Contribution of Immunoglobulins M and G, Complement, and Properdin to the Intracellular Killing of *Escherichia coli* by Polymorphonuclear Leukocytes

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The effect of immunoglobulins and complement (C) on phagocytosis and intracellular killing of *Escherichia coli* was studied in vitro. The incubation system consisted of monolayers of human polymorphonuclear leukocytes and C-resistant, [³H]thymidine-labeled *E. coli*. C source was human serum deprived of immunoglobulins and properdin by immunoabsorption. In the absence of C, only immunoglobulin G-coated bacteria were phagocytosed, whereas immunoglobulin M lacked opsonic activity. In the presence of C, phagocytosis was enhanced; however, immunoglobulin M was now more efficient than immunoglobulin G. Intracellular killing was notably augmented when C was activated by immunoglobulin G- or immunoglobulin M-coated bacteria; in contrast, the alternative activation of C by properdin had no effect on phagocytosis or intracellular killing. These results demonstrate the importance of immunoglobulins together with C not only for phagocytosis but also for efficient intracellular killing.

The cooperative action of humoral and cellular defense mechanisms against infections has been the subject of extensive studies. One of the best known cooperations is the opsonization of bacteria by humoral factors and the subsequent phagocytosis by leukocytes. The importance of the humoral component, i.e., antibody and complement (C), is well documented (for review, see reference 28). However, information is still lacking concerning their influence on the subsequent events of killing and digestion of bacteria. The importance of a heat-labile serum factor for efficient intracellular killing was already observed by Li et al. (17). Later Glynn and Medhurst (7) used C-resistant and C-sensitive strains of bacteria as well as C-intact and C-defective animals, and their results indicated the participation of a humoral factor, presumably C, in the intracellular killing even of C-resistant strains of bacteria. Similar results were obtained by Solberg and Hellum (25), however, without distinguishing between the action of antibody and C.

Details of this possible intracellular cooperation are as yet unknown. Recent experiments of Goldstein et al. (9) have shown that activated C3a is able to promote a release of degradative enzymes from the lysosomes through the plasma membrane, which may resemble the transfer of enzymes from the lysosomes to the phagosome during phagocytosis. The same authors (8, 10) demonstrated that a fragment of C, similar or identical to C5a, was able to enhance the nitro

blue tetrazolium reduction and superoxide generation in polymorphonuclear phagocytes (PMN), which are metabolic activities normally associated with phagocytosis.

Since C3 and the later components of C are activated by different mechanisms—the classical and the alternative pathway—the question arises whether these two mechanisms are similarly effective in the bactericidal activities of PMN. Also, we need to know whether the two main serum immunoglobulins—immunoglobulin M (IgM) and immunoglobulin G (IgG)—participate in these reactions according to their different abilities to activate C.

We have tried to answer these questions by using purified immunoglobulins of the classes M and G and by employing a C source which permitted the selective activation of C by the two known pathways.

MATERIALS AND METHODS

Medium. Hanks balanced salt solution was used throughout the experiments. It was buffered to pH 7.4 with *N*-2-hydroxyethyl piperazine-*N*'-2-ethanesulfonic acid (Serva, Heidelberg, Germany).

Bacteria. C-resistant *Escherichia coli* O8K27-(E56b) was used. A small inoculum of *E. coli* from a petri dish was incubated in 30 ml of standard nutrient broth (Merck AG, Darmstadt, Germany) for 2 to 3 h. At a concentration of approximately 5×10^8 *E. coli* per ml, 1 ml was transferred into 20 ml of new broth containing 100 μ Ci of [³H]thymidine (specific activity, 5 Ci/mmol). *E. coli* was labeled for 1 h. The final

concentration was 5×10^7 /ml, which yielded a radioactive label of 1.3×10^5 to 4.2×10^5 cpm/ 5×10^7 *E. coli*. The efficiency of labeling was tested for each experiment.

