of the cells of the peripheral blood of these rabbits were made at various times during the course of the experiment. In the control group there was no

essential change in the percentage or totals of the differential count. There was no tendency for a rise in monocytes. One normal animal, R 1607, receiving phosphatide intrameningeally, had a shower of monocytes for 1 day (22 per cent with a total of 3,058), but with this exception, the range of monocytes in this animal during the period of injections was 29 to 1,090 cells and the average total was only 906 monocytes, as compared with 928 before. The red cells remained normal. In the tuberculous group the blood changes were characteristic of advancing tuberculosis with an irregular rise in monocytes.

Precipitin tests (20) on the spinal fluid were negative in all cases. R 1882, which received a total of 12.0 mg. of phosphatide, had no precipitin reaction of the blood serum. This test was performed in order to find any evidence of precipitins to the phosphatide in the blood serum.

*The Reaction in Normal and Tuberculous Rabbits to Subarachnoid Injections of the Tuberculo-Protein, MA-100*

A normal rabbit, R 1639, was injected intrathecally with 1.0 mg. of tuberculo-protein MA-100. The animal appeared normal after the operation. The temperature rose from 102.8°F. before injection to a peak of 106.4°F. in 5 hours. The following day the temperature remained around 104.0°F., but on the 2nd day after injection it was below the level previous to the operation. 12 days later, the animal received a second dose of 1.0 mg. of MA-100. The temperature rose from 99.8° before injection to a peak of 105.4° in 3½ hours, but was normal the following day. The rabbit was then given protein every 3rd day. The third injection of 1.0 mg. gave a rise in temperature from 101.8° to 103.2° in 2 hours without a peak and remaining within the limits of normal variation. The fourth injection of the same 1.0 mg. dose on the 3rd day following gave only a variation in temperature within the normal limits (Chart 1). Apparently the animal had become refractory so that it failed to react to this dose of protein intrameningeally. Following this, on the 3rd day, the dose was increased to 2.0 mg. and the temperature reaction was similar to that after the original injections. The temperature rose from 101.8° before injection to a peak of 105.6° in 5 hours. A final injection of 4.0 mg. was given after 3 more days, with a rise in temperature from 102.6° before injection to a peak of 106.0° in 6 hours. The following day the temperature was normal (102.3°). 2 days after this last injection the animal appeared normal and healthy. It was killed by the intravenous injection of air. At autopsy the visceral organs were normal in the gross and on histological study. There were no hemorrhages in the tissues. The brain showed no hyperemia, edema, or hemorrhages. The dura was thickened. Supravital studies of the pia revealed a marked clasmatocytic response. The cells contained uneven sized vacuoles which varied from salmon pink to deep red. None of the typical coarse vacuole epithelioid cells, found after phosphatide, were seen. The predominant cells were lymphocytes and plasma cells, in sharp contrast to those seen after phosphatide.

