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The Roles of The Fc and C3 Receptors in the Phagocytosis and Killing of Bacteria By Human Phagocytes

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We have studied the influence of the *Escherichia coli* capsule on complement fixation and phagocytosis and the roles of the Fc and C3 receptors of human phagocytes in phagocytosis of encapsulated *E. coli*. We have focused on encapsulated bacteria because, for many bacterial species including *E. coli*, the capsule has been identified as a critical determinant of virulence; *in vivo*, pathogenic strains of these bacterial species are encapsulated.

We first examined the influence of the capsule on the attachment and ingestion stages of phagocytosis. We wanted to know if ingestion automatically follows attachment of *E. coli* to the surface of phagocytic leukocytes or if the specific serum ligands Ab and complement are additionally required. We also wanted to know if the presence of a capsule influences the fate of bacteria once they are attached.

To study these issues, we required a method of attaching encapsulated and unencapsulated *E. coli* to the surface of phagocytes without specific ligands, i.e., Ab and complement. We did this by using the plant lectin Concanavalin A (Con A) and *E. coli* strains whose capsular K and somatic O Ags are both composed of repeating polysaccharide subunits containing the specific carbohydrate combining site for Con A. The presence of Con A binding sites on the bacteria allowed us to attach the bacteria to the surface of mouse and human leukocytes with Con A and study bacteria-phagocyte interactions in the absence of the 2 major serum opsonins, Ab and complement. The 2 strains of *E. coli* used in our initial studies had the same somatic 0 Ag (09), but one was unencapsulated and the other had a capsule (K29).

We used resident and thioglycollate-elicited mouse peritoneal macrophages and human polymorphonuclear leukocytes (PMN). The PMN were purified (> 99%) by dextran sedimentation and centrifugation of the supernatant over a discontinuous gradient of ficoll and sodium diatrizoate.

We attached *E. coli* to resident or thioglycollate-elicited macrophages with Con A by coating the macrophages with Con A at 4 C and then incubating them with encapsulated or unencapsulated *E. coli* at 4 C. We then washed the macrophage-*E. coli* complexes to remove nonadherent bacteria. We used a similar method to attach *E. coli* to PMN except we pretreated the *E. coli* with Con A and then incubated them with PMN. With these methods, we attached 10 to 20 bacteria/PMN and 20 to 50 bacteria/macrophage.

When we attached unencapsulated *E. coli* to the surface of resident or thioglycollate-elicited macrophages or human PMN with Con A and incubated the complexes at 37 C, the bacteria were avidly ingested. In contrast, when we attached encapsulated *E. coli* to these phagocytes with Con A and incubated them at 37 C, the bacteria were not ingested (Fig. 1A). In fact, these bacteria multiplied on the surface of the phagocytes. At the end of 1, 2, or 3 hr of incubation, virtually all of the encapsulated *E. coli* were eluted from the phagocyte surface with α-methyl-D-mannopyranoside, a competitive inhibitor of Con A (Fig. 1B).

When we added fresh mouse serum from nonimmune mice to complexes of Con A-coated encapsulated *E. coli* and macrophages, the *E. coli* were still not ingested. However, when we added antibacterial or anti-Con A Ab, these encapsulated *E. coli* were ingested.

We concluded from these experiments that attachment by itself results in ingestion of unencapsulated but not encapsulated *E. coli;* serum ligands, in this instance Abs to bacterial surface Ags or

