PHAGOCYTOSIS OF BACTERIA IN THE ABSENCE OF ANTIBODY AND THE EFFECT OF PHYSICAL SURFACE

A Reinvestigation of "Surface Phagocytosis"

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According to current immunological concept, phagocytosis of virulent bacteria by polymorphonuclear leukocytes may occur in the absence of specific antibody or opsonin. This has been ascribed to the phenomenon of "surface phagocytosis," as described in detail by Wood and coworkers (1–4). They observed that pneumococci were phagocytosed by leukocytes of rat peritoneal exudates when preparations were placed upon a rough surface, but that no phagocytosis occurred when the same preparations were placed upon a smooth surface. This was largely the basis for the conclusion that a rough surface enhanced phagocytosis, with the subsequent deduction that physical factors were responsible for phagocytosis in the absence of antibody.

Since previous studies in this laboratory had shown that growth in certain culture media could sensitize bacteria for *in vitro* phagocytosis in the absence of opsonin (5–7) and in the absence of a rough surface, it was felt desirable to test the hypothesis that the effect of filter paper in Wood's experiments may have been a chemical stimulation rather than a physical surface phenomenon. It was subsequently shown that previous contact of either live or killed *Escherichia coli* (8) or *Brucella abortus* (7) with filter paper resulted in increased phagocytosis of these microorganisms by guinea pig or dog polymorphonuclear leukocytes when preparations were rotated in glass tubes. Similar effects were obtained by prior contact of the bacteria with chemically pure para-hydroxybenzoic acid, an active principle found in filter paper and reported by Davis as a new bacterial vitamin and antagonist for para-aminobenzoic acid (9, 10). Contact with fixed tissues had a slightly inhibitory effect on the phagocytosis of *E. coli* (8). These findings opened to question the conclusions of Wood and coworkers (1–4) that the presence of a rough surface such as that provided by moistened filter paper was necessary for the luekocyte to carry out effective phagocytosis of bacteria. Consequently, studies on the effects of filter paper and para-hydroxybenzoic acid on phagocytosis have been extended to the *Pneumococcus*.

Using previously employed methods of standardization and measurement

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(7, 8, 12), preliminary results with a *Pneumococcus* Type IV obtained from a routine human throat culture indicated that previous contact with para-hydroxybenzoic acid increased the phagocytosis of this strain by either washed guinea pig or rat blood cells in rotated preparations to a significant degree (Table I).

The *Pneumococcus* Type I A5 strain used by Wood, Smith, and Watson in their experiments describing surface phagocytosis (1, 2) was requested and was kindly supplied by Dr. M. R. Smith. When preparations were rotated in glass tubes under the standardized

TABLE I

Effect of Para-Hydroxybenzoic Acid Contact on Phagocytosis of Pneumococcus Type IV

Guinea pig and rat blood leukocytes

Test substance	No. of experiments	Per cent neutrophiles containing bacteria	
		Mean ± S.E.	Difference / S.E. difference
A. Saline	8	80.5 ± 4.9	
B. Para-hydroxybenzoic acid	8	97.5 ± 1.3	B-A 3.3

TABLE II

Effect of Filter Paper and Para-Hydroxybenzoic Acid Contact on Phagocytosis of Pneumococcus Type I A5

Guinea pig blood leukocytes

Test substance	No. of experiments	Per cent neutrophiles containing bacteria	
		Mean ± S.E.	Difference / S.E. difference
A. Saline	33	41.3 ± 5.4	
B. Filter paper	24	34.5 ± 5.7	B-A 0.86
C. Para-hydroxybenzoic acid	9	33.1 ± 8.9	C-A 0.79

conditions employed with other microorganisms, phagocytosis of the Type I A5 *Pneumococcus* by washed guinea pig blood cells was not increased by previous contact with filter paper in 24 separate experiments, or with para-hydroxybenzoic acid in 9 separate experiments (Table II). Actually, there was a slight decrease below control levels after contact with either of the two above substances, but this was not statistically significant. When washed rat blood cells were tested with rotated preparations as above, previous contact with filter paper did not significantly increase the phagocytosis of the Type I A5 *Pneumococcus* in 15 experiments (Table III).

