

## THE ROLE OF OPSONINS IN THE CLEARANCE OF LIVING AND INERT PARTICLES BY CELLS OF THE RETICULOENDOTHELIAL SYSTEM\*

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(Received for publication, April 17, 1961)

The function of the reticuloendothelial system in clearing the blood stream of living and inert particles has been well established and its importance as a host defence mechanism emphasized. (1-3). Recent *in vitro* and *in vivo* studies on the phagocytosis of Gram-negative and Gram-positive bacteria have shown that phagocytosis takes place only in the presence of serum and is greatly enhanced by addition of specific antibody to the serum. (4-8). Serum factors other than specific O antibody have also been shown to enhance phagocytosis. (7, 9, 10). Indeed it appears that the level of these uncharacterized opsonins may predetermine the susceptibility of the individual normal animal and its species to infection by a particular pathogen. (11, 12). *In vitro* experiments with perfused livers have shown that these same serum factors are also necessary for removal of bacteria by the fixed macrophages constituting the R.E.S. (13, 14).

The importance of these opsonins in determining the susceptibility or resistance of the host to infection cannot be too strongly emphasized since the efficiency of the phagocytic cells in removing bacteria and possibly events taking place within the cell after ingestion may depend on the titre of these factors at the time of infection. (7, 11).

This paper presents the results of a study of the removal of virulent and avirulent pairs of the same strain of bacteria (*i.e.*, having the same O antigen) by the R.E.S. of the mouse. The same methods when applied to the clearance of inert colloids show that even these particles depend, as do bacteria, on adsorption of serum factors for their removal by phagocytic cells. In fact the so called "R.E.S. blockade" by large doses of colloid appears to be due to depletion of these serum factors, and not to saturation of the phagocytic cells by the colloid as has been suggested (15).

### Material and Methods

**Bacterial Strains.**—The virulent and avirulent pairs of bacteria used were as follows: *Salmonella typhimurium* C5 (LD<sub>50</sub> for mice,  $2 \times 10^2$ ), M 206 (LD<sub>50</sub>,  $10^6$ ) (16), *Salmonella*

\* This work was supported by grant E. 3226 (C1), from the United States Public Health Service.

*gallinarum* 9240 ( $LD_{50}$ ,  $5 \times 10^8$ ), 9240 A ( $LD_{50}$ ,  $10^8$ ) (17), *Klebsiella pneumoniae* NCTC 5055 ( $LD_{50}$ ,  $5 \times 10^8$ ), and NCTC 5054 ( $LD_{50}$ ,  $10^7$ ).

For R.E.S. clearance studies the strains of bacteria were grown in a minimal medium supplemented with Difco cas amino acids (Difco Laboratories, Detroit) (8). To 50 ml of this medium 1 mc of  $P^{32}$  as orthophosphate was added. The inoculated medium was shaken at  $37^\circ C$  for 18 hours. The  $P^{32}$ -labelled bacteria were washed three times with 50 ml of saline and finally resuspended in the above medium to give a suspension of  $10^9$  bacteria/ml. Bacterial suspensions were kept at  $4^\circ C$  and not used for longer than 5 days.

*In Vivo Clearance Studies of Bacteria and Carbon by the R.E.S.*—The technique used was essentially that described by Biozzi, Benacerraf, and Halpern (18). Labelled bacteria were injected intravenously into mice at a concentration of  $10^9$  bacteria/100 gm. At suitable intervals blood samples (0.02 ml) were taken from the retro-orbital plexus and pipetted onto filter paper discs stuck to stainless steel planchettes. Each sample was assayed for radioactivity using a thin mica end-window Geiger counter installed in a Nuclear Chicago automatic sample changer C 110A (Nuclear Chicago, Chicago), with an automatic printing timer C 111, coupled to the model 183 scaling unit to record the results. This apparatus was modified by the insertion of a General Electric helium-filled thin-window Geiger counter tube (General Electric Co., Schenectady, New York) which increased the sensitivity of the machine threefold.