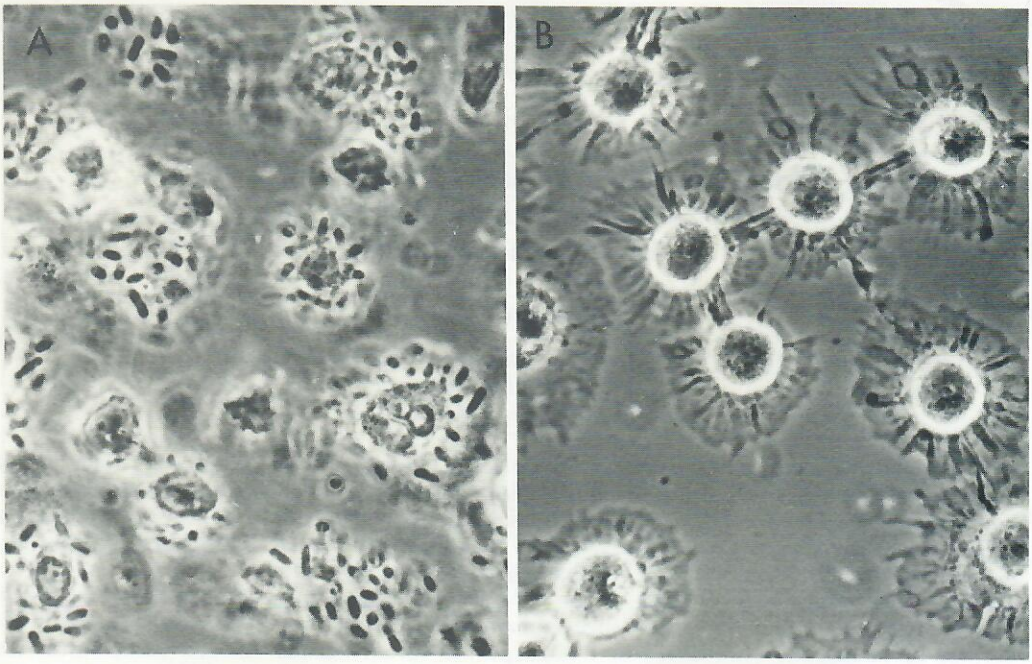

Fig. 1. Encapsulated *E. coli* attached to PMN surfaces with Con A. Note that the PMN did not phagocytose the *E. coli*. *E. coli* K^+ - PMN complexes were incubated in PBS at 37 C for 1 hr and examined by phase contrast microscopy before (A) and after (B) treatment with 100 mM α-methyl-D-mannopyranoside. X 1,350. Reprinted Horwitz and Silverstein (2).

to Con A on the bacterial surface, are required to promote phagocytosis of encapsulated *E. coli* even when these bacteria are bound to the surface of the phagocyte.

In experiments conducted without Con A, unencapsulated but not encapsulated *E. coli* were phagocytosed by macrophages when fresh mouse serum was added to the media. This suggested that unencapsulated but not encapsulated *E. coli* fix complement in the absence of Ab and consequently are phagocytosed in the absence of Ab. To test this hypothesis, we studied phagocytosis and killing by human PMN and monocytes of the same encapsulated *E. coli* (*E. coli* K^+) and an unencapsulated mutant of the same strain (*E. coli* K^-) (Fig. 2).

To assay killing of *E. coli* by PMN, we determined the number of colony-forming units before and after incubating the bacteria with PMN on a rotatory shaker in the presence of buffers and various

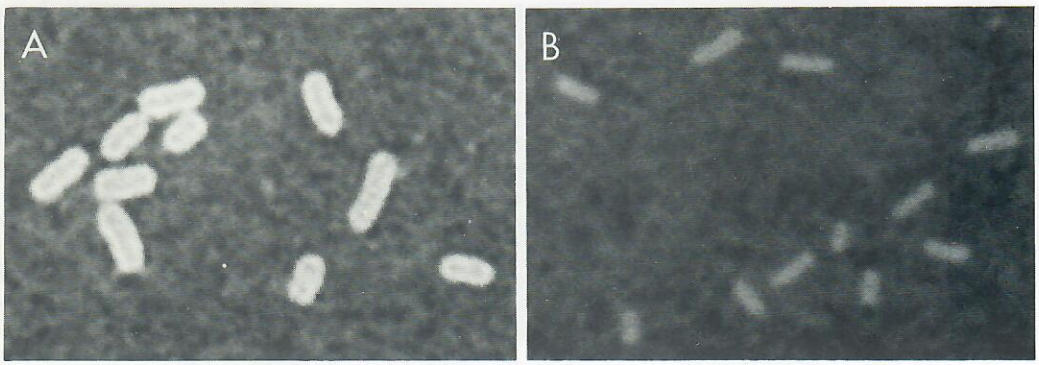

Fig. 2. India ink preparation of *E. coli* K^+ (A) and *E. coli* K^- (B). The bacteria were grown to mid-log phase and photographed at the same magnification by bright field microscopy. The capsule accounts for the difference in size. X 3,000.

serum preparations. To examine the role of complement in the absence of Ab, we absorbed normal human serum with *E. coli* K^+ and K^-. We used heat-inactivated human serum as a source of Ab. Later in this report, I shall present evidence that the normal human serum used in these studies contained Ab to the bacteria.

In the presence of normal serum, a source of both Ab and complement, PMN killed both *E. coli* K^+ and K^-, but in serum lacking Ab, PMN killed only *E. coli* K^- (Fig. 3). Without PMN, neither type of *E. coli* was killed in normal serum, even at concentrations as high as 50%.

We concluded from these experiments that complement is required for efficient killing of *E. coli* by PMN, and that, in addition, Ab is required for killing *E. coli* K^+.