Leukocytes. Human PMN were isolated from blood of different healthy donors by the method of

Chodirker et al. (1). In short: fresh venous blood was diluted 1/20 in saline at 4°C and centrifuged for 30 min at $1,200 \times g$. The cell sediment was then resuspended in 0.2% saline in the cold for 20 to 30 s, which lysed practically all erythrocytes. Isotonic conditions were restored by addition of an equal volume of 1.6% saline. This procedure was repeated if necessary to lyse remaining erythrocytes. White cells were washed twice and adjusted to 2.5×10^6 cells per ml. No heparin, dextran or Ficoll-Hypaque was used in the separation procedure to avoid any alteration of the cell membrane. The final cell suspension contained between 70 and 80% PMN.

Antibody. Specific antibodies were raised in rabbits. Three doses of 0.25, 0.5, and 1.0 ml of Formalin-inactivated *E. coli*, 10^7 /ml, were given intravenously at 5-day intervals. Animals were bled at day 13. IgG and IgM were purified by conventional chromatographic techniques as described (26). The activity of the two immunoglobulins was tested by indirect hemagglutination and bacterial agglutination. The content of specific immunoglobulin was quantitated by precipitation with *E. coli* O8 lipopolysaccharide kindly given by R. Jann, Max-Planck-Institut, Freiburg. The protein content was determined by the Folin method.

Properdin. Properdin (P) was isolated from human serum by the methods described previously (H. Jungfer, habilitation thesis, University of Heidelberg, Heidelberg, Germany, 1976). In short: 90 mg of immunoglobulin, isolated from rabbit anti-human P serum, was coupled to aminopropylsilyl-controlled pore glass (Serva, Heidelberg) by means of glutaric aldehyde. The immunoabsorbent was incubated with 400 ml of fresh human serum containing 0.02 M ethylenediaminetetraacetic acid for 60 min at room temperature. The absorbed material was then eluted with 3 M thiocyanate and further purified by inverse immunoabsorption on controlled pore glass coated with polyspecific anti-human antiserum. The final product was concentrated to 1 mg/ml and tested for purity in an acrylamide gel electrophoresis (Fig. 1).

C. Human serum was used as C source. Immunoglobulins and P were removed by immunoabsorption according to Jungfer (habilitation thesis).

Indirect C fixation test. This test followed the method described by Kabat and Mayer (15).

C3 binding assay. Anti-human C3 serum was raised in sheep and purified by immunoabsorption (Jungfer, habilitation thesis). Purified anti-C3 antibody (1 mg/ml) was labeled with 125 I by the Chloramin method. Free iodine was removed by a passage through Sephadex G25. Antibody-bound radioactive label was 1.1×10^6 cpm/mg. The C3 binding was tested under the same conditions as described for the phagocytosis experiments. Opsonized bacteria were then incubated for 1 h at 37°C with an excess of anti-C3 antibody and thoroughly washed, and the remaining radioactivity was determined in a gamma counter.

Immunoelectrophoresis. The two-dimensional

FIG. 1. Analysis of purified human P by polyacrylamide gel electrophoresis. Fifty microliters of a preparation containing 1 mg of protein per ml and 20% sucrose was applied between two gel columns (1). Following electrophoresis the gels were sliced longitudinally. One half was stained, and the other half was embedded in agarose. The trough (2) contained monospecific anti-P.

antigen-antibody crossed immunoelectrophoresis was carried out according to Laurell (16).

Opsonization. A total of 5×10^7 *E. coli* per ml were incubated with an antibody dilution of 1/20,000 for 30 min at 37°C. Bacteria were then washed and, in the case of C activation, incubated with C at a concentration of 4 CH₅₀ units/ml for 30 min at 37°C. After washing, they were resuspended in the same volume of Hanks balanced salt solution.

