THE FUNCTION OF MACROPHAGES IN LOCAL RESISTANCE TO BACTERIAL INFECTIONS.

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PLATE 3.

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That intraperitoneal or intrapleural injections of fatty oil bring about a marked mononuclear exudation is a well established fact.[1,2] Although the large mononuclear cells which predominate in the cellular population of the exudate were once considered as of the lymphoid series, a closer study by means of supravital technique has demonstrated them to be macrophages (clasmatocytes and monocytes).[3]

Metschnikoff[4] early distinguished the large mononuclear phagocyte under the name of macrophage from the polymorphonuclear leucocyte (microphage) on the ground that it does not phagocytize bacteria, except for the tubercle bacillus and the bacillus of leprosy, and engulfs only cellular débris. This conception has long since been found to be erroneous, and the phagocytic action of macrophages upon bacteria in general, in addition to that upon pigments, foreign particles, etc., is now a generally accepted fact. Furthermore, there are some indications pointing to the active rôle of macrophages in defensive processes, not only against chronic infections, but also against acute phenomena as well.[5,6] Gay and Morrison[7] only recently showed that in a certain streptococcus infection macrophages (clasmatocytes), with the exclusion of polymorphonuclear cells, are active agents

[1] Bergel, S., *Berl. klin. Woch.*, 1919, lvi, 915; *Ergebn. inn. Med. u. Kinderheilk.*, 1921, xx, 36.

[2] Nakahara, W., *J. Exp. Med.*, 1922, xxxv, 493.

[3] See, for a recent account of this subject: Sabin, F. R., Doan, C. A., and Cunningham, R. S., *Carnegie Institution of Washington, Pub. No. 361, Contributions to Embryology*, 1925, xvi, 127.

[4] Metschnikoff, E., *Virchows Arch. path. Anat.*, 1887, cvii, 209.

[5] Wallgren, A., *Beitr. path. Anat. u. allg. Path.*, 1899, xxv, 206. Zangemeister, W., and Gans, H., *Münch. med. Woch.*, 1909, lvi, 793.

[6] Buxton, B. H., and Torrey, J. C., *J. Med. Research*, 1906, xv, 55, 73.

[7] Gay, F. P., and Morrison, L. F., *J. Infect. Dis.*, 1923, xxxiii, 338.

in resistance. They noted that the reaction of effective immunity was characterized by a predominance of macrophages in the exudate, while a polymorphonuclear reaction was invariably an accompaniment of a fatal infection. Evidence was presented also that animals in which a rich polymorphonuclear exudate was produced by means of aleuronat, diatomaceous earth, etc., were actually less resistant than normal animals, whereas even a relatively slight macrophage exudate, such as can be produced by plain infusion broth, tended to protect the animal against many multiples of the fatal dose of streptococcus.

The method of inducing a marked macrophage reaction in the peritoneal cavity by means of oil injection offers an excellent opportunity for the study of the function of these cells, and advantage was taken of this method in experiments to be presented in this paper. With the hope of elucidating somewhat the significance of the macrophage reaction in resistance to bacterial infections, I have first attempted to determine the influence of the cellular reaction on the survival of bacteria and then to ascertain to what extent the local resistance thus induced would affect the course of the eventual general infection.

Production of Macrophage Reaction by Oil Injection.

For the purpose of inducing the cellular reaction commercial olive oil was used in all the experiments. This substance was injected intraperitoneally into mice in a single dose of 0.2 cc.

Following the oil injection, there is a considerable polymorphonuclear reaction. By the 48 hour period, however, the leucocytes are largely replaced by mononuclear elements. These latter, consisting chiefly of macrophages, are found in very large numbers during the next few days and this reaction gradually subsides in the course of 2 weeks. Some idea of the macrophage reaction may be obtained from the few examples cited below (Table I). The percentage changes are based on differential cell counts on smears obtained by the capillary method before and at intervals after oil injections. It should be mentioned that oil injections bring about no perceptible increase in the total cell counts. In our experience these latter varied between 100,000 to 180,000 cells per c. mm. of peritoneal fluid in normal and oil-injected mice alike. The total amount of fluid appeared to be slightly increased in the treated animals, however.