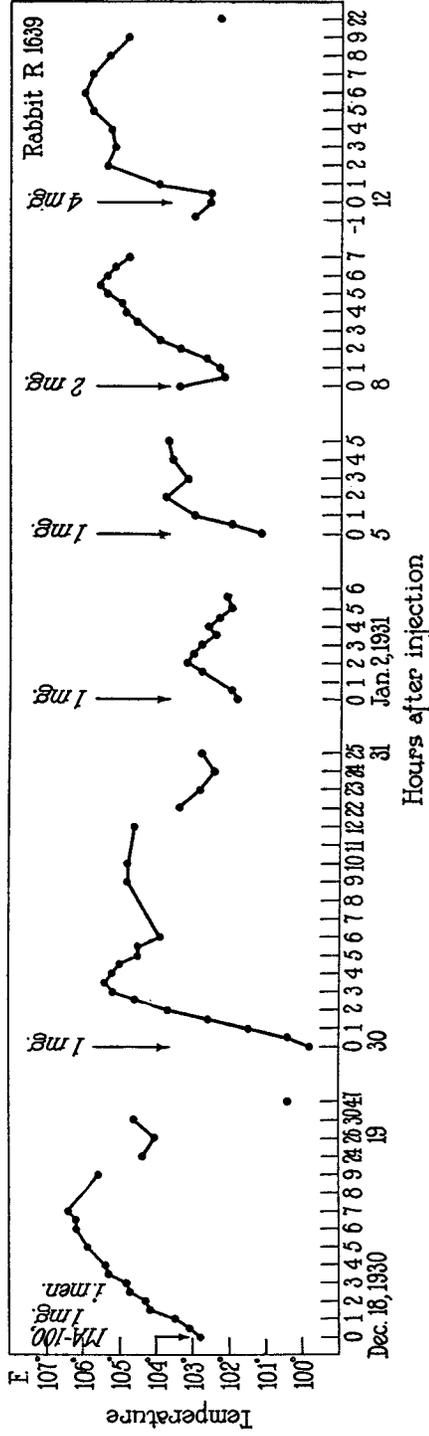


CHART 1. The changes in temperature in a normal rabbit, R 1639, after repeated intraneural injections, by cisternal puncture, of varying amounts of tuberculo-protein MA-100

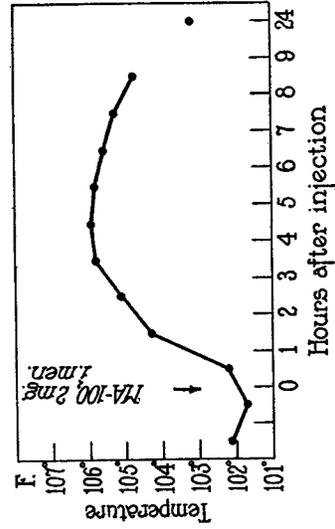


CHART 2. Fused temperatures of eight normal rabbits after intraneural injection, by cisternal puncture, of 2.0 mg. of tuberculo-protein MA-100.

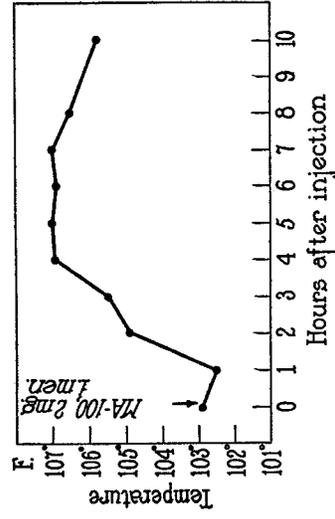


CHART 3. Fused temperatures of eight tuberculous rabbits after intraneural injection, by cisternal puncture, of 2.0 mg. of tuberculo-protein MA-100.

The response of the animal above was typical of the temperature reactions elicited in the normal animals. Chart 2 shows the fused temperature reactions of eight normal rabbits after a single injection of 2.0 mg. of MA-100 intrameningeally. The lowest peak was 105.2°, while the highest was 107.0°, in this group.

Eight tuberculous rabbits were given doses of 2.0 mg. of MA-100 intrameningeally. Six animals received one dose, one animal two doses, and one animal four doses. Each gave a temperature reaction and in each case of the tuberculous group, the rise was higher than that elicited in the normal animal. Chart 3 shows the fused temperature reaction after the first injection of 2.0 mg. of MA-100 in eight tuberculous rabbits. The lowest peak in the tuberculous animals was at 106.9°, and the highest 108.4° while the highest in the normal animals was 107.0°F. It would appear that the reaction to protein was more intense in tuberculous animals. One tuberculous rabbit, R 1810, after the fourth injection of 2.0 mg. of the MA-100, failed to exhibit a characteristic rise in temperature. This animal apparently had a response similar to that of the normal Rabbit R 1639. These tuberculous animals either died from the disease or were killed at varying intervals, so that the reaction was studied on the 1st, 3rd, 4th, 5th, 7th, 8th, and 30th days after injection.

Except for one animal, R 1809, which died from an accidental infection, the autopsy findings in the tuberculous animals were essentially the same at all of these periods.

