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# Participation of Immunoglobulins and Complement Components in the Intracellular Killing of *Staphylococcus aureus* and *Escherichia coli* by Human Granulocytes

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Immunoglobulins and complement components are required for optimal ingestion and optimal killing of microorganisms by granulocytes. The degree of opsonization of microorganisms necessary for their ingestion was lower than that required for the killing of these bacteria during the ingestion phase. Killing during this phase was found to depend mainly on the presence of heat-labile opsonins, probably C3b, present on the microorganisms. Extracellular immunoglobulin G (IgG) and C3b were indispensable for optimal intracellular killing after ingestion was complete. This was established with an assay permitting assessment of the course of the number of viable intracellular bacteria independent of the ingestion of new live bacteria. Maximal intracellular killing by human granulocytes of ingested catalase-positive (*Staphylococcus aureus* and *Escherichia coli*) or catalase-negative (*Streptococcus pyogenes* and *S. pneumoniae*) microorganisms was found only when fresh serum was present extracellularly. Killing was suboptimal in the absence of serum. With heat-inactivated serum, the killing index lay between the indices obtained in the presence and absence of fresh serum. The stimulatory activity of heat-inactivated serum was most probably due to the interaction of IgG with the Fc receptor on the granulocyte membrane, since IgG subclasses IgG1 and IgG3 as well as pFc fragments of IgG stimulated the intracellular killing to the same degree as heat-inactivated serum did. In addition, $(Fab^1)_2$ fragments of IgG did not stimulate killing, and reduced killing was observed in the presence of heat-inactivated serum after reduction of the number of Fc receptors. The extra stimulation of the killing process in the presence of fresh serum compared with heat-inactivated serum was due to the interaction between membrane receptors and complement—most probably C3b generated by both the classical and the alternative pathways of complement activation. This conclusion is based on results obtained with sera in which one or both complement pathways were blocked, on the restoration of the killing-stimulatory activity of C3-deficient serum after addition of fresh C3, and on the reduced killing observed in the presence of fresh serum after reduction of the number of C3 receptors by the use of pronase or antigranulocyte serum.

Optimal functioning of one of the most important host defense mechanisms, phagocytosis and intracellular killing of invading microorganisms, requires serum factors. Immunoglobulin G (IgG) alone or IgG and IgM together with complement component C3b can act as opsonins, thus facilitating the attachment and ingestion of bacteria by granulocytes, monocytes, and macrophages. Of these opsonins, primarily complement has been reported to enhance the killing of bacteria by polymorphonuclear leukocytes (2, 18, 19, 24, 25, 32). However, in most of these studies, methods were used that measure the killing of bacteria under the continuous ingestion of new live bacteria, which makes it uncertain whether the serum factor under study acts solely on the killing process or also on attachment and ingestion.

The present paper describes the effect of serum factors on the bactericidal activity of granulocytes during the ingestion phase as well as the effect on the intracellular bacteria after ingestion has been completed. For the latter, we used a method that makes it possible to measure intracellular killing independent of the continuous ingestion of new live bacteria (12, 13, 30).

## MATERIALS AND METHODS

**Reagents.** Stock solutions of 0.1 M ethylenediaminetetraacetate (EDTA; Sigma Chemical Co., St.

Louis, Mo.) and 0.1 M ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA; Sigma) containing 25 mM $MgCl_2 \cdot 6H_2O$ were prepared with Hanks balanced salt solution (HBSS), adjusted to pH 7.2 with dilute NaOH, and stored at 4°C for at most 14 days. Pronase (Sigma) was dissolved in phosphate-buffered saline (PBS; Bacto-Hemagglutination Buffer; Difco Laboratories, Detroit, Mich.) just before use. Phenylbutazone (Butazolidine; Ciba-Geigy, Switzerland) and sodium fluoride (Merck AG, Darmstadt, West Germany) were prepared with HBSS just before use.

**Granulocytes.** Blood was collected from healthy donors by venipuncture. Granulocyte suspensions were prepared by sedimentation of the erythrocytes with a 5% solution of dextran (molecular weight, 200,000) in PBS (3 ml of solution to 10 ml of blood). Leukocytes were collected by centrifugation (10 min, 110 × *g*), washed twice with PBS containing 0.5 U of heparin per ml, and suspended in HBSS with 0.1% gelatin (gelatin-HBSS) to a concentration of $1 \times 10^7$ to $2 \times 10^7$ granulocytes/ml. This suspension also contained $4 \times 10^5$ monocytes/ml and $1 \times 10^7$ to $3 \times 10^7$ lymphocytes/ml. The viability of the granulocytes, as checked with trypan blue, was always more than 98%.

**Microorganisms.** *Staphylococcus aureus* (type 42D) and *Escherichia coli* (O54), in both cases serum-resistant strains, were stored on agar slants at 4°C and transferred once every 2 weeks. *Streptococcus pneumoniae* and *S. pyogenes* were held on blood agar plates and transferred every other day. The microorganisms were cultured overnight in nutrient broth no. 2 (Oxoid Ltd., London), harvested by centrifugation for 10 min at 1,500 × *g*, washed twice with PBS, and suspended to a concentration of $10^7$ microorganisms per ml in gelatin-HBSS.