Carbon C 11/1431a (Gunther Wagner, Hanover, Germany) in 1 per cent gelatin was used at a concentration of 16 mg/100 gm. For blockade 32 mg/100 gm of carbon was injected intravenously and the clearance of 16 mg/100 gm of carbon followed at intervals after the blocking dose. Blood samples were pipetted into 3 ml of 0.1 per cent sodium carbonate and the concentration of carbon determined by optical density. Readings were taken in an Optican spectrophotometer CF4 (Optica, Milan, Italy) at a wave length of  $675 m\mu$ . The phagocytic index  $K$  was calculated from the equation:

$$K = \frac{\text{Log. } C1 - \text{Log. } C2}{T2 - T1} \quad (18)$$

where  $C1$  and  $C2$  are the concentrations of bacteria or colloid at times  $T1$  and  $T2$ . All mice used in the clearance studies were previously injected with 40 units of heparin. Both males and females of the L.A.B. strain were used weighing 18 to 20 gms. The results presented in the figures and tables are the summation of experiments performed in at least 3 mice and many are the summation of results obtained from 20 or more.

*Preparation of Radioactive Lipopolysaccharide.*— $P^{32}$ -labelled lipopolysaccharide was prepared from *Salmonella typhimurium* C5 as described in a previous paper (19). This preparation contained less than 5 per cent nucleic acid and had a specific activity of 500 count/ $\mu g$ /min. The unlabelled *Pasteurella pseudotuberculosis* lipopolysaccharide was kindly given by Dr. D. A. L. Davies, Porton, England.

*Opsonization of Bacteria and Colloids.*—Bacteria were opsonized either with pig serum which has previously been shown to possess a high titre of opsonic factors unrelated to specific O antibody (11), or with serum obtained from mice 48 hours after the injection of 25  $\mu g$  lipopolysaccharide. The lipopolysaccharides used were those obtained from *S. typhimurium* C5 and *P. pseudotuberculosis*. For opsonization 1 ml of the bacterial culture was mixed with 1 ml of serum at  $4^\circ C$  and kept at that temperature for 20 minutes, during which time there was no drop in the viable count and no visible agglutination. The mixture was finally centrifuged at 3,000 g for 15 minutes and resuspended in 1 ml of the supplemented minimal medium for injection. 1 ml of carbon at a concentration of 32 mg/ml in 2 per cent gelatin was added to 1 ml of the serum to be tested and incubated at  $37^\circ C$  for 20 minutes. Spectrophotometric analysis of the treated carbon did not reveal any agglutination. Following incubation 0.2 ml of this mixture was injected into the mouse and the clearance of the colloid followed. Lipopolysaccharide at various concentrations was opsonized in a similar fashion.

## RESULTS

*Removal of Virulent and Avirulent Bacteria by the R.E.S.*—Unopsonized labelled bacteria were injected intravenously and their clearance from the blood stream followed. It is apparent from the results illustrated in Fig. 1 and Table I, that the avirulent strain of every pair in the series under study was removed

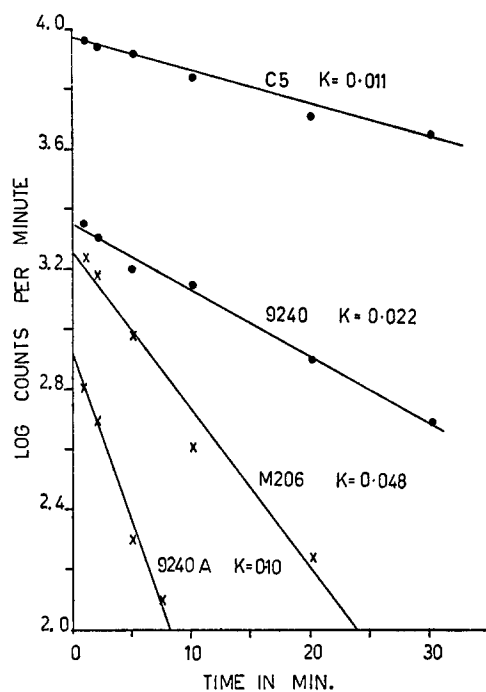


FIG. 1. Rates of clearance of virulent and avirulent *S. typhimurium* and *S. gallinarum* in mice. ●—●, virulent strains; X—X, avirulent strains.

TABLE I  
*Rates of Clearance of Living Virulent and Avirulent Strains of Bacteria by the R.E.S. as Measured by the Phagocytic Index K*

Bacterial strain	K
<i>S. typhimurium</i> C5 (V)	0.017
<i>S. typhimurium</i> M206 (AV)	0.058
<i>S. gallinarum</i> 9240 (V)	0.022
<i>S. gallinarum</i> 9240 A(AV)	0.10
<i>K. pneumoniae</i> 5055 (V)	0.005
<i>K. pneumoniae</i> 5054 (AV)	0.018

V = virulent.