The visceral organs showed advanced generalized tuberculosis. The protein is water-soluble and therefore may have passed from cerebrospinal fluid into the blood. There were no hemorrhages in any of the tissues. The brains appeared normal in the gross. There was no edema, hemorrhage, or exudate. Supravital studies of the pia showed a great increase in the number of lymphocytes and plasma cells. Typical clasmotocytes were seen in moderate numbers, but there were none of the coarse vacuole epithelioid cells such as are seen after the injection of phosphatide. A few polymorphonuclear leucocytes were present. The findings were the same in the animal killed on the day after injection of the protein, as in those killed later on.

*The Reaction to Tuberculo-Phosphatide A-3 and Protein MA-100  
Introduced into the Subarachnoid Space in Normal Rabbits*

1 mg. of bovine phosphatide A-3 was introduced into the subarachnoid space of two normal rabbits. One animal received an injection

of 1.0 mg. of protein MA-100 in the subarachnoid space on the 6th and one on the 14th day after the phosphatide; the other received one injection of the same dose on the 14th day. There was a typical rise in temperature after each injection of the protein but otherwise both rabbits appeared normal. They were killed on the 2nd day after the last injection of protein. At autopsy the visceral organs were normal. Both of the brains appeared slightly hyperemic. No edema or hemorrhages were present. Fresh preparations of the pia on supravital study contained no monocytes or fine vacuole epithelioid cells. There was a predominance of lymphocytes, plasma cells, and cells with large vacuoles of equal size and taking a uniform stain with neutral red. This picture seemed to be a combination of that found after intrameningeal injection of phosphatide and protein.

*The Reaction to Tuberculo-Polysaccharide Introduced into the Subarachnoid Space of Normal Rabbits*

Two normal rabbits were given tuberculo-polysaccharide into the subarachnoid space. This material was prepared by the H. K. Mulford Company from the media in which the organisms had been grown. One animal received 5.0 mg. in 0.2 cc. of freshly distilled water. The animal showed a rise in temperature similar to that occurring after protein. The temperature rose from 101.3° previous to injection to a peak of 105.6° in 6 hours. The rabbit was killed 22 days later, when in excellent condition. At autopsy, the findings were normal throughout.

The other rabbit received 2 doses of 5.0 mg. in 0.2 cc. of distilled water 1 month apart and the characteristic temperature response was elicited after each injection. It remained in excellent condition and was killed 3 months after the first injection. At autopsy the tissues were normal throughout.

The polysaccharide contains a small content of nitrogen to which the rise in temperature may be due (21).

DISCUSSION AND CONCLUSIONS

From the above description one can trace the histological changes that occur in the animal during experimental tuberculous and tubercular meningitis.

The introduction of 500,000 living tubercle bacilli into the sub-arachnoid space of rabbits produces tuberculous meningitis. The disease is characterized by an incubation period of about 15 days, during which time the animal appears normal. After this period the course is progressive from weakness in the hind quarters to paralysis and death. This confirms the findings of previous investigators.

The predominant cell in the fresh preparations of the pia is one with large vacuoles of equal size which take a uniform stain with neutral red. It is significant that this type of cell appearing after injection of living or dead bacilli is the same as that seen after the injection of tuberculo-phosphatide. It is clearly a cell which has engorged itself with a great amount of one type of material, and the vacuoles appear to contain a lipid since some of the cells show myelin-like figures, which is characteristic of the phosphatide (20, 21). That it is a phenomenon of phagocytosis and not of cellular degeneration is shown by the fact that the cells are alive and active; and that the vacuoles slowly lessen in size, while at the same time their number increases, until the cell corresponds to the epithelioid cell with coarse vacuoles. In our opinion it is a young connective tissue cell, possibly less differentiated than the monocyte, which has been stimulated to marked phagocytosis of a lipid. The evidence that monocytes and younger connective tissue cells phagocytize the tuberculo-phosphatide is more convincing in the reactions of the omentum. Here it can be seen that the cells of the milk spots phagocytize the phosphatide while the clasmatoocytes of the interspaces remain relatively inactive (11). The vacuoles of the cell now under discussion are larger than those of the cell described by Sabin (20, 24) and Smithburn and Sabin (21) as the coarse vacuolated, epithelioid cell, the second stage in development of this type in the reaction to phosphatide, but they are smaller and more even in size than the first stage of the epithelioid cell as formed in the omentum and subcutaneous tissues. This cell, which is the type of the early stage of the development of the epithelioid cell, as found in the meninges, is so constant a stage in the reaction to tubercle bacilli and tuberculo-phosphatide in this area, that we have designated it the meningeal first stage of the epithelioid cell. It may be that further study will prove this cell to be of sufficient specificity to be diagnostic of tuberculous meningitis.