Phagocytosis experiments. A diagram of the experimental procedure is shown in Fig. 2. It followed the method described previously (2). Phagocytosis was performed in Leighton tissue culture tubes. Two to three aliquots of PMN suspensions from a single donor containing 5×10^6 cells were allowed to form a mono-

layer on a cover slip (9 by 50 mm) in the tube. The nonadherent cells, comprising approximately 20% of the initial inoculum, were removed by rinsing. The remaining monolayer consisted of over 92% PMN, 3 to 4% monocytes, 2 to 3% eosinophils, and a few mononuclear cells. The number of adhering cells did not vary by more than 10% from cover slip to cover slip, as determined by measuring the protein content of adhering leukocytes. To the monolayers, 2 ml of [³H]-thymidine-labeled bacteria (opsonized or unopsonized) was added. The time of phagocytosis was always 20 min. The Leighton tubes were gently shaken on a Bellco rocker platform, 20 cycles per min, to increase contact between the monolayer and bacteria. The tubes were then cooled to 4°C to inhibit further phagocytosis and intracellular killing. The cover slips were removed from the tubes and rinsed six times in saline to remove extracellular or nonadherent bacteria.

The cover slips were then inserted into tubes containing 10 ml of sterile water. Ultrasonic treatment of these tubes for 15 min in an ultrasonic bath, 100 W (Branson), and thorough mixing on a whirlmix under hypotonic conditions destroyed the PMN and resuspended the ingested or cell-associated microorganisms. The viability of bacteria was not affected by these procedures. The total amount of ingested bacteria was measured by determining the radiolabel of the cell lysate after passing the suspension through a membrane filter (0.2 μm; Millipore Corp.). The filters were washed, dissolved in a scintillation cocktail, and counted in a Nuclear-Chicago liquid scintillation counter.

The number of surviving bacteria was measured by colony counting from appropriate dilutions of the cell lysate after growing overnight on agar plates.

RESULTS

Initial experiments were performed to determine the specificity of the anti-*E. coli* O8 antiserum. The amount of specific immunoglobulin was measured by quantitative precipitation with purified *E. coli* O8 lipopolysaccharide. The activities of the IgG and IgM fractions were tested by indirect hemagglutination and bacterial agglutination (Table 1). IgG contained 26.2 mg of protein per ml with 3.6 mg of specific IgG per ml and was essentially pure in an Ouchterlony diffusion test. IgM had 13.8 mg of protein per ml with 1.6 mg of specific IgM per ml. The IgM preparation showed a minor contamination with IgG in the order of 1% of the total protein content.

In all the following experiments, antibodies of either class were used in a subagglutinating dilution of 1/20,000. Under these conditions, IgM proved to be 12-fold more efficient than IgG on a molar basis, as tested by bacterial agglutination (Table 1). Neither amount of antibody was sufficient to fully activate 4 CH₅₀ units in a standard C consumption test. Table 2 demonstrates in more detail the quantity of C which was consumed by activation with IgG, IgM, and P as determined in the indirect C fixation test.

Table 2 shows also the binding of C3 to bacteria as measured by the C3 binding assay. Al-

FIG. 2. Experimental procedure to measure phagocytosis and intracellular killing of bacteria.

TABLE 1. Properties and activity of rabbit anti-*E. coli* O8 immunoglobulins

Anti- <i>E. coli</i> O8	Protein (mg/ml)	Specific immunoglobulin (mg/ml)	HA ^a (×10 ³)	BA ^b (×10 ³)	Antibody molecules/ <i>E. coli</i> ^c
Antiserum	48.9	0.8	8-16	ND ^d	
Immunoglobulins	7.3	0.8	8-16	ND	
G	26.2	3.6	2-4	20	7,200
M	13.8	1.6	20	20	530

^a Hemagglutination titer.
^b Bacterial agglutination titer.
^c Assuming that all available specific antibodies were bound to 10⁸ bacteria.
^d ND, Not done.

though C3 bound unspecifically to unopsonized bacteria, a much higher C3 binding was obtained when the classical C activation was initiated with IgG or IgM. Addition of P, however, resulted in no increase of C3 binding.