AV = avirulent.

at a faster rate than the virulent strain. Pretreating the virulent strains with pig serum greatly enhanced the clearance rate (Fig. 2). Similar results were obtained after treating the virulent strains with serum obtained from mice previously treated with lipopolysaccharide, though in this case the enhancement was not as marked (Fig. 3).

*Removal of Colloidal Carbon by the R.E.S.*—Though it has been generally assumed that the clearance of inert colloidal particles by the R.E.S. is independent of serum factors, the early work of Fenn on the phagocytosis of carbon

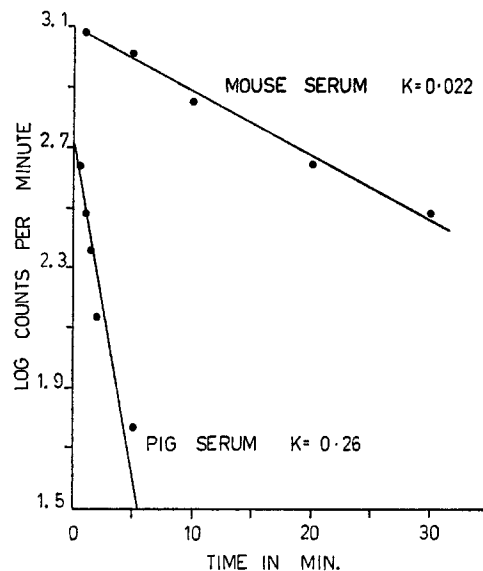


FIG. 2. The effect of pretreatment with pig serum and mouse serum on the rate of clearance of *S. typhimurium* C5 in normal mice.

and quartz particles by polymorphonuclear cells (20) and more recently the researches of Nelson and Lebrun (21) on the uptake of starch granules by similar phagocytes suggests that the ingestion of inert particles by these cells is dependent on the presence of serum opsonins.

The clearance of 16 mg/100 gm carbon, before and after opsonization with various animal sera was followed. It is apparent from the results given that four of the sera tested enhance the clearance of carbon: foetal pig serum, adult pig serum, rabbit antiserum to C5, and serum obtained from lipopolysaccharide-stimulated mice. (Table II). It is interesting that foetal pig serum, whilst increasing the rate of clearance of carbon did not have any effect on the clearance of bacteria. This suggests that some of the opsonic factors required by the two systems may be different.

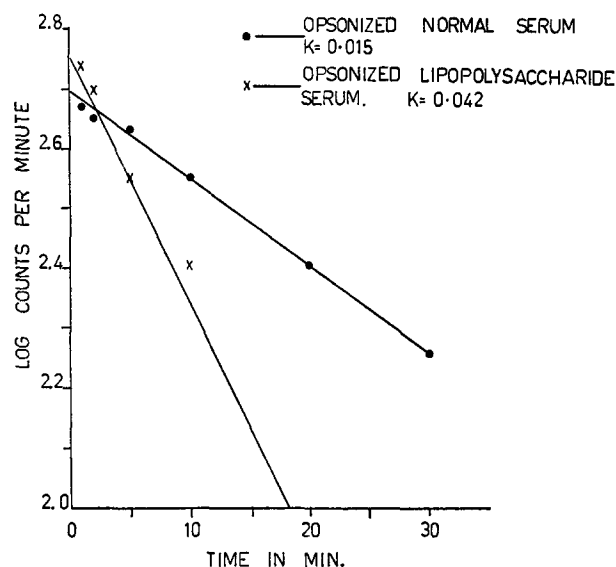


FIG. 3. The effect of pretreatment with serum from normal mice and from lipopolysaccharide-stimulated mice on the rate of clearance of *S. typhimurium* C5 in normal mice.

TABLE II

*Rate of Clearance of Carbon Particles after Opsonization with Various Animal Sera by the R.E.S. of the Mouse*

Rate of clearance measured by the phagocytic index  $K$  for a dose of 16 mg of carbon per 100 gm body weight.

Type of serum used to opsonize the carbon	$K$
Normal rabbit	0.030
Rabbit antiserum to C5	0.042
Normal adult pig	0.054
Normal foetal pig*	0.054
Normal chicken	0.031
Normal mouse	0.025
Lipopolysaccharide-treated mice	0.044

\* Foetuses obtained from adult pigs at slaughter.