Lymphocytes and plasma cells increase in number after the 3rd day and soon become the most numerous cells in the meninges following the inoculation with living tubercle bacilli in the subarachnoid space. This same cellular picture is obtained after the intrathecal injection of the tuberculo-protein MA-100. The results correspond with those of Miller (25) obtained by the intraperitoneal introduction of the tuberculo-protein in rabbits.

The theory of Webb and Williams (26, 27) and of Murphy and Sturm (28) and Murphy (29) that a high percentage of lymphocytes in the blood is correlated with increased resistance of the animal has been confirmed by the more recent work in experimental tuberculosis (30, 31). But an increase of lymphocytes in the meninges is not accompanied by increased local resistance.

There has been much speculation as to the route a meningeal infection takes in spreading into the brain substance. The present experiments do not suffice for a decision on the point but the microscopic sections reveal a progressive lymphocytic infiltration of the meninges. This occurs first at the upper end of the cord near the site of inoculation, gradually extends over the midbrain and finally involves all the meningeal surfaces, invading and filling the sulci. On the 15th day there is beginning tubercle formation in these areas. At this time the vessels near the surface of the brain show perivascular lymphocytic infiltration. By the 19th day the tuberculous process in the meninges is pronounced, the perivascular infiltrations are increased, and in some areas there is lymphocytic infiltration in the brain substance. In the last stages there is extensive tuberculosis of the meninges, some of the tubercles having caseous centers. The process involves not only the meninges and sulci, but the perivascular spaces, and areas in the brain near them are extensively involved with tuberculous tissue.

Heat-killed organisms are not innocuous in the meninges. They produce lesions similar to those obtained with living organisms, but with a greatly delayed period of reaction, over 2 months. It should be said that the inoculations of heat-killed organisms were all made from the same suspension. It might be thought that a few organisms may have remained alive and propagated during this long period, until there were a sufficient number to produce the disease. Against

this is the fact that the suspension was tested for viability by inoculation into rabbits. However, the experiment is being repeated, using series inoculated both with heat-killed bacilli obtained from Dr. S. A. Petroff and with a new suspension of bovine heat-killed organisms. These experiments are not completed at the present time but the first animals of both series show the same cellular response—first stage of the meningeal epithelioid cell—as in the experiments described in this paper.

Different cell strains in the meninges apparently react to specific chemical fractions of the bacilli. The tuberculo-phosphatide stimulates the monocytic strain of cells in the meninges, causing a relatively undifferentiated monocytic cell to phagocytize this material. This cell has been described above and termed the meningeal epithelioid cell. This characteristic type of cell is produced by the living bacilli and heat-killed bacilli as well as by the tuberculo-phosphatide. The protein of the bacilli causes a rise in temperature and a proliferation of lymphocytes and plasma cells. The temperature reaction is more pronounced in tuberculous rabbits than in normal rabbits after the injection of the tuberculo-protein MA-100, though the difference is quantitative and of a small degree. It is possible that this may be an allergic phenomenon. The recent work of Sommerfeld and Zishind (32), who found little difference between the reaction in tuberculous and normal guinea pigs after the intrathecal injection of varying dilutions of tuberculin, bears out this point. Numerous investigators have referred to the importance of the rôle of allergy in tuberculous meningitis. Rich (33), states that acute tuberculous meningitis represents a model example of the allergic inflammatory-necrotizing reaction. That allergy is present in the meninges as in the other tissues of sensitive animals, is probably true. One wonders, however, if it is the most sensitive tissue to the allergic phenomenon, for if this were true one would expect after the intrathecal injection of tuberculo-protein MA-100 in a sensitive animal, a very intense inflammatory necrotic response. This did not develop in these experiments. The predominant cells in the cerebrospinal fluid and meninges after tuberculo-protein MA-100 were lymphocytes and plasma cells. A few clasmatocytes and an occasional polymorphonuclear leucocyte were seen in the fresh preparations. None of the

