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Research Article

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Requirement of Extracellular Complement and Immunoglobulin for Intracellular Killing of Micro-Organisms by Human Monocytes

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ABSTRACT The role of serum factors in the intracellular killing of bacteria by monocytes was studied on the basis of an assay independent of phagocytosis. After 3 min of phagocytosis of preopsonized bacteria and removal of noningested bacteria, the monocytes containing bacteria are reincubated for various periods and the number of unkilled bacteria is determined by a microbiological method after lysis of the cells.

Evidence that this assay measures the killing of ingested bacteria was provided by scanning electron microscopy, lysostaphin treatment, and the effect on the rate of intracellular killing of inactivated serum lacking specific opsonic activity.

Intracellular killing of *Staphylococcus aureus*, *S. epidermidis*, and *Escherichia coli* by human monocytes does not occur or is low in the absence of serum, and maximal killing is only reached when fresh serum is present; intermediate values are obtained in the presence of heat-inactivated serum. These findings indicate that complement stimulates intracellular killing.

Isolated heterogeneous immunoglobulin (Ig)G, pFc fragments of heterogeneous IgG, and both IgG_1 and IgG_3 stimulate intracellular killing of *S. aureus* by monocytes to the same degree as heat-inactivated serum. Sphingomyelinase, which decreases the number of Fc receptors, and neuraminidase, which increases these receptors, respectively, decreased and increased the intracellular killing, whereas anti-monocyte serum completely abolished the stimulation of intracellular killing by inactivated serum. These results prove that interaction of the Fc receptor with the Fc part of IgG is required for the intracellular killing.

Inhibition of the activation of complement components via the alternative pathway gave a considerable reduction in the intracellular killing of *S. aureus*; impairment of the activation via the classical pathway had no effect. The addition of complement components to heat-inactivated serum showed that intracellular killing is maximal only when C3b is generated. Reduction of the number of C3b receptors in the membrane by trypsin or pronase decreased intracellular killing in the presence of fresh serum; anti-monocyte serum completely abolished the stimulation of intracellular killing by fresh serum. These results lead to the conclusion that intracellular killing is also dependent on the interaction between C3b and its receptor in the membrane.

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INTRODUCTION

The process of phagocytosis of particles by phagocytic cells has two phases: attachment of the particle to the cell membrane, followed by ingestion of the particle. Particles opsonized by IgG with or without C3 or immunoglobulin (Ig)M together with C3 are recognized by the Fc and C3 receptors on the cell membrane (1–5). After ingestion, most bacteria species are readily killed intracellularly.

The rate of intracellular killing is usually measured at the same time as the rate of phagocytosis in vitro and expressed as the difference between the total initial number of viable intracellular plus extracellular bacteria and the number of viable extracellular plus cell-associated bacteria (6–8). This combined assay of phagocytosis and intracellular killing has the disadvantage that the factors with an influence on intracellular killing cannot be studied apart from their effect on the phagocytis process.

In the present study the method generally used to measure the intracellular killing was modified to permit determination of the rate of this process separately

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from the rate of phagocytosis. This method makes it possible to study the factors influencing the intracellular killing by phagocytes. The results show that intracellular killing of various bacteria species by human monocytes is dependent on extracellular stimulation by several serum factors.

METHODS

Reagents. Stock solutions of 0.1 M EDTA (Sigma Chemical Co., St. Louis, Mo.) and of 0.1 M EGTA (Sigma Chemical Co.) containing 25 mM $MgCl_2 \cdot 6H_2O$ were prepared with Hanks' Balanced Salt Solution (HBSS)[1] adjusted to pH 7.2 with dilute NaOH, and stored at 4°C for at most 14 d. Solutions of human albumin (Behringwerke A. G., Marburg, West Germany), pronase (Merck, Darmstadt, West Germany), trypsin (Sigma Chemical Co.), and trypsin inhibitor (Sigma Chemical Co.) were prepared with phosphate-buffered saline (PBS) immediately before use. Stock solutions of sphingomyelinase C (kindly donated by Dr. P. Wilkinson, Department of Bacteriology and Immunology, University of Glasgow, Scotland), neuraminidase (*Vibrio cholerae*, Behringwerke A. G.), and lysostaphin (Sigma Chemical Co.) were prepared in PBS and stored at −20°C. The phenylbutazone (Butazolidine, Ciba-Geigy, Bern, Switzerland) solution was prepared with HBSS just before use.

Monocytes. Monocytes were collected by differential centrifugation of blood from healthy donors on Ficoll-Hypaque gradients (Ficoll, Pharmacia, Inc., Uppsala, Sweden; Hypaque, Winthrop Laboratories, Sterling Drug, Inc., New York) (9). The interface layer containing monocytes and lymphocytes was washed four times with PBS containing 0.5 U/ml heparin, and a cell suspension of $5–6 \times 10^6$ cells/ml in HBSS with 0.1% (wt/vol) gelatin was prepared. The percentage of monocytes in this suspension, which was determined in a Giemsa-stained cytocentrifuge preparation (10), amounted to 35±8 (mean±SD), and the percentage of granulocytes was <2. Viability of the suspended cells was checked in the experiments by trypan-blue exclusion and amounted to >95%. To prevent attachment of monocytes to the glass surface, all glassware was siliconized.

Micro-organisms. *S. aureus* (type 42D), *S. epidermidis* (serotype 4), and *E. coli* (054) were stored on agar slants at 4°C and transferred every 14 d. The micro-organisms were cultured overnight in Nutrient Broth No. 2 (Oxoid Ltd., London, England), harvested by centrifugation at 1,500 *g* for 10 min, and washed twice with PBS. A suspension containing 10^7 bacteria/ml was prepared in HBSS with 0.1% (wt/vol) gelatin.

Preopsonization of bacteria. Preopsonized micro-organisms were obtained by incubation of the suspensions (5×10^6 bacteria/ml) with 10% (vol/vol) AB serum for 25 min at 37°C under slow rotation (4 rpm) followed by centrifugation at 1,500 *g* for 10 min and 2 washings in ice-cold gelatin-HBSS.

The bacteria were then suspended in gelatin-HBSS to a concentration of $\cong 10^7$/ml. The presence of immunoglobulin on the surface of the bacteria was checked by an immunofluorescence procedure with fluorescein-labeled specific anti-human IgG and anti-human IgM sera (Nordic, Tilburg, The Netherlands). The opsonic activity of serum or serum fractions was determined as described elsewhere (10).