TABLE I.

Intraperitoneal Macrophage Reaction Induced by Oil Injection.

Mouse No.	1 day before oil injection.	48 hrs. after oil injection.	5 days after oil injection
	per cent	*per cent*	*per cent*
1	2.8	72.7	76.6
2	6.2	76.9	80.1
3	14.8	64.8	73.6
4	21.0	87.4	86.5
5	21.7	68.3	78.8
Average.....	13.3	74.0	79.1

We have previously noted that accompanying this local reaction there is a proliferation of the cells of the lymphoid germ centers, but that no material change in the blood picture occurs.[2]

Effect of Oil Injection on the Survival of Bacillus coli in the Peritoneal Cavity.

The first experiments were planned to test the effect on bacteria of the macrophage reaction induced by oil injection. In these experiments, as in others to be described later, olive oil was injected 3 or 4 days in advance of bacterial inoculation, this period being that of the maximum cellular reaction. For obvious reasons a relatively non-pathogenic bacterium was chosen as the test organism; namely, *Bacillus coli.*

Experiment 1.—60 normal white mice of approximately the same size and weight were divided into 4 groups:

Group 1 (20 mice) was injected intraperitoneally with olive oil, and 3 days later was inoculated, also intraperitoneally, with a suspension of *B. coli.*

Group 2 (20 mice) was inoculated intraperitoneally with the same suspension of *B. coli*, without the previous oil injection.

Group 3 (10 mice) was injected with olive oil but was not inoculated with *B. coli.*

Group 4 (10 mice) remained without oil injection or *B. coli* inoculation as normal controls.

24 hours growth of *B. coli* on 4 agar slants was suspended in 8 cc. of sterile normal salt solution, and 0.1 cc. of the suspension was inoculated into each mouse.

2 days after the bacterial inoculation, 10 mice each from Groups 1 and 2 and all the mice of Groups 3 and 4 were killed and cultures were taken from the peritoneal

exudate. 5 mice each from Groups 1 and 2 were killed 4 days after the inoculation and cultures were taken similarly. The remaining 5 mice each from these two groups were killed and cultures taken at the 6 day period.

After 24 hours incubation at 37°C., tubes were examined and growth of *B. coli* noted. The result was as follows (Table II):

TABLE II.

Effect of Oil Injection on the Recovery of B. coli Subsequently Inoculated.

Intervals after *B. coli* inoculation.	Group 1. Inoculated with *B. coli* 3 days after oil injection.	Group 2. Inoculated with *B. coli* alone.	Group 3. Injected with oil alone.	Group 4. Untreated controls.
days				
2	+++	+++	−	−
	++	+++	−	−
	++	+++	−	−
	++	+++	−	−
	++	++	−	−
	+	++	−	−
	+	++	−	−
	−	++	−	−
	−	++	−	−
	−	++	−	−
4	+	++		
	−	++		
	−	+		
	−	+		
	−	−		
6	−	++		
	−	−		
	−	−		
	−	−		
	−	−		

+++ indicates profuse growth; ++, several colonies; +, colonies present.

As is apparent from the above result, *Bacillus coli* dies off rapidly in the normal peritoneal cavity, but it disappears much more quickly from the cavity previously injected with oil. It may be noted that by the 4th day after inoculation cultures from the oil-injected mice were mostly negative, while those from the control animals showed, in the majority of instances, some growth of the organism. Even

2 days after there was a marked difference in the number of recovered organisms.

Experiment 2.—The above experiment was repeated with 40 mice, similarly divided into 4 groups. In this case, however, all the mice were killed and cultures taken 3 days after the *B. coli* inoculation. In Groups 1 and 2, cultures were also taken from the heart's blood. The dose of *B. coli* was the same as in Experiment 1. The growth of *B. coli* in the cultures after 24 hours incubation is shown in Table III.

TABLE III.

Effect of Oil Injection on the Recovery of B. coli Subsequently Inoculated.