It seemed likely that the differences in serum requirements for killing of *E. coli* K^+ and K^- by PMN reflected differences in requirements for phagocytosis. We examined this next by incubating bacteria with PMN on a monolayer in the presence of various serum preparations. At the end of a 20-min incubation at 37 C, we fixed the cells, examined them by phase contrast microscopy, and determined the percentage of PMN with vacuoles containing bacteria (Table 1).

The important point in Table 1 is that with normal serum, containing complement and Ab, PMN ingested both *E. coli* K^+ and K^-. With serum lacking Ab, PMN ingested *E. coli* K^- as avidly as with normal serum, but the PMN did not ingest *E. coli* K^+. Thus, the requirements for phagocytosis of *E. coli* by PMN precisely mirrored the requirements for killing; Ab and complement were required for phagocytosis of *E. coli* K^+ while complement alone sufficed for *E. coli* K^-. This suggested that Ab was required for fixation of complement onto *E. coli* K^+ but not K^-.

We examined this requirement directly by studying complement fixation onto the surface of the bacteria. We incubated the bacteria with normal serum or absorbed serum, washed them, incubated them with rhodamine-conjugated goat anti-human C3 antiserum, and examined them by fluorescence microscopy. In serum containing Ab, *E. coli* K^+ bound complement as evidenced by bright fluorescence of the bacteria (Fig. 4). This assay provides direct confirmation that the third component of complement is bound to the surface of the bacteria. In serum lacking Ab, *E. coli* K^+ did not bind complement. *E. coli* K^- bound complement in serum whether or not Ab was present.

Thus Ab was required for binding complement to *E. coli* K^+ but not K^-. The fact that, in the presence of complement, Ab was similarly required for phagocytosis and killing of *E. coli* K^+ but

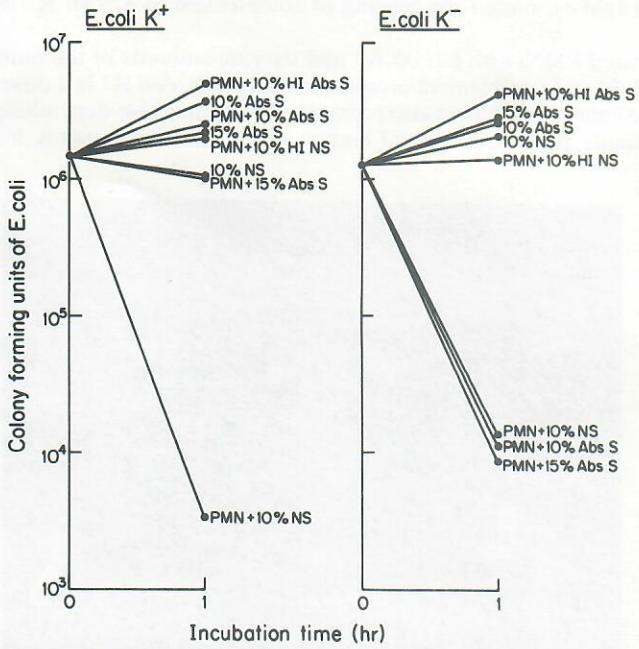

Fig. 3. PMN killing of *E. coli* K^+ and K^- in the presence of absorbed, heat-inactivated, or normal serum. PMN (5×10^6) were incubated with 1×10^6 *E. coli* K^+ (left) or *E. coli* K^- (right) at 37 C for 1 hr in the presence of the serum preparation indicated. Colony-forming units of *E. coli* were determined initially and at the end of the incubation. Reprinted from Horwitz and Silverstein (2).

TABLE 1

PMN Phagocytosis of *E. coli* K^+ and K^- in the Presence of Absorbed, Heat-Inactivated, or Normal Serum[a]

Serum preparation	Percent PMN Ingesting	
	E. coli K^+	*E. coli* K^-
None	0	0
Normal Serum (10%)	83	66
Heat-Inactivated Normal Serum (10%)	0	4
Absorbed Serum (10%)	0	65
Heat-Inactivated Absorbed Serum (10%)	0	1
Absorbed Serum (15%)	0	67

[a]PMN (1.25×10^5) in a monolayer on cover slips were incubated with 6.25×10^5 *E. coli* K^+ or K^- for 20 min at 37 C in the presence of the various serum preparations indicated. At the end of the incubation, the coverslips were washed, fixed, and examined by phase-contrast microscopy, and the percentage of PMN with intracellular bacteria was determined. This table was reprinted from Horwitz and Silverstein (2).

not K^- indicated that unless the bacteria were coated with complement, PMN could neither phagocytose nor kill them efficiently.