epithelioid cells with coarse vacuoles such as are seen after the introduction of the phosphatide and bovine tubercle bacilli were observed after the intrameningeal injection of the protein in normal animals. The histological sections showed only a slight quantitative difference between the cellular response in sensitive and non-sensitive animals. Nowhere was there found a fulminating necrotic reaction such as one would expect if the meninges were extremely hypersensitive. Both normal and tuberculous rabbits may fail to give a temperature response to a given dose of tuberculo-protein MA-100 after repeated intrameningeal doses of the same size. However, an increase in the size of the dose in these animals elicits a temperature response similar to that of the first injection. The present experiments suggest that the number of injected organisms play a greater rôle in tuberculous meningitis, than the phenomena of sensitization.

There seems to be a definite period elapsing between the time of infection and the onset of symptoms. It is to be supposed that this period varies with the interplay of a number of factors which are common to all tuberculous infections; namely, the number and virulence of the infecting organisms, the natural or acquired immunity of the animal, and the degree of allergy present. The work of Soper and Dworski (6, 7) shows definitely that the number of infecting organisms plays a very important rôle in the length of this period.

In these experiments the injection of the various extracts from the tubercle bacilli gave characteristic cellular reactions. The dosage of these extracts was small but in every case it was many times larger than the amount of extract present in the living bacilli introduced to produce the disease. No toxic effects followed the injection of these fractions which would account for the fatal outcome of tuberculous meningitis.

#### SUMMARY

1. Both living and dead tubercle bacilli, as well as tuberculo-phosphatide, give rise to lesions in which the same type of cell is found, a cell which may prove characteristic of the meningeal reaction. After the phosphatide this cell is produced by the phagocytosis of lipoidal material. The vacuoles of these cells are slowly broken into smaller and smaller size until they correspond to the type found in

the epithelioid cells with coarse vacuoles. This change is much slower in the cells of the meninges than in the cells of the omentum and subcutaneous tissue.

2. Intrathecal injections of tuberculo-protein produce a characteristic rise in temperature in normal and tuberculous rabbits. Both normal and tuberculous rabbits, after repeated intrameningeal injections of a given dose of tuberculo-protein, may fail to give a rise in temperature and later react to a larger dose.