Group 1. Inoculated with *B. coli* 3 days after oil injection.		Group 2. Inoculated with *B. coli*.		Group 3. Injected with oil.	Group 4. Normal controls.
Exudate.	Heart's blood.	Exudate.	Heart's blood.	Exudate.	Peritoneal fluid.
++	−	+++	++	−	−
+	−	+++	++	−	−
+	−	+++	+	−	−
+	−	+++	−	−	−
−	−	++	−	−	−
−	−	++	−	−	−
−	−	++	−	−	−
−	−	+	−	−	−
−	−	+	−	−	−
−	−	−	−	−	−

Does Oil Injection Affect the General Resistance?

The next experiment was carried out with the idea of determining whether the resistance induced by oil injection is confined to the peritoneal cavity or whether it involves a general change. The method of experiment adopted was to inject olive oil intraperitoneally as usual, and then to introduce *Bacillus coli* into the pleural instead of peritoneal cavity.

Experiment 3.—10 normal white mice (Group 1) were injected intraperitoneally with 0.2 cc. of olive oil. 3 days later these, together with another 10 mice (Group 2), were inoculated with an equal amount of a suspension of *B. coli* intrapleurally. All the mice, along with 10 untreated normal controls (Group 3), were killed and cultures were taken from the pleural fluid. Examination of the cultures after 24 hours incubation showed the following result (Table IV).

TABLE IV.

Effect of an Intraperitoneal Oil Injection on the Recovery of B. coli Inoculated Intrapleurally.

Group 1. Inoculated with *B. coli* after oil injection.	Group 2. Inoculated with *B. coli* alone.	Group 3. Normal controls.
++,+	++,+	−
+++	+++	−
+++	+++	−
+++	+++	−
+,++	+++	−
+++	+++	−
+++	+,+	−
+,+	++	−
++	++	−
−	+	−

This experiment was repeated with the result entirely in agreement with the above.

The observations seem sufficient for the conclusion that there is no perceptible increase in the general resistance of animals under the conditions of these experiments.

Effect of Local Resistance on the Generalization of Infection.

So far, we have confined ourselves to the study of the survival of relatively non-pathogenic bacteria in the peritoneal cavity as affected by previous oil injections. There is another way in which the question of the local resistance can be approached, and that is to test the effect of the treatment on the dissemination of highly virulent microorganisms from the locus of inoculation. The local resistance at the site of primary infection, if of sufficient degree, should modify the course of the eventual general infection.

The following experiments were performed with the above problem in mind, with *Staphylococcus aureus* and pneumococcus as test organisms.

Staphylococcus. Experiment 4.—40 normal white mice were divided into 2 groups:

Group 1, composed of 20 mice, was injected intraperitoneally with 0.2 cc. of

olive oil. 3 days later they were inoculated, also intraperitoneally, with 0.1 to 0.8 cc. of a suspension of *Staphylococcus aureus*.

Group 2 was not injected with olive oil but was inoculated with corresponding amounts of the same bacterial suspension.

The suspension was made by taking up 24 hours growth of the organism on 4 agar slants in 20 cc. of sterile salt solution.

The death rate and the proportion of surviving mice for different doses of staphylococci inoculated, as contrasted between the oil-injected and non-oil-injected groups, is summarized in Table V. These results were confirmed by another experiment of similar nature. Animals living 7 days after inoculation were considered to have survived the infection in these experiments.

TABLE V.

Effect of Oil Injection on the Resistance of Mice to Staphylococcus aureus.

Amounts of suspension of staphylococci inoculated.	Oil-injected mice.	Control mice.
cc.		
0.8	D. 24 hrs. " 48 " " 3 days. " 3 " S.	D. 24 hrs. " 24 " " 24 " " 24 " " 3 days.
0.4	D. 48 hrs. " 3 days. S. " "	D. 24 hrs. " 24 " " 3 days. S. "
0.2	D. 3 days. S. " " "	D. 48 hrs. " 48 " " 48 " " 3 days. S.
0.1	S. " " " "	D. 3 days. " 4 " " 5 " S. "

D., died; S., survived.

Pneumococcus.—Experiments similar to the above were carried out with a Type I pneumococcus of the Neufeld strain.[8]

The following protocol represents one of two experiments which I have performed, and which have given comparable results.