Having determined that phagocytosis and killing of *E. coli* K^+ was dependent upon both Ab and complement, we then sought to determine the roles of the C3 and Fc receptors of human PMN in this process. To study C3 receptor function independent of the Fc receptor, we used IgM to fix complement to the bacterial surface. To study Fc receptor function independent of the C3 receptor, we used IgG in the absence of complement.

We fractionated human serum into IgM and IgG fractions; the fractions were free of detectable contamination with each other by double immunodiffusion and immunoelectrophoresis. Using rhodamine-conjugated goat anti-human C3 antiserum and fluorescence microscopy, we confirmed that both IgG and IgM promoted the binding of complement to *E. coli* K^+ in a dose-dependent fashion.

When we incubated PMN with *E. coli* K^+ and varying amounts of the human IgM and/or IgG fractions, human IgM and complement promoted killing of *E. coli* K^+ in a dose-dependent fashion (Fig. 5). Human IgG and complement also promoted killing in a dose-dependent fashion. However, IgG alone, in amounts present in normal human serum, did not promote killing (not shown in Fig. 5).

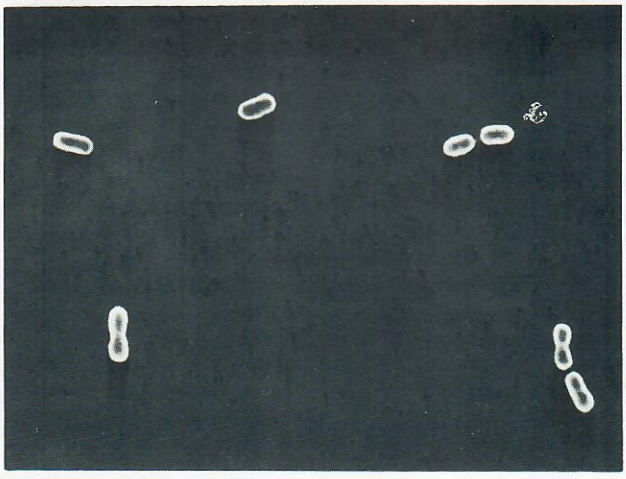

Fig. 4. Complement fixation to encapsulated *E. coli* in the presence of Ab and complement. *E. coli* K^+ were incubated in normal serum, washed, incubated in rhodamine-conjugated goat anti-human C3 IgG, and examined by fluorescence microscopy. X 1,200. Reprinted from Horwitz and Silverstein (2).

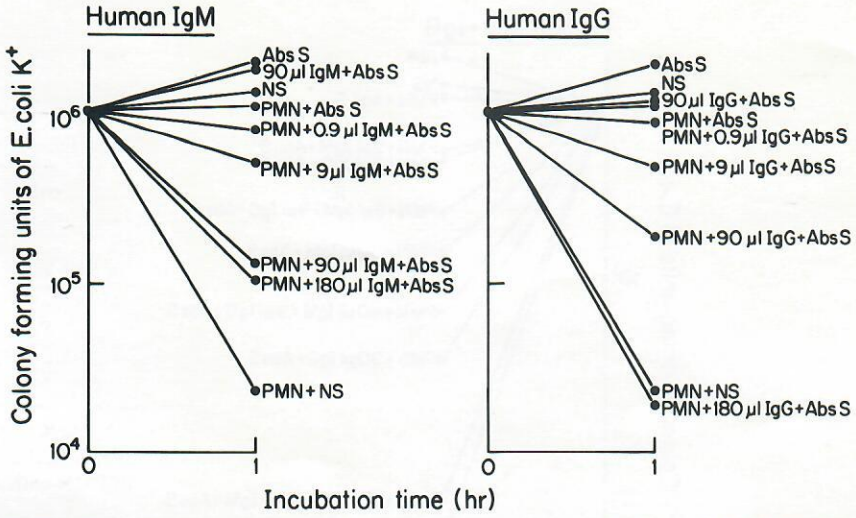

Fig. 5. PMN killing of encapsulated *E. coli* in the presence of human IgM and IgG. PMN (5×10^6) were incubated with 1×10^6 *E. coli* K^+ in the presence of absorbed serum and varying amounts of human IgM (left) or IgG (right) at 37 C for 1 hr. Colony-forming units were determined initially and at the end of the incubation. Reprinted from Horwitz and Silverstein (2).

When added together, human IgM and IgG acted synergistically (Fig. 6). For example, when a small amount (9 μl) of the IgM preparation was added to 90 μl of the IgG preparation, killing was enhanced by a log over that found with IgG alone. Bacterial killing was also augmented when 9 μl of IgG were added to 90 μl of IgM.