Intracellular killing assay. First, a suspension of 10^7 monocytes/ml was incubated with an equal volume of 10^7 preopsonized bacteria/ml for 3 min at 37°C under rotation (4rpm) in the absence of serum. Phagocytosis was stopped by shaking the tube through crushed ice, and the noningested bacteria were removed by differential centrifugation for 4 min at 110 *g*. Next, the cells were washed twice with ice-cold PBS containing 0.5 U/ml heparin to remove all free extracellular bacteria and any bacteria attached to monocytes. The cells were then resuspended in gelatin-HBSS to a concentration of 5×10^6 monocytes/ml and reincubated at 37°C under rotation (4 rpm) in the presence or absence of serum according to the experimental design. After various periods of reincubation, intracellular killing was stopped by adding 0.5 ml ice-cold HBSS to 0.5 ml of the cell suspension, after which the cells were spun down for 4 min at 110 *g*, the supernate was removed, and 1.0 ml distilled water containing 0.01% (wt/vol) bovine albumin was added to the cell pellet. Lysis of the monocytes was obtained by alternately freezing the suspension with liquid nitrogen (−170°C) and quickly thawing it in a waterbath (+37°C) three times. This procedure did not affect the viability of bacteria. Serial 10-fold dilutions in saline were made over a range assuring that at least one dilution will contain between 100 and 1,000 viable bacteria per ml. Aliquots (0.1 ml) of the three highest dilutions were pipetted onto each of two DST agar plates (Diagnostic Sensitivity Medium Agar, Oxoid Ltd.), the plates were incubated at 37°C for 18–24 h, and the number of colonies counted with a colony counter. The number of viable bacteria per ml was calculated from the means of the colony counts of duplicate plates of the two highest dilutions, providing the plate contained <500 colonies (10).

Serum. Serum prepared from the blood of healthy donors with bloodgroup AB was used in all experiments. The blood was clotted for 1 h at room temperature, centrifuged at 1,100 *g* for 20 min, and stored in 2-ml aliquots at −20°C for maximally 2 mo until use.

Adsorption of serum opsonins. Heat-inactivated serum (30 min at 56°C) was adsorbed by incubating 1 ml serum with 10^{10} *S. aureus* washed 3 times with PBS and concentrated by centrifugation (1,500 *g*). This procedure was repeated five times, after which the serum had no opsonic activity for *S. aureus* (as tested by a phagocytic assay [10]). This procedure decreased the concentration of IgG, IgM, and IgA by only 21, 28, and 19%, respectively, as determined by the single radial immunodiffusion method (11). The same adsorption method was used to prepare sera without opsonic activity for *S. epidermidis* and *E. coli*.

Immunoglobulins. IgG preparations devoid of IgM were prepared by batch-wise chromatography of AB serum on DEAE cellulose (DE 52, Whatmann, Biochemicals, Kent, England), according to Reif (12) or gel filtration, twice, on Sephadex G-200 (Pharmacia, Inc.), and concentrated by freeze-drying. The IgG concentration of this preparation was measured by the single radial immunodiffusion method according to Mancini et al. (11); the IgG preparation contained no aggregated IgG as shown by ultracentrifugation. pFc and $(Fab^1)_2$ fragments of human heterogeneous IgG were prepared by pepsin digestion according to Nisonoff et al. (13). These fractions no longer contained any IgG or aggregates as checked by ultracentrifugation. Pure preparations of the subclasses IgG_1, IgG_2, IgG_3, and IgG_4, isolated from sera with a high concentration of the respective paraproteins, were kindly donated by Dr. P. Goosen (Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands). Monoclonal IgM was kindly donated by Dr. F. Klein (Dept. of Epidemiology, Erasmus University, Rotterdam, The Netherlands). The preparation of this material is described elsewhere (14). Isolated IgE prepared from myeloma P.S. (originally obtained from Dr. K. Ishizaka) was kindly pro-

[1] *Abbreviations used in this paper*: HBSS, Hanks' balanced salt solution; PBS, phosphate-buffered saline.

vided by Dr. G. Delespesse (St. Pieters Hospital, Brussels, Belgium).

The purity of the preparations was checked by immunodiffusion according to Ouchterlony (15) and by immunoelectrophoresis performed with anti-human serum, anti-IgG, IgM, anti-IgA serum, and anti-IgG subclass sera (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam).

Complement-deficient sera and complement components. AB serum was inactivated by incubation at 56°C for 30 min, which resulted in total elimination of the hemolytic activity, a 99% decrease of complement component C1, 100% of C2, 48.1% of C3, 50.7% of C4, 47.9% of C5, and 100% of factor B activity, as compared with freshly frozen untreated AB serum. The various activities were measured as described below.

A serum deficient in factor D was prepared by gel filtration of 10 ml fresh AB serum on a 2.5 × 95-cm Sephadex G-75 superfine column in 0.01 M veronal-buffered saline containing 0.02 M EDTA (pH 7.5). The protein peak that appeared in the void volume of the column was concentrated by ultrafiltration (UM 10 membranes, Amicon Corp., Lexington, Mass.) to its original volume and reconstituted with Ca^{2+} and Mg^{2+} by dialysis against 0.01 M veronal-buffered saline containing calcium and magnesium. The absence of factor D activity in this preparation was tested by its capacity to sustain lysis of rabbit erythrocytes in the presence of 10 mM EGTA, as described elsewhere (16).

The methods used to prepare isolated complement component C3 and factors B and D have been described elsewhere (17–19). The purity of these preparations was assessed by alkaline disc electrophoresis in which each factor shows a single stained band corresponding to the position from which the functionally active protein was eluted from a replicate unstained gel. The protein concentrations of the factor B and C3 preparations were determined by the single radial immunodiffusion method, and that of the factor D preparation according to Lowry et al. (20) with human serum albumin as the reference standard. A preparation of guinea pig C1 (21) and a human C2 preparation (22) were made as described elsewhere, and the purity was assessed by functional tests (21, 22).

Anti-monocyte serum. A serum against monocytes was prepared by Dr. Brutel de la Rivière (Binnengasthuis, Amsterdam) by immunization of rabbits with monocytes from a patient with chronic monocytic leukemia, and adsorbed with platelets, erythrocytes, granulocytes, and lymphocytes as described elsewhere (23).

Receptors. The presence of Fc and C3 receptors on the monocytes was investigated with IgG-coated sheep erythrocytes and IgM- and complement-coated sheep erythrocytes as described elsewhere (24).

Scanning electron microscopy. Scanning electron microscopy was used to investigate monocytes after ingestion of *S. aureus* (bacteria:monocyte ratios of 1:1 and 10:1) for 3 min at 37°C. The monocytes were washed as described for the intracellular killing assay, sedimented at 1 g on silver grids fixed in 1.5% (vol/vol) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at 0–4°C, and dehydrated in graded solutions of ethanol. The samples were then critical point dried at room temperature and coated with a thin layer of gold (25). In each preparation at least 50 monocytes were examined.