Experiment 5.—50 normal white mice were divided into 2 groups of 25 each. Group 1 was injected intraperitoneally with 0.2 cc. of olive oil, Group 2 remaining untreated. 3 days later, both groups were inoculated intraperitoneally with 0.000,1 to 0.000,000,01 cc. of an 18 hour broth culture of pneumococci. The outcome of this experiment was as follows (Table VI):

TABLE VI.

Effect of Oil Injection on the Resistance of Mice to Pneumococcus.

Amounts of pneumococcus culture injected.	Oil-injected mice.	Control mice.
cc.		
0.000,1	D. 48 hrs. " 48 " " 48 " " 3 days. " 4 "	D. 24 hrs. " 24 " " 24 " " 48 " " 48 "
0.000,01	D. 48 hrs. " 48 " " 3 days. " 3 " " 4 "	D. 24 hrs. " 48 " " 48 " " 48 " " 48 "
0.000,001	D. 48 hrs. " 3 days. " 3 " " 3 " " 4 "	D. 48 hrs. " 48 " " 48 " " 48 " " 48 "
0.000,000,1	D. 3 days. " 4 " S. " "	D. 48 hrs. " 48 " " 48 " " 48 " " 3 days.
0.000,000,01	S. " " " "	D. 48 hrs. " 48 " " 48 " " 3 days. S.

[8] I am indebted to Dr. O. T. Avery for the cultures of this strain.

Failure of Olive Oil by Itself to Influence Infections.

A question naturally arises: Is not the apparent resistance observed attributable to the action of olive oil itself upon the organisms? We have attempted to answer this question by studying the effect of nearly simultaneous injections of oil and bacteria. If the amount of olive oil injected was sufficient by itself to influence the bacterial growth *in vivo*, this effect should be more strikingly brought out in these experiments than when the oil injection was given a few days in advance of bacterial inoculation, as we have heretofore done. It is also to be remembered that there is no marked macrophage reaction in the peritoneal cavity during the first 24 hours after oil injection.

Effect on the Survival of B. coli. Experiment 6.—20 normal white mice were inoculated intraperitoneally with equal amounts of a suspension of *B. coli*. Immediately afterward 10 of these mice were injected, also intraperitoneally, with 0.2 cc. of olive oil, the remaining 10 mice, not injected with olive oil, serving as controls. 3 days later all the mice were killed and cultures were taken from the peritoneal exudate. The growth of *B. coli* in these cultures after 24 hours incubation was as follows (Table VII):

TABLE VII.

Effect of an Oil Injection on the Recovery of B. coli.

Inoculated with *B. coli* immediately before oil injection.	Inoculated with *B. coli* alone.	Inoculated with *B. coli* immediately before oil injection.	Inoculated with *B. coli* alone.
+++	+++	+	++
+++	+++	+	+
+++	+++	+	+
+++	+++	−	−
++	++	−	−

Another experiment of similar nature was carried out with the same number of mice, and with entirely analogous results.

It is evident from the above results that the possible direct action of olive oil on *Bacillus coli* cannot account for the early disappearance of the organism from the oil-prepared peritoneal cavity as noted in the preceding section.

Effect on Pneumococcus Infection.—As a further check, an additional experiment was performed with pneumococcus, as follows:

Experiment 7.—30 normal white mice were divided into 3 groups of 10 mice each. Group 1 was injected with 0.2 cc. of olive oil and 4 days later was inoculated with 0.000,001 cc. of 24 hours growth of Type I pneumococci in broth. Group 2 was inoculated with the same amount of the same culture but was injected immediately before with 0.2 cc. of olive oil. Group 3 was simply inoculated with the same amount of the same culture. Oil as well as pneumococci was inoculated intraperitoneally in all instances.

The proportions of mice in each group surviving the pneumococcus infection were as follows (Table VIII):

TABLE VIII.

Effect of Oil Injection on the Resistance of Mice to Pneumococcus.