*Removal of Lipopolysaccharide by the R.E.S.*—It has been reported that lipopolysaccharide is unique in that compared with other colloids its rate of clearance by the R.E.S. does not follow an exponential curve. (22). However, these results may be accounted for by the small doses of colloid used in the investigations.

Various doses of lipopolysaccharide were injected intravenously and their

rate of clearance followed for 30 minutes. The results illustrated in Fig. 4, show that below a certain dose the clearance of lipopolysaccharide is non-exponential, but above that dose the clearance is exponential like that of any other colloid studied. Treatment of the lipopolysaccharide with specific rabbit antiserum or with pig serum increased the rate of removal, though with the

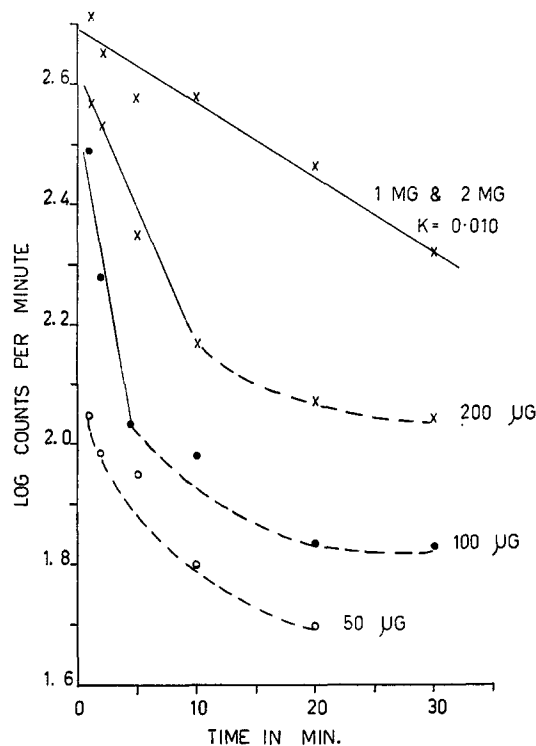


FIG. 4. Clearance of various doses of  $P^{32}$ -labelled lipopolysaccharide in normal mice

latter serum this was apparent only when small samples of the colloid were employed.

*Clearance of Carbon and Bacteria in Blocked Animals.*—Since the colloids studied depended to some extent on serum opsonins for their phagocytosis, the depressed rate of clearance observed in animals following injection of a large dose of colloid (the blocking dose), might be due in part to a removal of opsonins essential for phagocytosis rather than to a saturation of the phagocytic cells as has been suggested. (15, 18).

The following experiments were designed to test this hypothesis. Mice were injected intravenously with 32 mg/100 gm and at intervals after this dose the clearance rate of a second dose of carbon was followed. In some cases this second

dose was pretreated with pig serum and the other mice served as controls for untreated carbon. From Table III it is obvious that whilst the clearance of

TABLE III  
*Rates of Clearance of Opsonized and Unopsonized Carbon (16 Mg per 100 Gm Body Weight) in Mice at Various Times after Blockade with 32 Mg of Carbon per 100 Gm Body Weight*

Rate of clearance measured by the phagocytic index  $K$ .

Time after blockade with carbon  <i>min.</i>	$K$ values for	
	Opsonized carbon*	Unopsonized carbon
30	0.038	0.010
60	0.044	0.009
120	0.046	0.012
Controls (unblockaded) . . . . .	0.051	0.029

\* Carbon opsonized with foetal pig serum.

TABLE IV  
*Rate of Clearance of Opsonized and Unopsonized Bacteria ( $10^8$  per 100 Gm Body Weight), and Unopsonized Carbon (16 Mg per 100 Gm Body Weight) in Mice at Various Times after Blockade with 32 Mg of Carbon per 100 Gm Body Weight*

Rate of clearance measured by the phagocytic index  $K$ .