Calculations. All values represent the mean of at least three experiments. Intracellular killing at a given time-point is expressed as the percentage decrease of the initial number of viable intracellular bacteria according to the following formula: $K(t) = (1 - N_t/N_0) \times 100$, in which K(t) is the intracellular killing index at time t, N_0 is the number of viable intracellular bacteria at time 0 (i.e., after 3 min phagocytosis of bacteria and subsequent washing of the cells to remove extracellular bacteria), and N_t is the mean number of viable intracellular bacteria at time t. Statistical analysis was performed with Student's *t* test. The mean values and standard deviations are given in the text and tables.

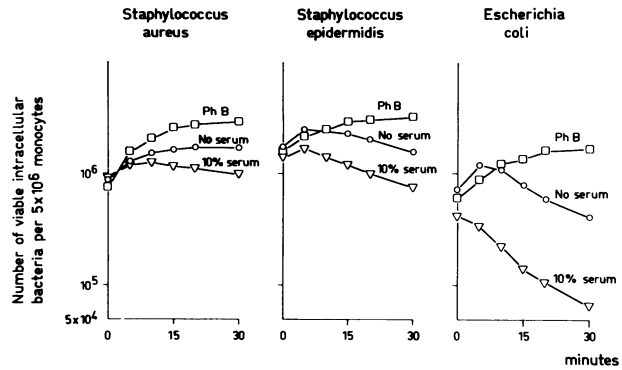

FIGURE 1 Course of the number of viable intracellular bacteria during phagocytosis of preopsonized bacteria (○), preopsonized bacteria plus 10% serum (▽), and preopsonized bacteria in the presence of 1 mg/ml phenylbutazone (PhB) and 10% serum (□). Bacteria:monocyte ratio of 1:1; bacteria present during the entire period of phagocytosis.

RESULTS

Number of viable cell-associated bacteria after ingestion. During the ingestion of preopsonized microorganisms by monocytes in medium without serum, the number of viable cell-associated bacteria increased rapidly during the first 5 min and then leveled off (*S. aureus*) or decreased (*S. epidermidis* and *E. coli*) (Fig. 1). Preopsonized bacteria ingested in the presence of 10% AB serum showed the same initial increase in the number of viable cell-associated *S. aureus* and *epidermidis*, but after the first 5 min the number of viable cell-associated bacteria decreased; the number of viable intracellular *E. coli* decreased from the start of the experiment onward (Fig. 1). These results indicate that intracellular killing is higher in the presence than in the absence of serum in the medium.

When preopsonized bacteria were phagocytosed in the presence of 1 mg phenylbutazone/ml, which has no effect on the rate of phagocytosis (26) (Unpublished results) but inhibits the intracellular killing by granulocytes and monocytes (26, 27), the number of viable cell-associated bacteria continued to increase (Fig. 1).

These results show that within 5 min of phagocytosis of bacteria at a bacteria:monocyte ratio of 1:1, the maximal number of viable cell-associated bacteria is reached. To obtain information on the number of bacteria killed within this initial 5-min period, the number of micro-organisms phagocytosed was determined. Table I shows that most of the bacteria phagocytosed

within this period were present intracellularly, the loss of viable bacteria amounting to only 10–30%. Although all handling of the cells after the ingestion period was performed at 4°C, some of the bacteria adherent to the monocyte surface might be ingested during the washing procedure and some already-ingested bacteria might be killed intracellularly. This could explain why the recovery (Table I) was not complete. In view of these findings, subsequent studies on intracellular killing were done with monocytes that had ingested preopsonized bacteria for only 3 min.

Demonstration of the absence of extracellular bacteria during the killing assay. To find out whether all bacteria included in the determinations were intracellular or some were still attached to the monocytes extracellularly at the beginning of the killing assay, several control experiments were carried out. For practical reasons, these were only performed with *S. aureus*.

To make certain that two washings of the monocytes are sufficient to remove the extracellular bacteria, a third washing was performed and the number of bacteria in the washing fluid was determined. After phagocytosis of preopsonized *S. aureus* at bacteria: monocyte ratios of 1:1 and 10:1, the number of viable bacteria in this washing fluid amounted to only 4.3% (range, 3.0–6.2%) and 5.6% (range, 3.9–8.3%) of the total number of viable cell-associated bacteria, in the cell pellet, respectively.

When monocytes that had ingested *S. aureus* at bacteria:monocyte ratios of 1:1 or 10:1 for 3 min were incubated with HBSS for 5 min, the number of viable bacteria determined after lysis of the monocytes amounted to $0.9–1.3 \times 10^6$/ml and $7–9 \times 10^6$/ml, respectively. When these monocytes that had ingested bacteria (at bacteria:monocyte ratios of 1:1 and 10:1) were incubated in the presence of 10 μg lysostaphin/ml, either under rotation (4 rpm) or stationary, the numbers of viable bacteria determined after lysis of the monocytes were $0.8–1.1 \times 10^6$/ml and $5–7 \times 10^6$/ml, respectively, which means a decrease of 12 (range, 5–20%) and 21% (range, 14–26%) in the number of viable bacteria.

Because lysostaphin kills *S. aureus* very efficiently (28) (incubation of 5×10^6 *S. aureus* with 10 μg lysostaphin/ml for 5 min at 37°C gave a reduction to 10^3 bacteria), these results indicate that at the beginning of the killing assay, after phagocytosis with a bacteria: monocyte ratio of 1:1, ≅12% of the bacteria are attached to the surface of the monocytes.

To rule out trapping of bacteria by lymphocytes, cell suspensions comprising >90% lymphocytes obtained from two patients with chronic lymphatic leukemia, were incubated with *S. aureus* (bacteria:cell ratio of 1:2) for 3 min. After washing, the cell pellets contained 1.0 and 2.5×10^5 bacteria/ml; and after lysostaphin treatment, only $0.5–1.0 \times 10^3$ bacteria/ml. These results indicate that after lysostaphin treatment hardly any bacteria are still attached to lymphocytes.

Scanning electron microscopy showed no extracellular bacteria on monocytes in suspensions studied after phagocytosis of *S. aureus* (bacteria:monocyte ratio of 1:1). Because after 3 min of phagocytosis at a bacteria:monocyte ratio of 1:1 only 26% of the monocytes had phagocytosed bacteria (Table II), these experiments were also performed with monocytes after ingestion of *S. aureus* at a bacteria:monocyte ratio of 10:1, which resulted in a mean of 71% monocytes with ingested bacteria (Table II). The scanning electron-microscopical picture of monocytes after phagocytosis at a bacteria:monocyte ratio of 10:1 showed that in each of two experiments in which 100 cells were examined, only 3 and 5 cells showed 1, 2, or 3 bacteria attached to the cell surface. In a monocyte suspension that had not been washed after phagocytosis, 30–40% of the cells had ≅8 bacteria on the surface. After incubation of such an unwashed cell suspension with 10 μg lysostaphin/ml, scanning electron microscopy showed no cells with attached bacteria. From the results of the

TABLE I
*Recovery of Viable Intracellular Bacteria after 3 min of Phagocytosis**

Phagocytozed micro-organisms	No. of bacteria phagocytozed by 5×10^6 monocytes‡	No. of bacteria within 5×10^6 monocytes§	Recovery of viable intracellular bacteria
	$\times 10^6$	$\times 10^6$	%
S. aureus	1.1–1.5	0.9–1.4	82–90
S. epidermidis	1.7–2.3	1.4–1.6	70–82
E. coli	1.0–1.9	0.7–1.3	68–70

* Phagocytosis performed at a bacteria:monocyte ratio of 1:1.
‡ Determined as a decrease in the number of viable bacteria in supernate.
§ Determined in the lysate of washed monocytes.