Since the C source was normal human serum, it was necessary for a selective activation of C

by these specific antibodies to eliminate natural antibodies and P by immunoabsorption. Normal human serum was first applied to an immunoabsorption column coated with anti-human immunoglobulin and then to one exposing anti-P. Complete removal of immunoglobulin could be verified in the Ouchterlony diffusion test, and removal of P was demonstrated immunochemically by the lack of precipitation with anti-P and functionally by restoration of absorbed serum with P. In the absorbed serum, the activation of C could be initiated either by immunocomplexes consisting of *E. coli* O8 bacteria and specific antibody or by the addition of highly purified human P. This was shown by two different assays, as follows.

Figure 3 demonstrates the separate activation of C by the classical and by the alternative pathways by immunochemical methods. Both types of C activation were followed by the conversion of C3 to C3c as shown in the crossed immunoelectrophoresis. It became evident that only the alternative C activation induced the formation of the C3 activator. A possible gener-

TABLE 2. Activation of C

Human serum (C) incubated for 30 min at 37°C	CH ₅₀ units of human C			Anti-C3 bound ^a (%)
	Added	Re-cov-ered	Con-sumed	
No bacteria	4	3.9 ^b	0.1	0.5 ± 0.1
<i>E. coli</i>	4	3.5	0.5	3.4 ± 0.3
<i>E. coli</i> , IgG ^c	4	2.6	1.4	5.1 ± 0.5
<i>E. coli</i> , IgM	4	1.5	2.5	7.2 ± 0.3
<i>E. coli</i> , P	4	2.6	1.4	3.7 ± 0.2

^a Mean ± standard deviation of four separate tests.

^b Measured by the indirect C-fixation test.

^c *E. coli*, 10⁸/ml, were opsonized either with immunoglobulin at a dilution of 1/20,000 and 4 CH₅₀ units of C or with 5 μl of P, 1 mg of protein per ml, and 4 CH₅₀ units of C.

FIG. 3. Activation of C by the classical or alternative pathway. The left part demonstrates in the crossed immunoelectrophoresis the splitting of C3 in human serum deprived of immunoglobulins and P. C3 was only activated by the addition of immunocomplexes of *E. coli* and anti-*E. coli* antibodies or by *E. coli* and P. The right part demonstrates that activation of the C3 proactivator (C3PA) to the C3 activator (C3A) occurred only in the presence of P.

ation of the C3 activator by the classical pathway via a feedback mechanism (19) could not be detected, which, however, does not exclude its formation in concentrations too low to be measured by these methods.

In Table 3 the activation of C in absorbed and unabsorbed serum is shown in a functional assay by using its hemolytic activity as indicator. Addition of *E. coli* O8 to unabsorbed serum led to a C activation in which the involvement of both pathways could not be separated. Only the absorbed serum permitted the selective activation of C by the classical (*E. coli* + anti-*E. coli* antibody) or the alternative (*E. coli* + P) pathway.

After characterizing antibodies, P and C, their capacity to promote phagocytosis and intracellular killing was studied. In preliminary experiments it was found that the rate and extent of phagocytosis depended mainly on the ratio of bacteria to phagocytes, which confirms the results of others (2). In our experiments, a ratio of 1×10^6 opsonized bacteria to 4×10^6 adherent leukocytes proved to be optimal to determine reproducibly phagocytosis. Figure 4 shows the influence of opsonization on the uptake and killing of microorganisms by PMN after 20 min of incubation. In subagglutinating concentrations, IgG had a good opsonizing capacity, whereas IgM failed to induce phagocytosis beyond that obtained without opsonization. However, the addition of C increased opsonization with both immunoglobulins, exhibiting a particularly striking effect with IgM, in which case C led to a more than 10-fold increase of ingestion. The activation of C by the alternative pathway (P) had only a slight effect on the uptake of bacteria, which was, however, less than the opsonization with C alone.