Group 1. Injected with oil 4 days before pneumococcus inoculation.	Group 2. Injected with oil immediately before pneumococci.	Group 3. Untreated mice inoculated with pneumococci.
S.	D. 48 hrs.	D. 48 hrs.
"	" 48 "	" 48 "
"	" 48 "	" 48 "
"	" 48 "	" 48 "
"	" 3 days.	" 48 "
"	" 3 "	" 3 days.
"	" 3 "	" 3 "
"	S.	" 3 "
"	"	S.
"	"	"

Is the Exudation Fluid Bacteriolytic?

At this point it is necessary to consider the possible rôle of the peritoneal fluid itself in the process of local resistance. It is not without reason to suspect that the soaps of fatty acids which may be formed in the peritoneal cavity after oil injection might be of some consequence. Of the three species of bacteria used in the preceding experiments pneumococcus is the only one known to be highly susceptible to the lytic action of sodium oleate, and, even in this case, the soap action is inhibited by the presence of serum.[9] Staphylococcus, on the other hand, is highly resistant to the germicidal action of soaps.[10] In spite of these facts it seems desirable to obtain direct experimental evidence. I have therefore tested peritoneal fluid from

[9] Lamar, R. V., *J. Exp. Med.*, 1911, xiii, 380.

[10] Walker, J. E., *J. Infect. Dis.*, 1924, xxxv, 557.

oil-injected animals for its action on pneumococcus, using rabbits, instead of mice, for technical reasons.

The following is one of two experiments which resulted in an identical outcome.

Experiment 8.—A normal rabbit was injected intraperitoneally with 20 cc. of olive oil. 4 days later the peritoneal exudate was collected and centrifuged, separating oil droplets and cells from the clear fluid. 0.5 cc. of this last mentioned fraction was mixed in a small test-tube with 0.05 cc. of 10 hour broth culture of a passage strain of Type I pneumococcus and placed in a water bath at 37°C.

Microscopical examinations were made of smears from the mixture taken at the end of 1, 3, and 6 hours of incubation. Before incubation smears showed, roughly, 1 organism in 5 to 10 microscopic fields. After 1 hour's incubation 1 or 2 organisms were seen in every field. A marked increase of pneumococci was noted after 3 hours incubation, at which period there were 10 to 20 organisms per field. The bacteria further increased during the next 3 hours of incubation, and as many as 50 of these were seen in a single microscopic field. The original undiluted culture showed as an average 4 or 5 pneumococci per field.

Cultures were also taken from the mixture after incubation for 1, 3, and 6 hours. All the cultures yielded luxuriant growths of pneumococci.

Finally, the mixture, after 6 hours incubation, was inoculated into mice in varying doses. 0.001 cc. of this material uniformly killed mice within 24 hours, and 0.000,01 cc. killed within 48 hours.

The evidence seems complete in demonstrating that the cell-free exudate from an oil-injected animal, instead of inhibiting the growth of pneumococcus, serves, on the contrary, as an excellent culture medium for this organism. This fact makes it certain that the properties of the fluid, including its soap contents, are not primarily responsible for the phenomena of local resistance.

Phagocytosis of Bacteria by Macrophages.

After eliminating the humoral factor from consideration there remains to be described the microscopical evidence of the action of macrophages upon bacteria.

A number of mice were injected with olive oil as in previous experiments and 3 or 4 days later they were inoculated with microorganisms. In inoculating virulent bacteria care was taken to use the amount of culture sufficient to kill normal animals and yet permit the oil-injected ones to survive. With *B. coli*, a small quantity of fairly thick suspension was used. All the animals were killed from

2 to 4 hours after the inoculation of bacteria, and smears were taken from the peritoneal exudate for microscopical examination.

Figs. 1 to 6 illustrate the findings. There were some variations in the relative number of bacteria found in the smear, depending, no doubt, upon the amount of cultures originally injected, upon varying time after the inoculation, etc., but the evidence of phagocytosis of bacteria by macrophages was a very frequent and constant observation. In Figs. 1 and 2 are shown a number of *Bacilli coli* in, as well as about, macrophages; a fair number of *Staphylococcus aureus* are shown phagocytized in Figs. 3 and 4, while several pneumococci engulfed by macrophages are illustrated in Figs. 5 and 6. It was noted that the cells phagocytizing all these microorganisms were chiefly macrophages, and only occasionally were polymorphonuclear leucocytes seen containing any bacteria.