TABLE II
*Monocytes Containing Ingested Bacteria after 3 Min of Phagocytosis**

Phagocytozed micro-organisms	Phagocytosis at bacteria:monocyte ratio 1:1	10:1
	%	
S. aureus	26	71
S. epidermidis	41	88
E. coli	20	72

* Determined in Giemsa-stained preparations.

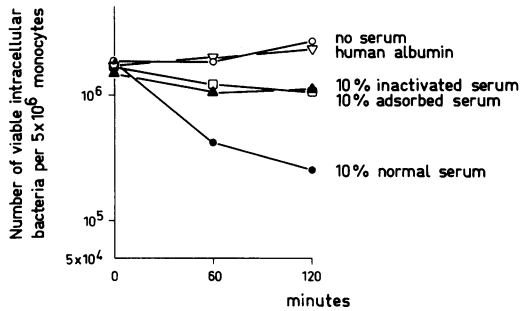

FIGURE 2 Intracellular killing by human monocytes after 3 min ingestion of preopsonized *S. aureus*. Bacteria:monocyte ratio of 1:1. The intracellular killing assay was performed without serum (○), in the presence of 10% normal serum (●), 10% heat-inactivated serum (▲), 10% adsorbed serum (□), or 2 mg/ml human albumin (▽).

TABLE III
*Effect of Serum on the Intracellular Killing of S. Aureus by Monocytes**

Incubation of monocytes containing bacteria with serum	Intracellular killing‡		No. of experiments
	60 min	120 min	
%	%	%	
0	−6.0±21.0	−65.0±31.1	20
1.0	31.3±10.8	14.1±30.3	4
2.5	64.1±15.0	73.2±21.5	4
5.0	74.0±12.7	85.1±12.3	4
10.0	75.3±9.5	83.5±11.1	25
25.0	76.1±13.8	81.3±10.8	3

* After phagocytosis at a bacteria:monocyte ratio of 1:1 for 3 min at 37°C.

‡ Expressed as $K(t) = (1 - N_t/N_0) \times 100\%$ (see Methods).

experiments in which a bacteria:monocyte ratio of 10:1 was used, it may be calculated that for a monocyte-lymphocyte suspension of 1.4×10^7 cells/ml of which an average 4% carry maximally 3 bacteria on their surface, no more than 1.7×10^6 bacteria/ml are extracellular. Because after lysis of the washed monocyte-lymphocyte suspension $8–9 \times 10^6$ viable bacteria were found, at most 21% of the bacteria in this suspension will be extracellular.

From these results it may be concluded that the number of viable bacteria measured after lysis of the monocytes represents mainly ingested micro-organisms; a maximum 20% of the bacteria will lie attached to the surface of the monocytes.

Effect of serum on the intracellular killing of S. aureus. The effect of serum on the intracellular killing of *S. aureus* was determined after phagocytosis of bacteria:monocyte ratios of 1:1 and 10:1. Figs. 2 and 3 show that the intracellular killing of *S. aureus* is dependent on the presence of serum. After phagocytosis at a bacteria:monocyte ratio of 1:1, the decrease in the number of viable intracellular bacteria amounted to 75.3±12.2% at 60 min and to 83.5±11.2% at 120 min, when the killing assay was performed in the presence of 10% serum. Incubation of monocytes containing bacteria in the absence of serum gave no decrease in the number of viable intracellular bacteria at 60 min and even an increase at 120 min. Incubation of monocytes in the presence of 1.0 or 2.5% serum gave intermediate values (Table III).

Incubation of monocytes after phagocytosis of preopsonized micro-organisms at a bacteria:monocyte ratio of 10:1 gave similar results: without serum, almost no intracellular killing was observed (15.3±15.7%), whereas with 10% serum, killing amounted to 60.6 ±13.4% at 60 min and 59.5±12.9% at 120 min of incubation (Fig. 3).

To make certain that this effect was the result of the serum and not to phagocytosis of the remaining attached bacteria, a killing assay was performed after removal of these extracellular preopsonized bacteria by incubation of the monocytes with 10 μg lysostaphin/ml for 5 min. Fig. 3B shows that after such treatment the effect of serum on the intracellular killing was the same as without. Incubation of monocytes with ingested bacteria in the presence of 10% adsorbed serum, which no longer had any opsonic activity, gave the same decrease in the number of viable intracellular bacteria as was obtained by incubation of these cells with inactivated serum (Fig. 2). Thus, we may conclude that the effect of serum on intracellular killing is not because of phagocytosis of attached preopsonized extracellular bacteria.

When monocytes that had ingested preopsonized bacteria were incubated without serum for 30 or 60 min, no killing occurred, but this process started immediately after the addition of 10% serum and to the

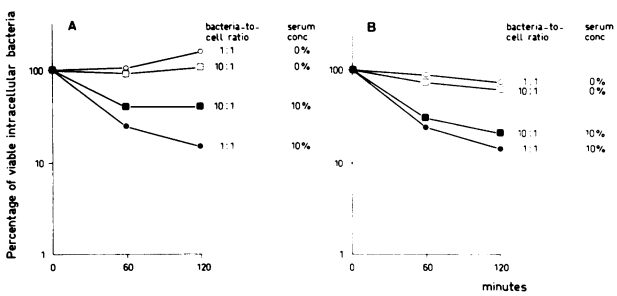

FIGURE 3 (A) Intracellular killing of bacteria by human monocytes in the presence or absence of serum (conc, concentration) after phagocytosis of preopsonized *S. aureus* by monocytes for 3 min at bacteria:monocyte ratios of 1:1 and 10:1. (B) As for A, but after exposure of monocytes with ingested bacteria to 10 μg lysostaphin/ml for 5 min at 37°C before the start of killing assay.