The capacity of IgM and complement to promote killing of *E. coli* K^+ by PMN indicates that these bacteria are phagocytosed via PMN complement receptors. This finding was surprising in view of the report of Ehlenberger and Nussenzweig (1) that the C3 receptor of PMN mediates attachment but not phagocytosis of complement-coated sheep RBC and that Fc receptor function is required for efficient phagocytosis of sheep RBC. To confirm our results, we repeated the killing experiments with high-titer rabbit anti-*E. coli* K^+ IgM and IgG. We fractionated the serum into IgM and IgG fractions; the fractions were free of detectable contamination with each other by double immunodiffusion. As an extra precaution against trace contamination of the IgM fraction with IgG, we heavily absorbed the IgM fraction with protein A-sepharose which complexes with rabbit IgG.

With a relatively low dose of either protein A-absorbed rabbit IgM or rabbit IgG, complement was required for PMN to kill *E. coli* K^+ (Fig. 7). Without complement, PMN did not kill with either IgM or IgG. With complement and both IgM and IgG, PMN killed significantly more *E. coli* K^+ than with either Ig alone.

These results confirm that the complement receptor of PMN mediates killing of *E. coli* K^+; Fc receptor function is not required.

With a relatively high dose of Igs, the results were similar except that PMN killed *E. coli* with IgG in the absence of complement (Fig. 8). This indicates that the Fc receptor was capable of mediating killing. However, even then, killing was not nearly as great as when complement was also present.

The left graph in Figure 8 shows that there was no difference in the IgM fraction before or after absorption with protein A, indicating that IgG was absent from this fraction even before absorption with protein A.

To confirm that PMN killing of bacteria with rabbit Ig was a consequence of phagocytosis of *E. coli*, we measured ingestion of these bacteria by PMN. The assay used was set up exactly as the killing assay, except that the incubation was stopped at 30 min and the cells were cytocentrifuged onto glass slides and stained. This assay allowed us to determine the number of intracellular bacteria as well as the percentage of PMN ingesting bacteria.

As expected, efficient phagocytosis occurred under the same conditions as killing, i.e., in the presence of complement and either IgM or IgG (Table 2). Figure 9 shows PMN that have phagocytosed *E. coli* K^+ in the presence of rabbit IgM and complement.

We repeated these killing and phagocytosis experiments with human blood monocytes instead of

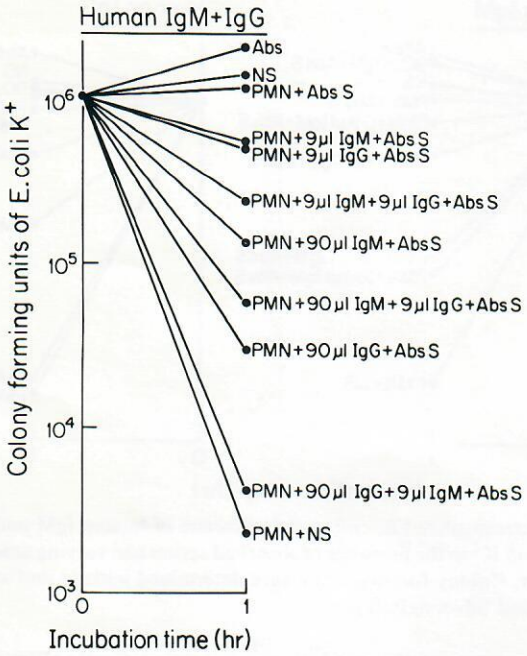

Fig. 6. PMN killing of encapsulated *E. coli:* synergy of human IgM with IgG in the presence of complement. PMN (5 x 10^6) were incubated with 1 x 10^6 *E. coli* K^+ in the presence of absorbed serum (complement source) and varying amounts of IgM and/or IgG at 37 C for 1 hr. Colony-forming units were determined initially and at the end of the incubation.