Time after blockade with carbon  <i>min.</i>	$K$ values for		
	Bacteria		Carbon
	Unopsonized	Opsonized*	Unopsonized
30	0.014	0.11	0.008
60	0.018	0.11	0.012
90	0.021	0.08	0.017
120	0.027	0.12	0.010
Control (unblockaded) . . . . .	0.021	0.10	0.028

\* Bacteria opsonized with adult pig serum.

unopsonized carbon was retarded, the phagocytic cells still cleared opsonized carbon at a rate similar to that observed in untreated mice. These experiments were repeated using opsonized and unopsonized bacteria for the second challenge. (Table IV). Once again whilst the clearance rate of unopsonized bacteria was depressed that of opsonized bacteria was not affected. It is interesting to

note that whilst the clearance of unopsonized bacteria gradually returned to normal in the blocked animals during the time period studied, the rate of clearance of carbon at the end of the experiment was still slower than that observed in the untreated control mice.

*Clearance of Bacteria in Lipopolysaccharide-Treated Mice.*—Mice were injected intravenously with 1 mg of lipopolysaccharide obtained either from *P. pseudotuberculosis* or from *S. typhimurium* C5. 15 minutes after injection

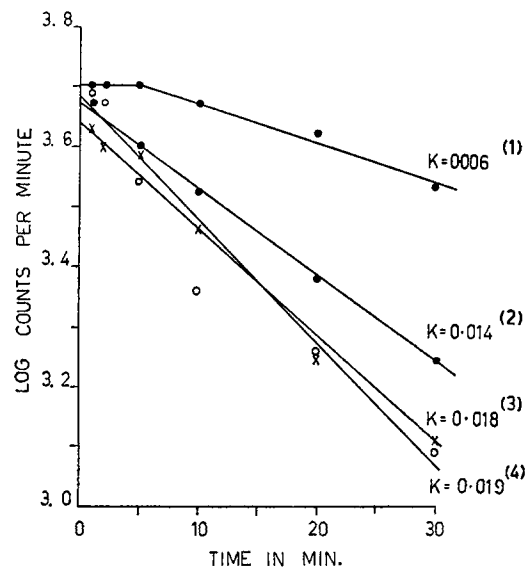


FIG. 5. Effect of previous injection of 1 mg *P. pseudotuberculosis* lipopolysaccharide on the rate of clearance of *S. typhimurium* C5 from the blood of mice. (1) Bacteria injected 15 min. after the injection of lipopolysaccharide; (2) Bacteria + 0.2 ml of serum from lipopolysaccharide-stimulated mice injected 15 min. after injection of lipopolysaccharide; (3) Lipopolysaccharide and bacteria injected together; (4) Control, bacteria injected into untreated mice.

the mice were challenged with labelled unopsonized *S. typhimurium* C5 and the rate of clearance of the bacteria followed. A further series of mice were injected at zero time with lipopolysaccharide plus bacteria whilst as a control other mice were injected with unopsonized bacteria only. Examination of the results in Fig. 5 suggests that the slow rate of clearance of bacteria in the mice receiving lipopolysaccharide 15 minutes prior to challenge is due to depletion of serum opsonins. That this may be so is further indicated by the finding that the clearance rate of unopsonized bacteria is not affected in the lipopolysaccharide-treated mice, if at the time of challenge with bacteria the animals are injected with serum from lipopolysaccharide-stimulated mice, which has previously been shown to have an increased titre of serum opsonins. (Fig. 3).



## DISCUSSION

Studies on the clearance of bacteria from the circulation such as those described in the present work measure at the best only a part of the phagocytic activity of the R.E.S., *i.e.*, fixation of bacteria by these cells. However, it is apparent that virulent and avirulent mutant pairs of the same bacterial strain are removed at different speeds, the virulent member being removed more slowly than the avirulent one. Treating the virulent strain with a serum rich in opsonins unrelated to specific O antibody (11) greatly increases its rate of removal. Previous *in vivo* and *in vitro* studies have shown some correlation between the rate of removal of bacteria, their subsequent fate within mononuclear cells, and survival of the experimental animal to infection. (7, 23, 24). It seems that there are instances in which the rate of phagocytosis is limiting the whole process of bacterial destruction and an increase in this rate is accompanied by increased chance of survival. On the other hand, whilst rapid phagocytosis is necessarily a prerequisite for rapid bacterial destruction, the one does not always follow the other (25). With some pathogens, for example *S. typhimurium*, virulence may depend on the ability of the bacteria to multiply within reticuloendothelial cells, and here the correlation between rate of the phagocytosis and survival of the animal may not be so apparent. Reasons for this have been discussed elsewhere. (7). However, even in these instances, providing a suitably small challenge dose is chosen, it is possible to show correlation between phagocytic rate and survival of experimental animal. (24).