To determine an additional effect of immu-

TABLE 3. Activation of C in human serum by the classical or alternative pathway

Human serum (C) incubated for 30 min at 37°C	Absorbed ^a (%)	Unabsorbed (%)
Control	100 ^b	100
P	96	97
<i>E. coli</i>	97	6
<i>E. coli</i> + P	8	5
<i>E. coli</i> + anti-tibody ^c	10	6

^a From the absorbed serum, immunoglobulins and P were removed by immunoabsorption. The total hemolytic activity was adjusted to the same titer in the absorbed and unabsorbed serum.

^b Percentage of hemolytic activity recovered.

^c Subagglutinating dilution of unfractionated anti-*E. coli* serum.

FIG. 4. Phagocytosis (total bars) of *E. coli* O8 by and survival (hatched bars) in human PMN (*n* indicates PMN of different human individuals). After various opsonization procedures, 1.0×10^6 *E. coli* O8 in 2 ml were fed to 4×10^6 PMN. The incubation was terminated after 20 min, and the amount of cell-associated bacteria was determined by the amount of radioactive label. The values are expressed as the mean $\pm$ standard deviation of the results obtained with the PMN of *n* individuals. Survival of ingested bacteria was determined by colony counting on agar plates.

noglobulins and C on the intracellular killing of bacteria, the survival of ingested microorganisms was measured under the same conditions as described above. By using colony counting on agar plates, the number of surviving bacteria after 20 min of phagocytosis differed markedly, as shown in Fig. 4. Unopsonized bacteria and those opsonized with IgM or with P and C were little phagocytized, and consequently, little killing occurred. Although IgG alone enhanced phagocytosis, the intracellular killing was much less when compared with the circumstances in which activation of C occurred on IgG- or IgM-coated bacteria (Table 4). It became apparent that activation of C by IgM or IgG resulted in a 98% or 95% killing, respectively. In contrast, an activation of C by the alternative pathway showed no enhanced killing of bacteria.

DISCUSSION

The participation of antibodies and C in phagocytosis and killing of bacteria by PMN has been

TABLE 4. *Effect of various opsonins on the killing of E. coli by PMN*

<i>E. coli</i> opsonized ^a with:	No. of <i>E. coli</i> killed ^b ($\times 10^4$)
$\emptyset$	2.7 $\pm$ 2.2
IgG	21.9 $\pm$ 10.1
IgG + C	58.6 $\pm$ 19.2
IgM	1.6 $\pm$ 1.2
IgM + C	73.5 $\pm$ 17.6
C	26.2 $\pm$ 13.5
C + P	6.2 $\pm$ 2.3

^a Opsonization as described in Table 2.^b Difference between ingested and surviving bacteria as shown in Fig. 4.

studied in various experimental systems (13, 20, 27). In our investigation, cellular monolayers were employed for several reasons. First, white cell suspensions from venous blood usually contain only 70% PMN. By the formation of a monolayer, PMNs could be enriched to over 90%, since nonadhering lymphocytes could be washed off. Second, phagocytosing cells in suspension adhere to each other and easily form cell clumps together with bacteria. Preliminary experiments showed that, particularly at high rates of ingestion, these clumps contained an undefinable number of cells and bacteria, which did not permit an exact quantitation of both phagocytosis and killing. Third, cellular monolayers offer the additional advantage of an easier separation of cell-associated bacteria from free bacteria by vigorous washing procedures. However, this system demanded a gross excess of offered bacteria in the supernatant to get measurable amounts of bacteria in contact with PMN for engulfment. The extent of phagocytosis, i.e., the percentage of ingested bacteria calculated from the total amount of added bacteria, was therefore less than 2%.

In this investigation, an effort was made to delineate a possible cooperation of humoral factors in the postphagocytic phase, i.e., during intracellular killing of ingested bacteria. We are aware of the difficulty in most phagocytosis experiments that all so-called phagocytosed bacteria—even after extensive washings of the leukocytes—are truly intracellular. From further studies, however, we know that more than 70% of the phagocytosed bacteria are closely associated with the granular fraction of PMN (manuscript in preparation). The study was facilitated by the availability of pure immunoglobulins, P, and a C source rendered free of immunoglobulins by immunoabsorption.