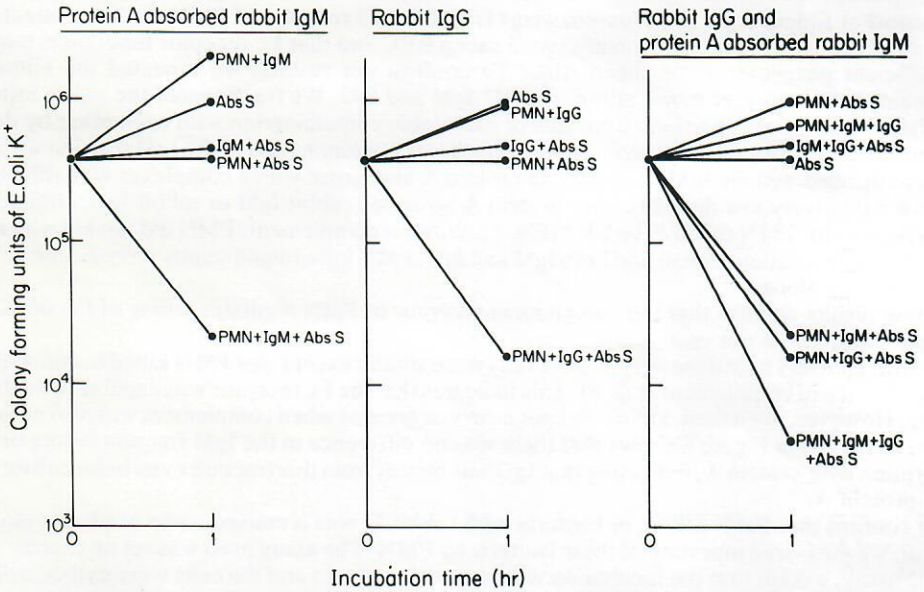

Fig. 7. PMN killing of *E. coli* K^+ in the presence of a relatively low dose of rabbit IgM and IgG. PMN (2.5 x 10^6) were incubated with 4 x 10^5 *E. coli* K^+ at 37 C for 1 hr in the presence of absorbed serum (as a source of complement) and 0.008 agglutinating doses of protein A-absorbed rabbit IgM and/or rabbit IgG. Colony-forming units were determined initially and at the end of the incubation. An agglutinating dose was the minimal concentration of Ab required to agglutinate 1 ml of a mid-log phase *E. coli* suspension containing 2.65 x 10^8 colony-forming units/ml.

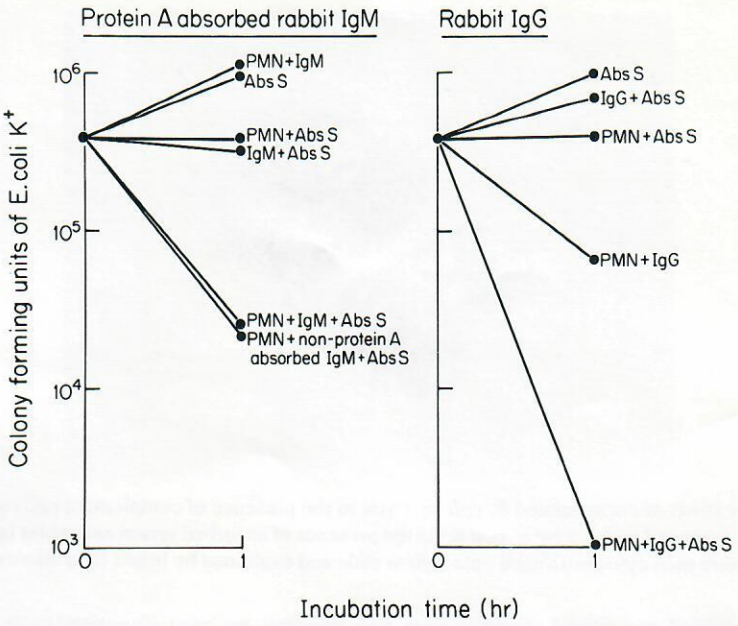

Fig. 8. PMN killing of *E. coli* K^+ in the presence of a relatively high dose of rabbit IgM and IgG. PMN (2.5×10^6) were incubated with 4×10^5 *E. coli* K^+ at 37 C for 1 hr in the presence of absorbed serum and 0.04 agglutinating doses protein A-absorbed rabbit IgM or rabbit IgG. Colony-forming units were determined initially and at the end of the incubation. A control sample contained 0.04 agglutinating doses of rabbit IgM not absorbed with protein A.

TABLE 2

PMN Phagocytosis of *E. coli* K^+ in the Presence of Rabbit IgM and IgG[a]

Opsonin(s) Added	PMN Ingesting *E. coli* (%)	Number *E. coli*/ PMN Ingesting	Phagocytic Index[b]
Normal Serum (Human)	96	5.6	538
Absorbed Serum (Human)	2	1.0	2
IgM (0.04 AD)[c]	0		0
IgM (0.04 AD) + Absorbed Serum	92	4.8	441
IgG (0.04 AD)	3	1.3	4
IgG (0.04 AD) + Absorbed Serum	85	2.8	239
HI Rabbit Serum (0.04 AD)	21	3.3	70
HI Rabbit Serum (0.04 AD) + Absorbed Serum	99	5.9	585

[a]PMN (2.5×10^6) were incubated with 1×10^7 *E. coli* K^+ for 30 min at 37 C in the presence of normal human serum (10%) or in the presence of absorbed human serum (10%) and/or 0.04 agglutinating doses of IgM, IgG, or the heat-inactivated rabbit antiserum from which the immunoglobulins were fractionated. At the end of the incubation, the cells were cytocentrifuged onto glass slides, fixed, stained, and examined by bright field microscopy and the number of PMN ingesting bacteria and the number of such bacteria per ingesting PMN determined. This table was reprinted from Horwitz and Silverstein (2).