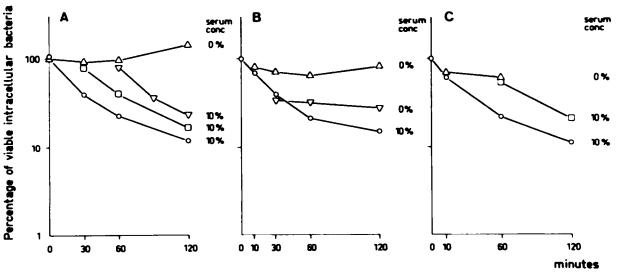

FIGURE 4 Effect of the presence of serum (conc, concentration) on the rate of intracellular killing of preopsonized *S. aureus* by human monocytes, determined after phagocytosis at a bacteria:monocyte ratio of 1:1. (A) Effect of the addition of serum. Incubation of monocytes containing bacteria in the absence of serum did not lead to intracellular killing (△). Serum was added after 30 or 60 min of incubation without serum (□, ▽). (B) Effect of the removal of serum. After incubation in the presence of serum (○), during 10 or 30 min the monocytes were washed and reincubated in a medium without serum (△, ▽). (C) Effect of the presence of serum. After incubation of monocytes containing bacteria in the presence of serum (○) for 10 min, the monocytes were washed and reincubated for 50 min without serum (△), and at 60 min fresh serum was added (□).

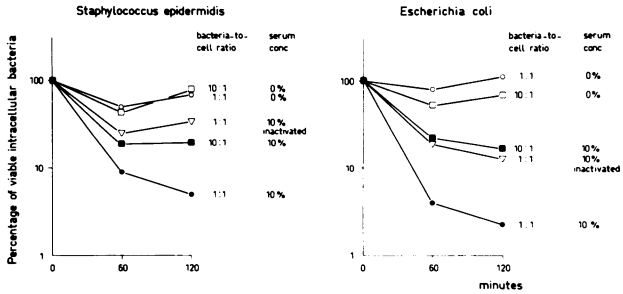

FIGURE 5 Effect of serum (conc, concentration) on the intracellular killing of preopsonized *S. epidermidis* and preopsonized *E. coli* by human monocytes. Phagocytosis was performed at bacteria:monocyte ratios of 1:1 and 10:1.

same degree as when serum was added at time 0 (Fig. 4A). When intracellular killing by monocytes was allowed to continue for 10 or 30 min in the presence of serum before the cells were washed twice with ice-cold HBSS and then reincubated in the absence of serum, the intracellular killing ceased completely as soon as the serum was removed (Fig. 4B). Monocytes containing bacteria were incubated in the presence of serum for 10 min, washed, and then reincubated without serum for another 50 min before serum was added again. Intracellular killing resumed as soon as serum was present extracellularly (Fig. 4C). From these findings it may be concluded that extracellular serum has an effect on the process of intracellular killing.

TABLE IV
Effect of Serum and Chelating Agents on the Growth of S. Aureus at 37°C

Treatment of bacteria	Incubation in the presence of	No. of bacteria at 0 min	60 min	120 min
		× 10^6/ml	× 10^6/ml	× 10^6/ml
Preopsonization	10% Serum	2.1	2.5	5.0
Preopsonization, 3 min phagocytosis and lysis of monocytes	10% Serum	1.6	1.6	3.8
Preopsonization	10% Serum + 10 mM EDTA	1.5	1.6	0.6
Preopsonization	10% Serum + 10 mM EGTA, 2.5 mM $MgCl_2$	1.8	1.8	2.4

To check whether serum has a direct effect on the ingested preopsonized bacteria, bacteria obtained by the lysis of monocytes after 3 min of phagocytosis were incubated in HBSS containing 10% serum. The number of bacteria did not decrease during an incubation period of 120 min but roughly doubled (Table IV). Similar results were obtained when preopsonized bacteria were incubated with 10% serum (Table IV).

Effect of serum on the intracellular killing of S. epidermidis *and* E. coli. To find out whether the effect of serum on the intracellular killing observed for *S. aureus* is a more general phenomenon, other microorganisms were studied. Fig. 5 shows the decrease in the number of viable intracellular bacteria when monocytes with other preopsonized bacteria species ingested during 3 min of phagocytosis at bacteria:monocyte ratios of 1:1 and 10:1 were incubated in the presence or absence of serum. When serum was present the intracellular killing of *S. epidermidis* and *E. coli* was more rapid than without serum, as was also found for *S. aureus*. As for *S. aureus*, the killing index in the presence of adsorbed serum, showing no opsonic activity for either *S. epidermidis* or *E. coli*, was similar (71.3 and 79.3%, respectively) at 60 min to that found for nonadsorbed inactivated serum. From these experiments it may be concluded that the intracellular killing of *S. epidermidis* and *E. coli* by monocytes is dependent on the presence of serum too.

Effect of various immunoglobulins and IgG fragments on the intracellular killing of S. aureus. Incubation of monocytes with ingested bacteria in the presence of IgM (concentrations of 120–500 μg/ml) or IgE (concentrations of 10^{-2} up to 10 μg/ml) showed that no intracellular killing occurred during the 1st h of incubation and there was even an increase in the

TABLE V

Effect of Immunoglobulins on the Intracellular Killing of S. Aureus by Monocytes after Phagocytosis at a Bacteria:Monocyte Ratio of 1:1

Incubation of monocytes containing bacteria with	Concentration	Intracellular killing at* 60 min	Intracellular killing at* 120 min	No. of experiments
	μg/ml	%		
IgG	210	26.3±7.8	−3.0±18.2	5
IgG	425	32.2±8.6	9.0±13.5	6
IgG	850	30.1±13.1	19.6±7.9	5
pFc	50	26.5±7.8	−2.0±15.3	3
pFc	100	32.0±13.5	−9.0±13.2	3
pFc	200	34.4±7.4	19.0±11.1	3
$(Fab')_2$	200	7.1±6.3	−7.2±19.1	3
$(Fab')_2$	400	6.3±9.7	10.1±12.2	3
$(Fab')_2$	600	0.3±14.3	5.4±13.2	3
IgG_1	500	32.2±9.1	ND‡	3
IgG_2	500	7.6±7.2	ND	3
IgG_3	500	25.7±11.3	ND	3
IgG_4	500	6.0±5.6	ND	3

* Expressed as $K(t) = (1 - N_t/N_0) \times 100\%$ (see Methods).
‡ND, not done.

number of viable intracellular bacteria during the 2nd h of incubation.

Incubation of monocytes with ingested bacteria in the presence of various concentrations of IgG showed that after 60 min the number of bacteria decreased by ≅30% (Table V). The effect of pFc fragments of heterogeneous IgG, which had been demonstrated to lack opsonic activity for *S. aureus*, on intracellular killing proved to be similar to that of IgG ($P > 0.1$) (Table V). Incubation of monocytes containing bacteria with $(Fab')_2$ fragments of IgG gave no decrease in the number of viable intracellular bacteria (Table V). During incubation of similar monocytes in the presence of the subclasses IgG_1, IgG_2, IgG_3, or IgG_4, lacking opsonic activity for *S. aureus*, a decrease in the number of viable intracellular bacteria was only seen for IgG_1 and IgG_3 compared with incubation without serum ($P < 0.01$) (Table V).