DISCUSSION.

It is well known that virulent microorganisms injected into the peritoneal cavity are rapidly disseminated into the circulating blood, and that the organisms, if allowed to proliferate, lead to the eventual death of animals. At the same time, there is no doubt that many bacteria are disposed of in the peritoneal cavity. The ultimate recovery of the animal may therefore depend to a certain extent upon how far the local destruction of bacteria is carried out. Despite the fact that polymorphonuclear cells are generally accepted as the major agent in these local processes, there are a few observers who consider these cells to be of little or no importance in disposing of bacteria in the peritoneal cavity (Buxton and Torrey,[6] Simon,[11] etc.). On the other hand, it has been suggested that macrophages might be active agents under these conditions (Gay and Morrison[7]).

It is not our intention to discuss here how important a part the macrophage plays in resistance to infections as they occur spontaneously in man and animals. Nor is it within the scope of this paper to question the function of polymorphonuclear cells. We have merely demonstrated in the present investigation the ability of macrophages to successfully dispose of pathogenic bacteria with but slight, if any, cooperation of polymorphonuclear elements.

[11] Simon, F. B., *Centr. Bakt., 1. Abt.*, 1901, xxix, 113.

SUMMARY.

Coincident with the marked macrophage reaction induced in the peritoneal cavity by oil injection, there is an increased resistance of this location to bacterial infections. Animals so prepared dispose of *Bacillus coli* in a much shorter time than normal animals, and survive multiples of the fatal doses of staphylococci and pneumococci.

The amount of oil injected is in itself incapable of inhibiting bacterial growth, nor is cell-free exudate from an oil-injected animal potent in this regard. The macrophages in the exudate, on the other hand, actively phagocytize bacteria. These facts lead to the conclusion that the increased resistance is due primarily to the action of macrophages.

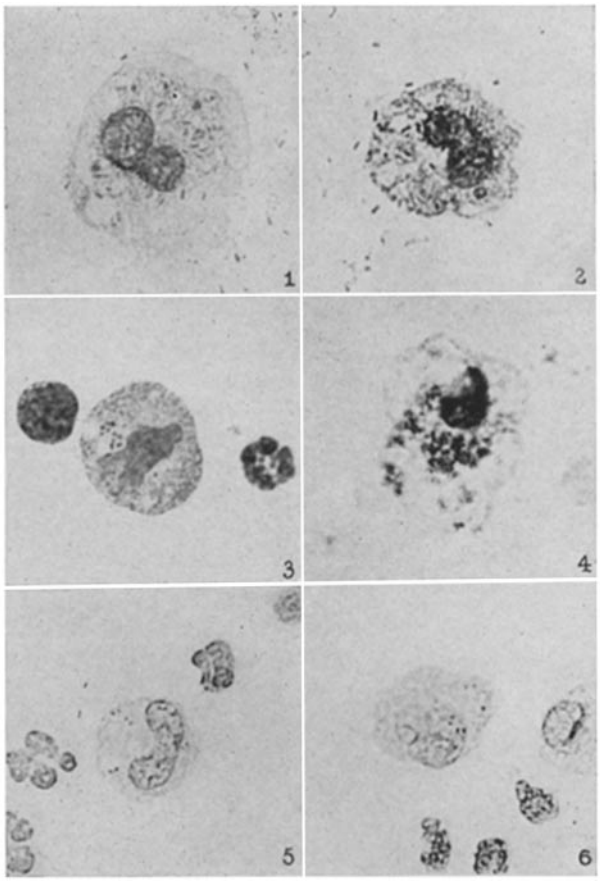
EXPLANATION OF PLATE 3.

Photomicrographs showing phagocytosis of bacteria by macrophages. Specimens (smears) were taken from oil-injected mice from 2 to 3 hours after they were inoculated with bacteria.

FIGS. 1 and 2. *B. coli.*

FIGS. 3 and 4. *Staphylococcus aureus.*

FIGS. 5 and 6. Pneumococcus.



(Nakahara: Macrophages in resistance to infection.)