After finding that prior contact with filter paper or an active principle therefrom could stimulate the phagocytosis of *E. coli*, *B. abortus*, and *Pneumococcus* Type IV, but had no such effect upon the Type I A5 *Pneumococcus*, an attempt was made to reproduce exactly the experimental conditions de-

scribed by Wood *et al.*, with this last named microorganism. The Type I A5 strain was used in all the following experiments. The procedure outlined by Wood *et al.* (2, 11) was followed, and comparison was restricted to the *in vitro* surface effects of glass and moistened filter paper (12). Since it was found that the techniques of estimating numbers of bacteria and leukocytes and the measurement of phagocytosis described in Wood's work were unsatisfactory for obtaining reproducible quantitative measurements, later experiments were improved by quantitative methods of estimation of phagocytosis and control of numbers of bacteria and leukocytes.

Pneumococcus Suspensions.—Type I A5 *Pneumococcus* was obtained in lyophilized form and initially grown out in tryptose broth, plated on blood agar, and was then carried routinely on sheep, guinea pig, and rabbit blood agar slants. Inoculation was made from a 24 hour blood agar slant into tubes containing 10 ml. of beef infusion broth enriched with 1.0 per cent defibrinated rabbit blood and 0.05 per cent dextrose. Growth was for 4 to 6 hours at 37°C. (11). The bacteria were harvested by decanting, centrifugation, and were suspended in Krebs-gelatin solution (13, 14). Krebs-gelatin solution is a modified Krebs-Henseleit solution (15) containing 3 per cent gelatin,[1] and has been employed successfully for the washing and manipulation of white and red blood cell preparations and bacterial suspensions. The bacteria were again centrifuged and resuspended in fresh Krebs-gelatin solution. Dilutions for chamber counting were made in the same solution. Counts of the live bacteria were made in a standard Petroff-Hauser chamber of 0.02 mm. depth. The numbers of bacteria were first kept at the levels described by Wood *et al.*, *i.e.*, 25 to

TABLE III

Effect of Filter Paper Contact on Phagocytosis of Pneumococcus Type I A5
Rat blood leukocytes

Test substance	No. of experiments	Per cent neutrophiles containing bacteria	
		Mean ± S.E.	Difference / S.E. difference
A. Saline	15	33.5 ± 7.3	
B. Filter paper	15	35.9 ± 7.3	B-A 0.23

[1] The composition of Krebs-gelatin solution (13–15) is as follows:

4	parts	0.154 M KCl
3	"	0.11 M $CaCl_2$
1	"	0.154 M KH_2PO_4
1	"	0.154 M $MgSO_4$
4.6	"	0.592 M $NaHCO_3$
50	"	Gelatin—Knox P-20, "for intravenous use," in 0.9 per cent NaCl
50	"	0.9 per cent NaCl

This differs from the gelatin-Locke's solution used by Wood (2, 11) and described by Robertson *et al.* (16), by the addition of $MgSO_4$ and the increased concentrations of $NaHCO_3$ and gelatin.

30 pneumococci per oil immersion microscopic field; but later, in order to standardize procedure further and to obtain reproducible numbers of bacteria in concentrations most favorable for phagocytosis, the suspensions were standardized to 1×10^{10} organisms per ml. Thus their absolute concentration and relative proportion to leukocyte numbers were kept the same for all experiments in a series.