If monocytes were incubated after phagocytosis with a bacteria:monocyte ratio of 10:1 instead of 1:1, similar results were obtained (Table VI). Almost no killing occurred in the presence of IgM or $(Fab')_2$ fragments of IgG ($P > 0.1$ compared with incubation without serum), and a suboptimal killing comparable to that obtained with inactivated serum was found with IgG (500 μg/ml) or pFc fragments (100 μg/ml) ($P > 0.1$). Incubation of these monocytes with IgG subclasses showed that intracellular killing only occurred in the presence of IgG_1 and IgG_3 (for both $P < 0.01$ compared with incubation without serum); the killing index found in the presence of IgG_2 and IgG_4 did not differ significantly from the index without serum ($P > 0.1$). These results indicate that IgG stimulates intracellular killing via an interaction of its Fc fragment and the corresponding receptors for IgG_1 and IgG_3 on the monocyte membrane.

Effect of complement inactivation on intracellular killing. To investigate the effect of complement on intracellular killing, monocytes with ingested bacteria were incubated in a medium containing heat-inactivated serum in various concentrations. The results (Table VII) show that the intracellular killing of *S. aureus* was significantly lower ($P < 0.01$) than with normal fresh serum. The presence of 2.5% or more heat-inactivated serum in the medium gave a killing index similar to that found with the IgG preparation (Table V). For *S. epidermidis* and *E. coli*, too, the intracellular killing was significantly lower ($P < 0.05$) in the presence of heat-inactivated serum than with normal fresh serum (Fig. 5).

When the killing assay was performed in the presence of fresh serum containing 10 mM EDTA, which blocks both the classical and the alternative complement pathways, the decrease in the number of viable intracellular bacteria was only 40% at 60 min and 67.3% at 120 min (Table VII). The latter value must be viewed in light of the decrease in the number of viable bacteria during the 2nd h of incubation ($P < 0.05$) (Table IV) found in control experiments with preopsonized bacteria incubated in the presence of serum

TABLE VI

Effect of Immunoglobulins on the Intracellular Killing of S. Aureus by Monocytes after Phagocytosis at a Bacteria:Monocyte Ratio of 10:1

Incubation of monocytes containing bacteria with	Concentration	Intracellular killing at 60 min*	No. of experiments
	μg/ml	%	
No serum		19.4±11.5	8
10% Fresh serum		63.5±7.0	8
10% Inactivated serum		36.8±7.2	5
IgM	500	8.7±12.3	2
IgG	500	36.1±5.7	6
pFc	100	40.8±6.8	6
$(Fab')_2$	400	27.6±14.7	6
IgG_1	500	38.9±8.6	6
IgG_2	500	22.3±6.1	6
IgG_3	500	40.6±6.6	5
IgG_4	500	26.7±5.8	6

* Expressed as $K(t) = (1 - N_t/N_0) \times 100\%$ (see Methods).

with 10 mM EDTA. It is conceivable that during the killing assay EDTA affects the viability of the intracellular bacteria, which would result in a higher killing index.

Inhibition of the classical pathway alone was brought about by adding 10 mM EGTA supplemented with 2.5 mM $MgCl_2$ to the medium containing 10% serum. Under these conditions intracellular killing did not differ significantly from that in the presence of normal serum ($P > 0.1$) (Table VII). The control experiment, in which preopsonized bacteria were incubated in medium containing EGTA, showed no reduction in the viability of the micro-organisms ($P > 0.1$) (Table IV).

In factor D-deficient serum, the alternative complement pathway is impaired. An intracellular killing assay performed with such serum gave a killing index of only 19.0% at 60 min (Table VII); with 10%, heat-inactivated, factor D-deficient serum, the index was 16.3% at 60 min.

Thus, it is clear that when only the classical complement pathway has been blocked the intracellular killing is roughly similar to that in the presence of 10% serum, but the killing index is greatly diminished when both pathways or only the alternative pathway are blocked. It may therefore be concluded that it is mainly factors belonging to the alternative complement pathway that affect the intracellular killing of bacteria.

Effect of various complement factors on intracellular killing. Heat-inactivated serum (which is totally devoid of factors C1, C2, and B and partially deficient in factors C3, C4, and C5) was supplemented with various combinations of isolated complement factors. The amount of each of these factors was chosen such that the final concentration in the reconstituted serum was equal to that in normal fresh serum. Intracellular killing in the presence of heat-inactivated serum reconstituted with factors C1, C2, and C3 in various combinations did not differ ($P > 0.1$) from that found with heat-inactivated serum alone (Table VIII). However, when factor B was added to heat-inactivated serum or to inactivated serum supplemented with factors C1, C2, and C3, the rate of intracellular killing was significantly higher ($P < 0.02$) than in the presence of heat-inactivated serum alone and not much different ($P > 0.05$) from that obtained with normal serum (Table VIII).

Incubation in the presence of inactivated serum, of monocytes containing ingested bacteria after phagocytosis at a bacteria:monocyte ratio of 10:1, gave a killing index of 41.3±11.4%; the addition of factors C3 + B or factor B to inactivated serum led to a killing index of 51.2±11.6% and 59.0±10.9%, respectively. The last value is significantly higher than those obtained with inactivated serum ($P < 0.05$) and does not differ from that obtained in the presence of fresh serum ($P > 0.1$).

Because the addition of factor B promotes cleavage of C3 by the alternative pathway and also results in the generation of C3b by amplification, the results of these experiments suggest that C3b stimulates the intracellular killing of *S. aureus*.

TABLE VII

*Effect of Complement Inactivation on the Intracellular Killing of S. Aureus by Monocytes**

Incubation of monocytes containing bacteria	Intracellular killing‡		No. of experiments
	60 min	120 min	
	%		
Inactivated serum, %			
0	−6.0±21.0	−65.0±31.1	20
1.0	20.3±15.3	−14.0±13.8	4
2.5	26.6±14.5	11.8±16.9	4
5.0	34.1±14.8	21.0±11.0	4
10.0	35.6±11.2	20.3±12.3	15
25.0	34.3±13.0	24.1±7.8	3
Normal serum			
10%	75.3±9.5	83.5±11.1	25
10% and 10 mM EDTA	40.3±17.5	67.3±21.1	5
10% and 10 mM EGTA 2.5 mM $MgCl_2 \cdot 6H_2O$	67.3±11.3	76.0±9.8	3
10% − factor D	19.0±14.5	ND	3

* After phagocytosis at a bacteria:monocyte ratio of 1:1.
‡ Expressed as $K(t) = (1 - N_t/N_0) \times 100\%$ (see Methods).
ND, not done.