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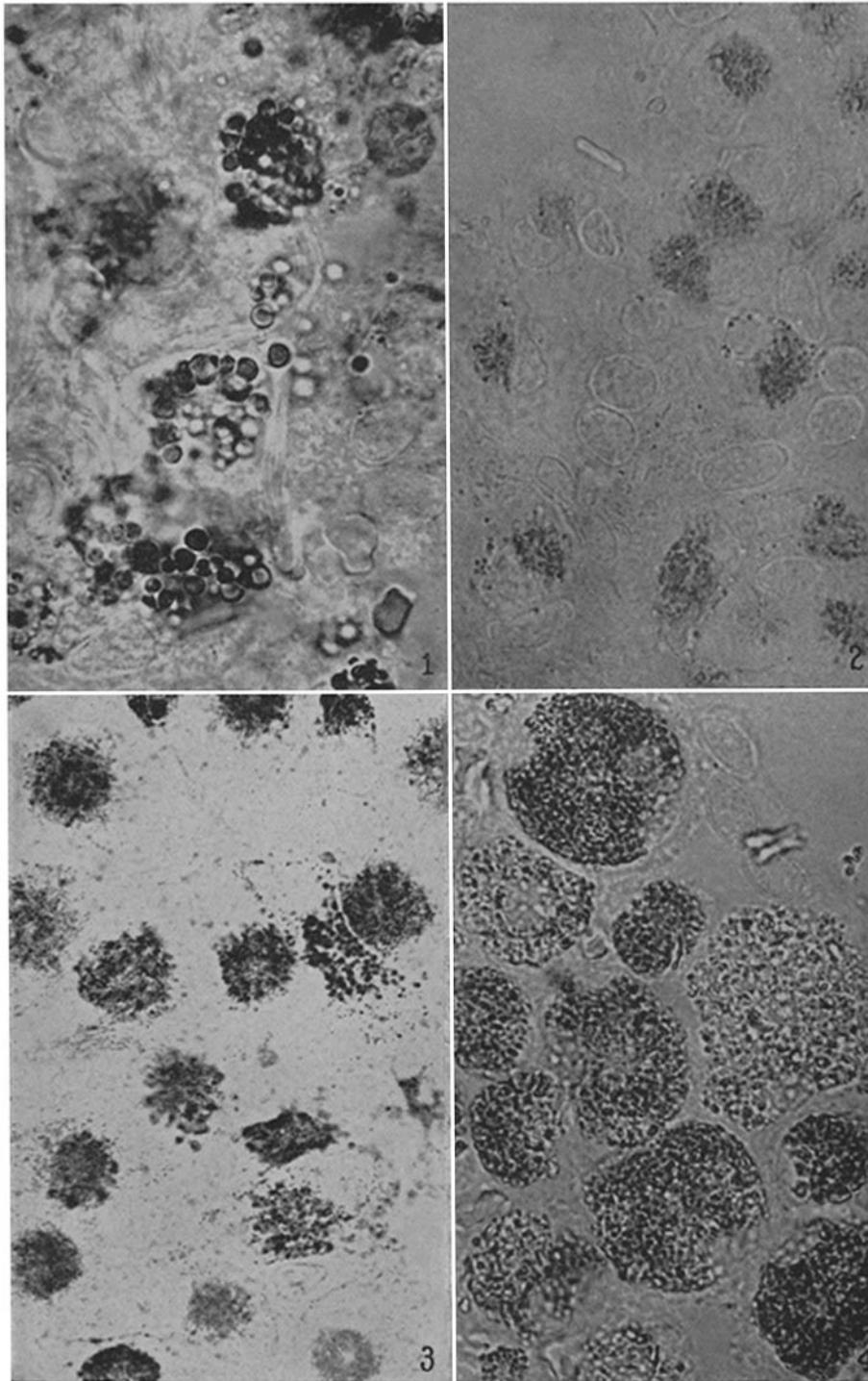
## EXPLANATION OF PLATE 1

FIG. 1. Film of pia of Rabbit R 1782, 1 month after an intrameningeal injection of heat-killed organisms, photographed while the cells were living. Stained in vital neutral red and Janus green dye. It shows the meningeal type of cell with vacuoles of even size stained uniformly with neutral red. The reason that the vacuoles do not appear to be of even size and uniform stain is that they were all not in the same focus.  $\times 1,200$ .

FIG. 2. Scraping from over the pons of Rabbit R 2020, 6 days after it had received a half-million living bovine organisms intrameningeally, photographed while the cells were living. Stained in vital neutral red and Janus green dye. It shows monocytes, stimulated monocytes, and epithelioid cells.  $\times 1,000$ .

FIG. 3. Film of living cells in the cerebrospinal fluid taken from the fourth ventricle of R 1783 after injection of heat-killed tubercle bacilli. It shows stimulated monocytes and epithelioid cells. Stained with vital neutral red and Janus green dye.  $\times 1,000$ .

FIG. 4. Scraping from over the pons of Rabbit R 1739, 3 months after the intrameningeal injection of 2.0 mg. of bovine phosphatide. It shows the monocytic cells which have phagocytized the phosphatide, until they appear as coarse vacuole epithelioid cells. Living cells stained with vital neutral red and Janus green dye.  $\times 1,000$ .



Photographed by Louis Schmidt

(Bickford: Reactions to extracts from tubercle bacilli)