Leukocyte Preparations.—Young adult white rats were injected intraperitoneally with 5 ml. of a wheat flour–starch mixture in tryptose broth, prepared by autoclaving a mixture of 5 gm. of wheat flour and 3 gm. of starch in 100 ml. of distilled water and adding this to an equal volume of sterile tryptose broth. 20 to 24 hours later these animals were injected intraperitoneally with 5 ml. of sterile saline solution containing 0.5 mg. heparin, and immediately sacrificed. The saline solution containing leukocytic exudate was removed from the rat peritoneal cavity by pipette, and a white cell count was done immediately, using a standard hemocytometer. Prophylactic sulfapyridine was not administered (as Wood *et al.* had done to prevent infection of the peritoneal exudate), but all exudates were examined for bacterial contamination, and the very rarely contaminated preparations were discarded. Nearly all animals so treated showed a mild, localized, sterile peritonitis. The saline suspensions of leukocytes were centrifuged, the saline discarded, the cells were washed twice in Krebs-gelatin solution, resuspended in the same, and standardized to concentrations of 1×10^7 to 1×10^8 per ml. (10,000 to 100,000 per mm.[3]). Equal volumes of bacterial and white cell suspensions were mixed for the subsequent tests. This gave a ratio of approximately 100 to 1000 pneumococci per leukocyte (or approximately 200 to 2000 pneumococci per polymorphonuclear neutrophile).[2]

Glass and Filter Paper Surfaces.—Discs of Whatman No. 42 filter paper were placed in the bottom of 15 × 100 mm. Petri dishes, and 22 × 50 mm. glass coverslips were placed upon the filter paper discs. A 20 × 40 mm. strip of Whatman No. 42 filter paper was placed upon every other glass coverslip. The filter paper strips and the filter paper discs were moistened with Krebs's solution without gelatin. 0.1 to 0.2 ml. of the pneumococcus-leukocyte mixture was pipetted onto the surface of the glass coverslips and onto the surface of the moistened filter paper strips placed on every other glass coverslip. The Petri dishes were closed and these preparations were incubated for 30 to 180 minutes at 37°C., after which period both impression and pulled smears were made and stained with Jenner-Giemsa stain.[3]

Estimation of Phagocytosis.—Instead of expressing phagocytosis as simply positive or negative, as Wood and associates had done, a quantitatively reproducible method of measuring phagocytosis was employed, as described by Victor *et al.* (18). This expresses amount of phagocytosis as the per cent of polymorphonuclear neutrophiles containing bacteria, and has been successfully applied to the quantitative measurements of opsonins for *Brucella suis* (18–20), *B. abortus* (7, 12, 18), *E. coli* (5, 6, 8, 12), and *Coxiella burnetii* (21).

Rotated Preparations.—Comparison of stationary preparations with rotated preparations

[2] The ratio used by Wood; namely, 5 to 10 phagocytic cells and 25 to 30 penumococci per oil immersion field, was first used. It was found not only that it was extremely difficult if not impossible to obtain quantitatively reproducible numbers of either component by this method, but that the concentration of bacteria was below the critical range for phagocytosis, so that all quantitative phagocytosis measurements were at equally low levels, regardless of surface employed or of whether preparations were rotated.

[3] The Jenner-Giemsa stain for blood smears is modified after Cowdry (17) by using buffer instead of distilled water, as follows:—

1. Flood slide with Jenner stain 3 minutes.
2. Flood slide with equal volume of McJunkin-Haden buffer 1 minute.
3. Drain.
4. Stain in jar of Giemsa (50 drops of stock to 50 cc. same buffer) 15 minutes.
5. Wash in running H_2O.

was also made. 0.2 ml. amounts of bacteria-leukocyte mixtures were pipetted into 8 mm. diameter test tubes, placed in a rotator revolving at 24 or 36 R.P.M. (22) in an incubator at 37°C. for 30 minutes, and smears were prepared, stained, and counted in the usual manner.

Effect of Glass and Filter Paper Surfaces on Phagocytosis by Rat Exudate Leukocytes

In 33 experiments, each of which included duplicate to sextuplicate comparisons, the phagocytosis of live Type I A5 *Pneumococcus* by rat exudate leukocytes was not significantly different when preparations were placed on glass or on moistened filter paper surfaces (Table IV). The mean per cent of neutrophiles containing bacteria and its standard error was 18.4 ± 2.1 for the glass surface, and 16.7 ± 2.3 for the filter paper surface; slightly lower for the rough surface.