[b]Phagocytic Index is the percentage of PMN with ingested *E. coli* K^+ multiplied by the average number of *E. coli* K^+ ingested per PMN times 100.

[c]See legend to Figure 7.

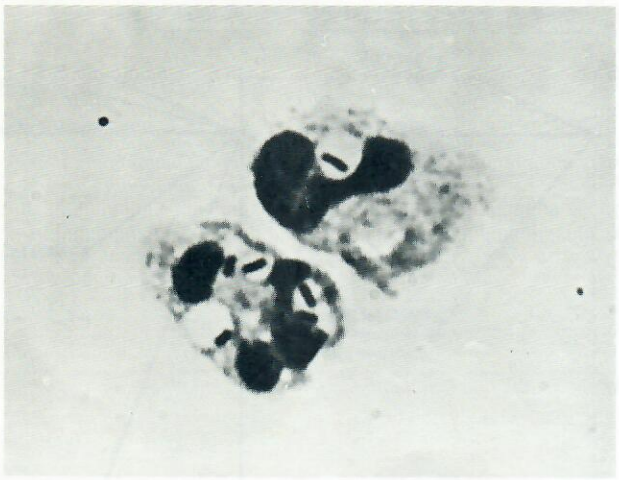

Fig. 9. Phagocytosis of encapsulated *E. coli* by PMN in the presence of complement and rabbit IgM. PMN (2.5×10^6) were incubated with 1×10^7 *E. coli* K^+ in the presence of absorbed serum and rabbit IgM at 37 C for 30 min. The cells were then cytocentrifuged onto a glass slide and examined by bright field microscopy. X 1,100.

PMN. We obtained the blood mononuclear cell fraction by centrifugation over ficoll-sodium diatrizoate and adjusted the monocyte concentration to the required level on the basis of a differential cell count.

Monocytes, like PMN, required complement and either IgM or IgG for effective phagocytosis (Table 3) and killing (Fig. 10) of encapsulated *E. coli*.

We concluded from these studies that the complement receptor of human PMN and monocytes mediates phagocytosis of complement-coated encapsulated bacteria and is the primary mediator of phagocytosis and killing of these bacteria.

In obtaining monocytes, we found by chance that if the blood was dextran-sedimented before application to ficoll-sodium diatrizoate instead of being applied directly as usual, the mononuclear fraction thus obtained was heavily contaminated with PMN and, by chance, consisted of nearly

TABLE 3

Monocyte Phagocytosis of *E. coli* K^+ in the Presence of Rabbit IgM and IgG[a]

Opsonin(s) Added	Monocytes Ingesting *E. coli* (%)	Number *E. coli*/ Monocyte Ingesting	Phagocytic Index[b]
Normal Serum (Human)	83	4.5	374
Absorbed Serum (Human)	1	1.0	1
IgM (.04 AD)[c]	0	0	0
IgM (.04 AD) + Absorbed Serum	49	3.2	157
IgG (.04 AD)	11	2.6	29
IgG (.04 AD) + Absorbed Serum	53	3.0	160
HI Rabbit Serum (.04 AD)	21	2.2	46
HI Rabbit Serum (.04 AD) + Absorbed Serum	79	4.9	389

[a]Monocytes (1×10^6) were incubated with 1×10^7 *E. coli* K^+ under the same conditions as PMN in Table 2 and phagocytosis was assayed in the same way as described for PMN in Table 2. This table was reprinted from Horwitz and Silverstein (2).

[b]See footnote *b*, Table 2.

[c]See legend to Figure 7.