Our phagocytosis experiments using various immunoglobulins and C confirmed results of others (5, 22–24). IgG proved to be a good opsonin, as demonstrated by the more than 10-fold

increase of bacteria ingestion when compared with nonopsonized bacteria (Fig. 4). In contrast, a poor opsonic effect could be demonstrated with IgM alone, resulting in phagocytosis which was not significantly different from the one obtained with nonopsonized bacteria. The most likely explanation is a functional deficiency of IgM receptors on granulocytes to induce phagocytosis (12, 24, 30). A completely different picture evolved when both immunoglobulins were combined with C. With IgG, a moderate enhancement of bacteria uptake became apparent. In contrast, addition of C to IgM-opsonized bacteria strikingly increased their ingestion by PMN.

The enhancing effect of activated C became even more apparent when its influence on intracellular killing was examined. Whether bacteria were opsonized with IgG or with IgM, in both cases the presence of activated C markedly augmented intracellular killing when compared with the effects of either immunoglobulin alone (Fig. 4 and Table 4). In particular, when IgM was employed which itself lacked a significant opsonic potency, an almost complete intracellular killing was detected in the presence of C.

To assess further the role of C in phagocytosis and intracellular killing, C was also activated by the alternative pathway (11, 19). The basis was the assumption that in the case of a bacterial infection, an antibody-independent C activation could provide a first line of defense before the advent of specific antibodies. Although an opsonic activity of products of the alternative C activation has been documented previously (3, 4, 29), an increase of phagocytosis and intracellular killing was observed only in the presence of purified C, whereas the combination of P with C proved to be inefficient when compared with controls (Fig. 4). At present no explanation can be forwarded to interpret the opsonizing effect of added purified C. Although the alternative pathway of C activation can be initiated in the absence of P (20), no information is available concerning the opsonizing qualities of these C products and their affinity for bacterial surfaces. Our results indicate that a low activation of C by antibody-free bacteria may either generate more efficient and stable opsonins or may affect their deposition on bacteria.

An attempt to quantitate the amount of C3 bound to differently treated bacteria was only partly successful (Table 2). Although bacteria in the absence of immunoglobulins bound a substantial amount of C3, a higher binding was obtained when IgG or IgM was added. However, with the C3 binding assay employed here, it is not possible to differentiate between opsonically active C3c, inactive C3d, or unspecifically bound

C3. Since deposition of C3 alone on the surface of particles does not promote phagocytosis (22), one could only speculate that the higher IgG- or IgM-induced C3 deposition reflected attachment of the opsonically active form of C3.

Details of the mechanism by which C, activated by the classical pathway, enhance phagocytosis and intracellular killing are still unknown. In favor of a combined action of immunoglobulins and activated C on the bacterial surface (6) are experiments in which *Salmonella typhi* were opsonized either directly by anti-*S. typhi* antibodies or indirectly by antiphage antibodies using phage-covered bacteria. Only the former were killed following phagocytosis, whereas the latter multiplied rapidly (14). These observations would imply the necessity of a direct C activation on the bacterial surface. By which mechanisms activated C on bacterial surfaces promotes more efficient intracellular killing remains unknown. It may be speculated that fusion of lysosomes with phagosomes is facilitated by bacteria-bound C3b or C5b. Also, an enhancing effect of activated C on various intracellular bactericidal systems may be possible.

The poor effect of the alternative C activation on phagocytosis and intracellular killing remains a puzzling finding. It is conceivable that the alternative C activation may lead to a more random distribution of C3 on the surface of the bacterium than the classical activation where clusters of C3 have been demonstrated (18). This assumption would indicate a decisive role for activated C only in the case of a local accumulation and in combination with IgG or IgM.

Our results clearly indicate the importance of the classical C activation for phagocytosis and intracellular killing. Moreover, evidence has been presented that low amounts of IgM are sufficient in the presence of activated C to initiate efficient phagocytosis and destruction of bacteria. These findings may be of particular relevance during the early phases of an infection where immunoglobulins are mainly of the M class.

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