TABLE IV

Effect of Surface on Phagocytosis of Pneumococcus Type I A5 by Rat Exudate Leukocytes
Filter paper, glass, and rotated tubes

Test surface	No. of experiments	Per cent neutrophiles containing bacteria	
		Mean ± S.E.	Difference / S.E. difference
A. Filter paper	33	16.7 ± 2.3	
B. Coverglass	34	18.4 ± 2.1	B-A 0.55
C. Rotated tube	34	16.7 ± 2.8	C-A 0
			B-C 0.49

Comparison of Stationary and Rotated Preparations in Phagocytosis by Exudate Leukocytes

During the foregoing experiments portions of the same exudate-leukocyte mixtures that were used in the filter paper and coverglass comparisons were placed in 8 mm. test tubes and rotated at 37°C. for the same time period. In 34 experiments, the phagocytosis of pneumococci by rat exudate leukocytes was at the same level in rotated preparations, 16 ± 2.8 per cent, as it was in either of the stationary preparations (Table IV), with no significant difference. This finding, that rotation did not increase the level of phagocytosis over that observed in stationary preparations, was contrary to most experiences with phagocytosis, and it was therefore felt that the exudate leukocyte might not have been physiologically active or might have been damaged during rotation, despite careful handling. Consequently, the same experiments were repeated using washed blood leukocytes:—

Effect of Glass and Filter Paper Surfaces on Phagocytosis by Rat Blood Leukocytes

Blood was obtained by cardiac puncture from the same rats which were used for the exudate leukocyte experiments. The plasma was removed by centrifugation, the packed

cells were resuspended in Krebs-gelatin solution, and washed twice in this solution—the same treatment which the exudate preparations had received. These were then mixed with the same *Pneumococcus* preparations.

In 10 experiments, each of which included duplicate to quadruplicate comparisons, the phagocytosis of pneumococci by rat blood leukocytes was not significantly different when preparations were placed on glass or moistened filter paper surfaces (Table V). The mean per cent of neutrophiles containing

TABLE V

Effect of Surface on Phagocytosis of Pneumococcus Type I A5 by Rat Blood Leukocytes
Filter paper, glass, and rotated tubes

Test surface	No. of experiments	Per cent neutrophiles containing bacteria	
		Mean ± S.E.	Difference / S.E. difference
A. Filter paper	10	26.6 ± 4.2	
B. Coverglass	10	30.5 ± 6.3	B-A 0.52
C. Rotated tube	10	52.2 ± 5.3	C-A 3.81
			B-C 2.66

TABLE VI

Phagocytosis of Pneumococcus Type I A5 by Rat Blood and Exudate Leukocytes
Stationary mixtures

Source of leukocytes	No. of experiments	Per cent neutrophiles containing bacteria	
		Mean ± S.E.	Difference / S.E. difference
A. Circulating blood	20	28.6 ± 3.6	
B. Peritoneal exudate	67	17.6 ± 1.5	B-A 2.82

bacteria and its standard error was 30.5 ± 6.3 for glass surface, and 26.6 ± 4.2 for filter paper surface; again slightly lower for the rough surface.

Comparison of Stationary and Rotated Preparations in Phagocytosis by Blood Leukocytes

The same blood-leukocyte mixtures were rotated in parallel with the surface experiments just described. In 10 experiments, rotation markedly increased levels of phagocytosis by blood leukocytes over the levels observed in stationary preparations, the mean per cent phagocytosis and its standard error in rotated preparations being 52.2 ± 5.3, as compared to 26.6 ± 4.2 and 30.5 ± 6.3 for stationary preparations (Table V).