Whilst there is considerable evidence to show that serum factors are required for phagocytosis of bacteria (4-8, 10) little is known in this respect about the uptake of inert colloidal particles. The early work by Fenn (20) demonstrated that only in the presence of serum would polymorphonuclear cells ingest carbon and quartz particles. More recently Nelson and Lebrun (21) have reported that these same cells phagocytose starch granules only in the presence of serum. It has also been observed that following the injection of a large amount of carbon, the clearance of a second dose of carbon injected some time later proceeds at a much slower rate compared with that observed in control animals. This has led to the assumption that the reduced rate of clearance of the second dose is due to saturation or "blockade" of the phagocytic cells by the first injected dose. In view of the present studies, the reduced rate of clearance in blockaded animals could be explained by depletion of serum opsonins. In the blockaded animals of our series the clearance of unopsonized carbon was slow as compared with that seen in control animals, but carbon pretreated with foetal pig serum was cleared in the blockaded mice as fast as it was in control mice. Similar results were obtained using opsonized and unopsonized bacteria. When lipopolysaccharide obtained from *P. pseudotuberculosis* was injected into mice, the clearance rate of *S. typhimurium* was markedly reduced, when measured 15 minutes after the injection of lipopolysaccharide. However, if serum

obtained from lipopolysaccharide-stimulated mice containing an increased level of opsonic factors was injected into the treated mice together with the bacteria, the rate of clearance of the labelled bacteria was similar to that observed in untreated control mice. The finding that when the blockading dose of lipopolysaccharide and bacteria were injected simultaneously the rate of clearance of bacteria was not affected and was exponential over the time period studied, is further support for the argument that depletion of serum factors necessary for phagocytosis occurs when the lipopolysaccharide is injected alone. Thus the blockade observed with large doses of carbon is in the nature of a humoral blockade rather than a cellular one.

It would be of interest to know if the phenomenon of preferential phagocytosis of colloids (and the variations in the rates of clearance of different colloids in the same dose range), is related to the absorptive properties of the colloids for serum opsonins. (15, 26, 27). That this might be so is suggested by the work of Fenn, who found that polymorphs in the presence of serum ingested carbon particles more rapidly than quartz. This difference might be a result of the different absorptive properties of the two particles. (20).

If we accept the findings that the initial depression of reticuloendothelial function which follows an injection of most colloids, is due at least in part to exhaustion of the serum contribution, then we are led to reexamine the reasons for the functional stimulation of the reticuloendothelial system that invariably follows the initial depression. (15, 28). The present work does not contribute much to our understanding of this aspect except in the finding that serum taken from animals at the time of reticuloendothelial stimulation with lipopolysaccharide possesses greater opsonic activity for both inert and living particles. It seems there is a possibility that this rise in serum opsonins might be an important factor in determining the increased activity of the R.E.S. The apparently greater number of phagocytes observed to rapidly arise at this time might in fact be due to stimulation of preexisting cells as suggested by Smulders (29) rather than to an actual increase by division in the number of phagocytes. We must admit, however, that the preliminary investigations of Kelly (30) on DNA synthesis (as measured by the incorporation of radioactive phosphate), in the liver of mice following injection of blockading doses of colloid, suggest that the increase in the number of phagocytes was due to division. This work requires further extension since the cells responsible for DNA synthesis were not defined.

#### SUMMARY

An investigation of the clearance of bacteria and colloids from the blood stream of mice has shown that both living and inert particles require serum factors (opsonins) in order that they may be phagocytosed by the macrophages of the reticuloendothelial system. It has been demonstrated that after the

injection of a large dose of colloid there is a depletion of these serum opsonins which appears to account for the reduced rate of clearance of a second dose of colloid or living bacteria, since replacement of these factors leads to normal clearance. The significance of these results is discussed and it is suggested that in "blockaded" animals there is a depletion of serum opsonins rather than a saturation of phagocytic cells.

We are very grateful to Dr. B. Benacerraf, Department of Pathology, New York University Medical School, for a generous gift of carbon.

We wish to acknowledge the excellent technical assistance of Mrs. Alexa McAskill and Mr. A. Sparrow. The gift of automatic counting equipment from Charles Pfizer, Inc., Brooklyn, New York, is gratefully acknowledged.

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