TABLE VIII

*Effect of Complement Factors on the Intracellular Killing of S. Aureus by Monocytes**

Incubation of monocytes containing bacteria	Intracellular killing at 60 min‡	No. of experiments
	%	
Normal serum	70.1±11.3	8
Inactivated serum§	29.3±9.8	6
+ C1	27.1±12.1	3
+ C2	30.1±11.3	3
+ C1 + C2	26.7±14.3	3
+ C3	34.5±6.8	3
+ C1 + C2 + C3	44.0±14.7	3
+B	55.0±9.5	3
+ C3 + B	59.0±8.4	3
+ C1 + C2 + C3 + B	59.1±11.1	4

* After phagocytosis at a bacteria:monocyte ratio of 1:1.
‡ Expressed as $K(t) = (1 - N_t/N_0) \times 100\%$ (see Methods).
§ In all experiments, the serum concentration was 10% and the various complement factors were supplemented to the normal levels in fresh serum.

Effect of enzymatic alteration of membrane receptor activity on intracellular killing. Incubation of monocytes with 1 mg/ml trypsin or pronase for 20 min at 37°C after the ingestion of preopsonized *S. aureus* (after which the trypsin activity was inhibited by adding 2 mg/ml trypsin inhibitor) gave a decrease in the number of monocytes with complement receptors ($P < 0.002$) and in the mean number of erythrocytes per rosette (Table IX), whereas Fc-receptor activity remained unimpaired ($P > 0.1$). Incubation of these enzyme-treated monocytes with 10% fresh normal serum showed a decrease of the killing index as compared with untreated cells ($P < 0.002$) (Table IX), whereas the intracellular killing in the presence of 10% inactivated serum was similar to that of untreated cells ($P > 0.1$). These results indicate that a decrease in the number of C3 receptors leads to a decrease in intracellular killing.

Incubation of monocytes with ingested bacteria with 6×10^3 U/ml sphingomyelinase C for 30 min at 37°C gave a slight decrease (11.7%) in the C3-receptor activity, whereas the Fc-receptor activity decreased by 24%. Incubation of these cells with 10% fresh or inactivated serum resulted in a slight decrease in the killing index as compared to untreated cells (Table IX).

Incubation of monocytes with ingested bacteria with 50 U/ml neuraminidase for 30 min at 37°C gave an increase in the mean number of Fc receptors per cell, as indicated by the increase in the mean number of erythrocytes per monocyte (Table IX); no significant difference in the number of complement receptors was found ($P > 0.1$). Incubation with 10% fresh or heat-inactivated serum resulted in a killing index slightly but not significantly higher than that of untreated monocytes (Table IX).

From these experiments it may be concluded that normal functioning of Fc and C3b receptors in the cell membrane is obligatory for the intracellular killing process.

Effect of anti-monocyte serum on intracellular killing. Incubation of monocytes with anti-monocyte serum leads to binding of antibody to the cell membrane, as shown by immunofluorescence staining (29) and a cytotoxicity assay (23). Because treatment of monocytes with anti-monocyte serum for 20 min at room temperature followed by incubation with fresh serum causes cell lysis, our killing experiments were performed with inactivated serum supplemented with factor B, which leads to generation of C3b.

Incubation of monocytes with ingested bacteria first with anti-monocyte serum for 30 min at room temperature and then with 10% inactivated AB serum with or

TABLE IX

Effect of Enzymatic Inhibition of Membrane Receptor Activity on the Intracellular Killing of S. Aureus by Monocytes

Treatment of monocytes containing bacteria*	EIgG				EIgMC				Reduction of viable intracellular bacteria‡	Reincubation with	Intracellular killing at 60 min§	
	Monocytes with ingested erythrocytes		Mean number of erythrocytes per monocyte		Monocytes with rosettes		Mean number of erythrocytes per rosette					
	%	n‖	%	n	%	n	%	n	%		%	n
HBSS	96.9±2.6	8	5.9±0.8	8	82.7±5.5	8	19.1±3.3	8		10% Serum	70.4±8.4	12
										10% Inactivated serum	30.1±9.5	6
Trypsin, 1 mg/ml	95.2±1.6	4	4.8±0.7	4	65.0±8.3	4	16.4±3.6	4	5±5	10% Serum	43.5±13.2	8
										10% Inactivated serum	32.0±7.8	4
Pronase, 1 mg/ml	91.7±7.0	4	5.5±0.9	4	10.0±11.1	4	7.2±4.1	4	14±5	10% Serum	31.4±6.8	6
										10% Inactivated serum	31.2±6.6	4
Sphingomyelinase, 6×10^3 U/ml	71.5±6.7	3	4.8±1.1	3	71.7±7.6	3	20.9±4.9	3	8±7	10% Serum	62.3±5.3	3
										10% Inactivated serum	23.2±6.1	4
Neuraminidase, 50 U/ml	96.6±3.4	3	10.0±2.1	3	80.6±6.1	3	25.3±4.7	3	13±7	10% Serum	77.1±14.6	5
										10% Inactivated serum	44.7±11.1	5

* After phagocytosis at a bacteria:monocyte ratio of 1:1, cells are next incubated for 20 min at 37°C with enzymes followed by three washings.
‡ Compared with number of viable bacteria in cells with Hanks' solution (B_H) and expressed as $(1 - B_t/B_H) \times 100\%$, in which B_T is the number of viable intracellular bacteria in enzyme-treated cells.
§ Expressed as $K(t) = (1 - N_t/N_0) \times 100\%$ (see Methods).
‖ *n*, Number of experiments.

without factor B for 1 h at 37°C did not lead to stimulation of intracellular killing (Table X). The killing index of these monocytes in the presence of HBSS was higher than normally found during incubation without serum (Table X), possibly because of a slight stimulation by the anti-monocyte serum; however, this effect was not found for monocytes treated with normal rabbit serum. In control experiments in which monocytes were first incubated with inactivated rabbit serum and then reincubated with 10% inactivated AB serum with or without factor B, the intracellular killing was roughly similar to that found for monocytes pretreated with HBSS (Table X).

From these findings it may be concluded that anti-monocyte serum reduced the accessibility of the receptors on the cell membrane to IgG and the complement components required for optimal intracellular killing.

DISCUSSION

The main conclusions to be drawn from this study are that intracellular killing by human monocytes does not occur or is extremely low in the absence of serum and that both IgG and C3b activate this process.