Comparison of Blood and Exudate Leukocytes in Phagocytosis

In all stationary preparations examined, on either filter paper or cover-glass surfaces, blood leukocytes showed a markedly increased capacity for phagocytosis of pneumococci. A total of 67 experiments with exudate leukocytes gave a mean per cent phagocytosis and standard error of 17.6 ± 1.5, while a total of 20 experiments with blood leukocytes gave a mean per cent and standard error of 28.6 ± 3.6 (Table VI). The difference and its standard error, 11.0 ± 3.9 per cent, was significant.

When preparations were rotated, allowing for more efficient contact of bacteria and leukocytes, and subsequently higher levels of phagocytosis, the difference was even more striking. In 34 experiments with exudate leukocytes,

TABLE VII

Phagocytosis of Pneumococcus Type I A5 by Rat Blood and Exudate Leukocytes
Rotated mixtures

Source of leukocytes	No. of experiments	Per cent neutrophiles containing bacteria	
		Mean ± S.E.	Difference / S.E. difference
A. Circulating blood	10	52.2 ± 5.3	
B. Peritoneal exudate	34	16.7 ± 2.8	B-A 5.93

the mean per cent phagocytosis and its standard error was 16.7 ± 2.8, while in 10 experiments with blood leukocytes, the mean per cent phagocytosis was 52.2 ± 5.3 (Table VII). The difference and its standard error, 35.5 ± 6.0 per cent was highly significant.

DISCUSSION

Evidence has been accumulated that the stimulation of phagocytosis of certain bacteria by filter paper is a chemical effect rather than a physical surface effect. The phagocytosis of live or killed *E. coli*, *B. abortus*, and *Pneumococcus* Type IV was enhanced by previous contact with either filter paper or liquid from moistened filter paper, or by para-hydroxybenzoic acid, a chemically pure compound found to be present in filter paper (7, 8, 12). The bacteria were definitely sensitized by prior contact with these substances, which were not present during the phagocytosis tests. The stimulatory effect of para-hydroxybenzoic acid on the phagocytosis of both *E. coli* and *B. abortus* was in linear ratio to the logarithm of its concentration, and was effective over a wide range of concentration (7, 8). These findings cast further doubt upon the postulated necessity for a rough surface to which the leukocyte may anchor itself or against which the bacteria may become entrapped, with result in

more effective phagocytosis. The ability of chemical agents to enhance phagocytosis has long been known, and stimulation of phagocytosis by such diverse substances as ions, salts, histamine, lipoids, etc., has been recorded (23, 24).

Prior contact with filter paper or with para-hydroxybenzoic acid did not enhance the phagocytosis of the Type I A5 strain of *Pneumococcus* used by Wood *et al.* No explanation is offered for this finding, but it would be remarkable to encounter a uniform response to artificial sensitization among all strains or species of microorganisms examined. However, in view of this failure of response to the chemical type of stimulation, which had been considered as a possible explanation for Wood's results, an attempt was made to reproduce exactly the experimental conditions described by Wood *et al.*, using the Type I A5 *Pneumococcus*. When this was done contact with filter paper surfaces did not result in increased phagocytosis over contact with glass surfaces. Since the numbers of leukocytes and bacteria employed by the workers cited were estimated as numbers per oil immersion field, the more rigid criteria employed in our previous work (5–8, 12) were introduced; namely, exact counts of bacteria and of white cells, and adjustment of the bacteria: white cell ratio so as to be most favorable for phagocytosis as also constant throughout a series of experiments. The importance of the relationship between the concentration of leukocytes and the concentration of bacteria, the need to exceed the critical range, and the need for an excess of bacteria, have been stressed by many workers in the past (18, 24–29). Since phagocytosis had been expressed merely as positive or negative by the workers just mentioned, the more exact and quantitative expression of percentage of leukocytes ingesting bacteria was employed. Finally, when blood leukocytes, which were more effective in general for phagocytosis, were used, there was still no increase in phagocytosis caused by a rough surface. Under all the conditions tested, there was no enhancement of *in vitro* phagocytosis by a filter paper surface.