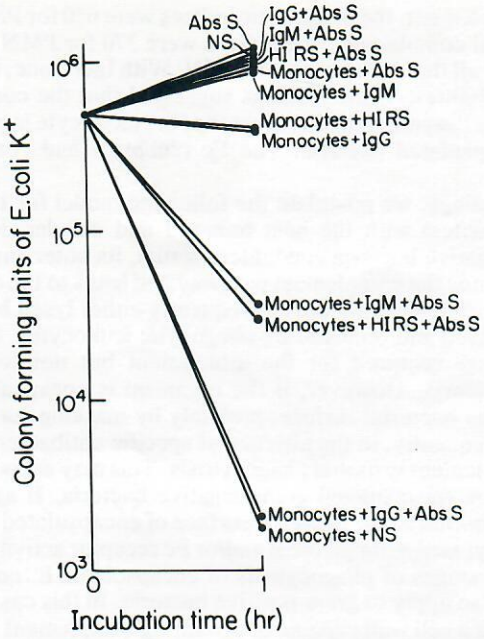

Fig. 10. Monocyte killing of encapsulated *E. coli* in the presence of rabbit IgM and IgG. Monocytes (2.5×10^6) were incubated with 5×10^5 *E. coli* K^+ in the presence of absorbed serum and rabbit IgM or IgG at 37 C for 1 hr. Colony-forming units were determined initially and at the end of the incubation. Reprinted from Horwitz and Silverstein (2).

equal numbers of PMN and monocytes. This allowed a comparison of the phagocytic capabilities of PMN and monocytes obtained in comparable numbers under identical conditions (Table 4).

The essential information in Table 4 is contained under the column entitled "PMN Phagocytic Index" and "Monocyte Phagocytic Index." As when the phagocytes were present alone, significant phagocytosis occurred only when both Ab, IgM or IgG, and complement were present. However, the PMN was strikingly superior to the monocyte in phagocytic capability. In the

TABLE 4

Comparison of the Capacities of PMN and Monocytes to Phagocytose *E. coli* K^{+a}

Opsonin(s) Added	PMN Ingesting *E. coli* (%)	Number *E. coli*/ PMN Ingesting	PMN Phagocytic Index	Monocytes Ingesting *E. coli* (%)	Number *E. coli*/ Monocyte Ingesting	Monocyte Phagocytic Index
Normal Serum (Human)	85	5.1	433	10	2.2	21
Abs Serum (Human)	10	1.5	15	3	1.3	4
IgM (0.04 AD)[b]	0		0	0		0
IgM (0.04 AD) + Absorbed Serum	88	7.1	620	31	2.9	89
IgG (0.04 AD)	6	1.8	11	14	2.2	31
IgG (0.04 AD) + Absorbed Serum	78	4.8	370	26	2.2	57
HI Rabbit Serum (0.04 AD)	11	3.0	33	10	4.1	40
HI Rabbit Serum (0.04 AD) + Absorbed Serum	72	4.2	300	19	1.9	36

[a]PMN (1×10^6) and Monocytes (1×10^6) were isolated together on a ficoll-sodium diatrizoate gradient and incubated with 4×10^6 *E. coli* K^+ under the same conditions, and phagocytosis assayed in the same way as in Tables 2 and 3.

[b]See legend to Figure 7.

presence of IgM and complement, the phagocytic indices were 620 for PMN and 89 for monocytes; in the presence of IgG and complement, the indices were 370 for PMN and 57 for monocytes; in these preparations, nearly all the bacteria were in PMN. With IgG alone, both PMN and monocytes had low phagocytic capabilities. These findings suggested that the complement receptor of the PMN is more efficient than the complement receptor of the monocyte in promoting phagocytosis of complement-coated encapsulated bacteria. The Fc receptors had comparably low phagocytic capabilities.

On the basis of our findings, we postulate the following model for the interaction of bacteria (except intracellular parasites) with the host humoral and cellular defense system. When an unencapsulated, gram-negative bacteria encounters serum, its outer surface, a membrane rich in lipopolysaccharide, activates the complement pathway and leads to the deposition of complement onto the bacterial surface. The bacteria are consequently either lysed by late-acting complement components or phagocytosed and removed by phagocytic leukocytes. Complement and complement receptor activity are required for the attachment but not for the ingestion stage of phagocytosis of these bacteria. However, if the organism is encapsulated, the capsule blocks complement fixation to the bacterial surface, probably by masking surface components such as lipopolysaccharide. Consequently, in the absence of specific antibacterial Ab, encapsulated bacteria are resistant to complement lysis and phagocytosis. This may account, at least in part, for the enhanced pathogenicity of encapsulated gram-negative bacteria. If specific IgM or IgG Ab is available, complement is bound to the bacterial surface of encapsulated bacteria, and the bacteria are either lysed or phagocytosed. Complement and/or Fc receptor activity are required for both the attachment and ingestion stages of phagocytosis of encapsulated *E. coli*.

These principles may also apply to gram-positive bacteria. In this case, the outer surface of the unencapsulated bacteria is a cell wall capable of activating complement in the absence of Ab. The capsule of gram-positive bacteria may similarly impose a requirement for specific antibacterial Ab for complement fixation and phagocytosis.

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