These results could be obtained because an assay method was developed that made it possible to investigate factors influencing intracellular killing apart from their effect on the phagocytosis of preopsonized bacteria. It was necessary, however, to demonstrate that the intracellular killing assay really measures the killing of bacteria that are intracellular at the beginning of the assay. The additional uptake of any appreciable number of extracellular preopsonized bacteria that are attached to the monocytes and are killed intracellularly during reincubation in the presence of serum had to be excluded. Scanning electron microscopy of suspensions of monocytes with ingested bacteria, performed before the killing assay was started, showed that almost no bacteria were present on the surface of the monocytes. Furthermore, treatment of these monocytes with lysostaphin, which lyses extracellular *S. aureus*, showed that at time 0 of the killing assay, an average 12% of the bacteria were extracellular and that the rate of intracellular killing of lysostaphin-treated monocytes was similar to that of monocytes not treated with this drug. Furthermore, incubation of monocytes with ingested bacteria in the presence of adsorbed serum, pFc fragments of heterogeneous IgG, or subclasses of IgG_1 and IgG_3, none of which showed opsonic activity for *S. aureus*, promoted intracellular killing at a level similar to that induced by heat-inactivated serum or the IgG fraction of that serum. From these control experiments it may be concluded that the decrease in time of the number of viable bacteria determined after lysis of monocytes that have ingested bacteria accurately reflects the intracellular killing of bacteria after a certain time.

For the three bacteria species investigated, intracellular killing by monocytes is stimulated by both normal fresh serum and heat-inactivated serum; however, the rate of this process is much higher in the presence of fresh serum than in the presence of inactivated serum. Because the complement activity is totally absent in inactivated serum, these results indicate that other serum proteins stimulate the intracellular killing. Because monocytes carry receptors for the Fc fragments of IgG (30), the effect of isolated

TABLE X

Effect of Anti-Monocyte Serum on the Intracellular Killing of S. Aureus by Monocytes

Treatment of monocytes containing bacteria*	Reincubation with‡	Intracellular killing at 60 min§	No. of experiments
		%	
HBSS	HBSS	6.0±10.0	3
	10% Inactivated AB serum	36.0±11.2	3
	10% Inactivated AB serum + factor B	63.7±14.3	4
Anti-monocyte serum, 25%	HBSS	15.0±7.2	3
	10% Inactivated AB serum	16.8±17.0	3
	10% Inactivated AB serum + factor B	21.7±9.1	4
Inactivated rabbit serum, 25%	HBSS	3.0±10.0	3
	10% Inactivated AB serum	30.7±18.4	3
	10% Inactivated AB serum + factor B	52.3±16.6	4

* After phagocytosis at a bacteria:monocyte ratio of 1:1, the cells are incubated for 30 min at room temperature with various sera followed by two washings.

‡ For 60 min at 37°C.

§ Expressed as $K(t) = (1 - N_t/N_0) \times 100\%$ (see Methods).

heterogeneous IgG and the pFc fragment of IgG was studied. Both stimulated intracellular killing to the same extent as inactivated serum, whereas $(Fab')_2$ fragments of IgG were completely inactive in this respect. This finding was confirmed by the observation of stimulation of the intracellular killing process by IgG_1 and IgG_3, whereas IgG_2 and IgG_4 were inactive, which is consistent with the fact that monocytes only have receptors for IgG_1 and IgG_3 (31).

Treatment of the monocytes with sphingomyelinase, resulting in a moderate decrease of the number of Fc receptors (32), gave a slight decrease of the intracellular killing during incubation in the presence of inactivated serum. Pretreatment of the monocytes containing ingested bacteria with anti-monocyte serum resulted in a considerable decrease of intracellular killing during incubation in the presence of inactivated serum, conceivably a result of interference with the interaction between IgG and its Fc receptor in the membrane. From these results it may be concluded that the interaction of the Fc-part of IgG with the Fc receptor on the monocyte membrane leads to the stimulation of intracellular killing.

The difference between the rates of intracellular killing in the presence of fresh and inactivated serum indicates that complement components are also involved. Inhibition of the classical pathway has little effect on the rate of intracellular killing. However, when the generation of complement components by the alternative pathway was impaired, the rate of intracellular killing was reduced.

In substitution experiments with inactivated serum supplemented with isolated complement factors only those combinations (all including factor B) that led to the generation of C3b by cleavage of C3 via the alternative pathway gave stimulation of the intracellular killing similar to that seen with fresh serum. Because monocytes have receptors for C3b in the cell membrane (30), the stimulation probably occurs via these receptors. Treatment with pronase, which strongly decreases the number of C3b receptors (33), resulted in a marked decrease of the intracellular killing by fresh serum. Furthermore, treatment of monocytes containing ingested bacteria with anti-monocyte serum resulted in a marked decrease in the intracellular killing in the presence of complement. Thus, intracellular killing by human monocytes is also stimulated by the interaction of C3b and its C3b receptors on the monocytes.

Because fresh serum contains native C3 but no C3b, it must be assumed that C3b is generated, conceivably via the alternative pathway, during the incubation of monocytes containing bacteria.

The activation of intracellular killing by serum factors could be demonstrated in the present study after a short period of ingestion of preopsonized bacteria. The measurement of intracellular killing according to the classical method (6–8) involves continuous phagocytosis for 1 or 2 h, which requires the presence of serum factors (e.g., IgG or IgM and complement factors). This technique obscures the effect of such factors on the intracellular killing process. It has been reported that IgG and complement components, bound to the surface of particulates, stimulate the bactericidal activity of phagocytes. Solberg and co-workers (34, 35) and Christie et al. (36) found an influence of IgG and complement bound to bacteria on the bactericidal activity of human granulocytes, but because these authors measured phagocytosis and intracellular killing simultaneously, their results do not permit definite conclusions. Intracellular killing in the absence of serum, but in the continuous presence of opsonized bacteria, was also observed in the present study (Fig. 1). Others have shown that particles opsonized with fresh serum stimulate the production by granulocytes and monocytes of two bactericidal agents, H_2O_2 and O_2^- (37–42), and similar results were obtained with serum-treated Sepharose beads (Pharmacia, Inc.) and IgG-coated micropore filters, neither of which can be phagocytozed (38, 39). However, latex particles without surface-bound IgG or complement also stimulate intracellular metabolism (41–42).

An effect of IgG and complement on the release of lysosomal enzymes was also found, because the combination of surface-bound IgG and C3b stimulates the release of lysozyme and β-glucuronidase by human and rabbit granulocytes (38, 43). Furthermore, Schorlemmer and Allison (44) described the release of β-glucuronidase, β-galactosidase, and *N*-acetyl-β-D-glucosaminidase by murine macrophages in the presence of soluble C3b.

All previous studies, except one (44), made use of IgG and complement components fixed to the surface of particulate material, but the presence study has shown for the first time that in solution both IgG and C3b stimulate intracellular killing via specific receptors on the cell surface. The recognition of this mechanism may have importance for the study of impaired cellular functions in various diseases, because it might mean that such defects are not intrinsic but a result of a serum-factor deficiency or some change in specific receptor functions.

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