Under the experimental conditions described, some of which were identical with those of Wood *et al.*, and some of which were more quantitatively controlled, *in vitro* "surface phagocytosis" was not evident. It is possible that the impression that a filter paper surface had a phagocytosis-enhancing effect may have arisen from variability in concentrations of bacteria-leukocyte preparations, or from lack of quantitative standards of measurement of phagocytosis.

Although no effect of filter paper surface was observed in the above experiments, this does not negate the existence of a "surface phagocytosis" phenomenon. There is much evidence from the excellent direct microscopic *in vivo* and *in vitro* observations of Wood *et al.* (2, 3, 31, 32) and from our own phase microscopic observations of living *in vitro* preparations, that bacteria may become entrapped on rough or adherent surfaces and then be phagocytosed, and that leukocytes occasionally may anchor themselves to a rough surface before engulfing microorganisms. It is, however, obvious that *the presence of a*

rough surface is not necessary for phagocytosis to occur in the absence of specific antibody.

It was further demonstrated that the polymorphonuclear neutrophiles of the circulating blood were more actively phagocytic for the *Pneumococcus* than were those from peritoneal exudates in either stationary preparations on glass or on filter paper, or in rotated preparations in glass tubes. The importance of agitation or rotation of bacteria-leukocyte mixtures has been repeatedly emphasized by many workers, to assure a constant and thorough mixing of leukocytes and bacteria during incubation, and the failure to secure uniform mixing of cells and test particles has been cited as an important source of error in the quantitative estimation of phagocytosis (13, 18, 22, 24, 26, 27). Thus, it is also apparent that the exudate leukocyte, which is used nearly universally in investigations of this nature, is inferior to the leukocyte from the circulating blood of the same animal, when employed for quantitative measurements of phagocytosis.

Addendum.—Since the preparation of this manuscript, Sawyer, Smith, and Wood (30) have stated that previous work by Lerner and Victor (8) disregarded the fact that all direct observations on the phagocytic mechanism were done in the *absence* of filter paper; *i.e.*, in formalin-fixed tissue sections mounted on glass coverslips, and they refer to their work (31, 32). We were not unaware of the direct microscopic observations by Wood and coworkers (2, 3, 31, 32), and, in our own phase microscopic studies, noted this phenomenon. Rechecking of these publications, and particularly of the experimental procedures under discussion (*J. Exp. Med.*, **84**, page 390), has yielded no mention of whether the compared test substances were actually in contact with filter paper, nor was there any indication in these articles that a coverglass was placed between the material tested and the filter paper.

A misunderstanding may possibly have arisen from lack of sufficiently detailed description of the experimental procedure, but both the earlier experiments with *E. coli* and *B. abortus*, and the present experiments with Type IV *Pneumococcus* unequivocally demonstrate a stimulating factor of chemical nature in filter paper. For the tests with the Type I A5 *Pneumococcus* either a moistened filter paper strip upon a coverglass was employed, or a coverglass alone, upon which the bacteria-leukocyte mixtures were placed. Thus, any possible objections to the previous experimental conditions utilized for study of "surface phagocytosis" do not obtain in the present case.

SUMMARY

The present experiments have shown that phagocytosis occurs in the absence of specific antibody and in the absence of a "suitable physical surface", as further that the presence of a rough surface does not increase the *in vitro* phagocytosis of pneumococci by polymorphonuclear leukocytes. This held true during repetition of Wood's experiments, as well as when more controlled quantitative techniques were employed, when conditions were made optimal

for phagocytosis by increasing bacterial concentrations, and when blood leukocytes were substituted for exudate leukocytes.

Evidence has been presented previously that the stimulation of phagocytosis of *E. coli*, *B. abortus*, and Type IV *Pneumococcus*, after contact with filter paper or an active compound present in filter paper, is a chemical effect rather than a physical effect. This type of stimulation did not occur with the Type I A5 *Pneumococcus*.

The leukocyte of the circulating blood was found to be definitely superior to the exudate leukocyte in phagocytic capacity, under all the experimental conditions tested.

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