When streptococci were used, the number of colony-forming units was determined on blood agar plates.

**Preopsonization of bacteria.** Preopsonization of microorganisms was performed by incubating $5 \times 10^6$ bacteria per ml with serum in the concentration under study for 25 min at 37°C under rotation. Next, the excess serum was removed by centrifugation at 1,500 × *g* and two washes with ice-cold gelatin-HBSS. Finally, the bacteria were resuspended to a concentration of $10^7$/ml in gelatin-HBSS.

**Sera.** AB serum prepared from the blood of healthy donors was used in all experiments. This serum did not possess bactericidal activity for the various microorganisms (15, 30) and did not induce clumping or agglutination of bacteria, since the mean number of bacteria per colony-forming unit hardly rose during a 120-min incubation period (15). The blood was clotted for 1 h at room temperature and centrifuged for 20 min at 1,100 × *g*, and the serum was stored in 2-ml portions at −70°C for up to 3 months. AB sera deficient in complement factors B (14), D (13), and C4 (14) were prepared as described in the indicated papers. C3-deficient serum, obtained from a child with a C3 deficiency, was a gift from J. Roord, Wilhelmina Kinderziekenhuis, Utrecht.

IgG and IgM and the pFc and $(Fab')_2$ fragments of immunoglobulin G were prepared as described elsewhere (13). Pure preparations of the subclasses IgG1, IgG2, IgG3, and IgG4 were kindly donated by P. Goosen (Central Laboratory of the Blood Transfusion Service, Amsterdam).

Heat-inactivated serum was prepared by incubation of serum for 30 min at 56°C. Adsorbed heat-inactivated sera freed of specific antibodies against *S. aureus* of *E. coli* were prepared as described earlier (1, 13).

An antiserum against granulocytes was prepared by A. von dem Borne (Central Laboratory Blood Transfusion Service, Amsterdam) by immunization of rabbits (1). The presence of antigranulocyte antibodies on the granulocytes was demonstrated by immunofluorescence staining (31), which was kindly performed by F. H. J. Claas (University Hospital, Leiden).

**Intracellular killing assay.** Intracellular killing of microorganisms was determined as described elsewhere (12, 13, 30). In short, $5 \times 10^6$ preopsonized bacteria per ml were incubated with $5 \times 10^6$ granulocytes per ml for 3 min at 37°C under rotation (4 rpm); phagocytosis was stopped by shaking the tube through crushed ice, and the noningested bacteria were removed by differential centrifugation (4 min, 110 × g) and two washes. After this procedure, no decrease in the number and the viability of the granulocytes was found. The granulocytes containing ingested bacteria were suspended in HBSS to a concentration of $5 \times 10^6$/ml and reincubated at 37°C under rotation. After various intervals, a 0.5-ml sample was taken in which intracellular killing was stopped by adding 0.5 ml of ice-cold HBSS, and the suspension was then centrifuged for 4 min at 110 × *g*. After removal of the supernatant, distilled water containing 0.01% bovine albumin was added to disrupt the granulocytes, and the number of viable intracellular bacteria was determined with a microbiological plate method.

**Receptors.** The presence of Fc receptors on granulocytes was detected with IgG-coated erythrocytes. Coating of the sheep erythrocytes was done with rabbit anti-sheep erythrocyte serum as described elsewhere (4), after which the number of cells with receptors was determined according to Scribner and Fahrney (22). The presence of complement (C3b) receptors was similarly detected, but with sheep erythrocytes coated with the IgM fraction of rabbit anti-sheep erythrocyte serum and subsequently with purified human C1, C2, C4, and C3.

**Calculations.** Intracellular killing at a given time point is expressed as the percentage decrease in the initial number of viable intracellular bacteria according to the formula $K(t) = (N_0 - N_t)/(N_0 \times 100\%)$, where $N_t$ is the number of viable intracellular bacteria at time $t = t$ and $N_0$ is the number of viable intracellular bacteria at time $t = 0$. Values in text and tables are means and standard deviations of at least three experiments performed with different cell preparations on different days. Statistical analysis was performed with Student's *t*-test.

## RESULTS

**Intracellular killing of microorganisms during ingestion by granulocytes.** The method used to study the effect of extracellular serum factors on the intracellular killing of microorganisms by granulocytes includes the reincubation of granulocytes containing ingested

bacteria after a short period of ingestion. However, preopsonized bacteria might already have been killed by interaction with the granulocytes during this period. This interaction, and especially the effect of serum on it, was investigated by incubating preopsonized bacteria and granulocytes in the presence and absence of phenylbutazone, a drug which inhibits the killing process but has very little effect on ingestion (12, 23, 26).

A 15- or 30-min of incubation of granulocytes with preopsonized *S. aureus* or *E. coli* at a bacteria-to-granulocyte ratio of 1:1 led to a larger number of viable cell-associated bacteria when the incubation was performed in the presence of phenylbutazone than in the absence of this drug (Table 1). Comparison of the numbers of viable cell-associated bacteria preopsonized with 10% fresh serum or 10% heat-inactivated serum and ingested in the presence of 2 mg of phenylbutazone per ml showed that more bacteria were ingested after preopsonization with fresh serum (Table 1, column B). However, the total numbers of cell-associated bacteria in the absence and presence of phenylbutazone indicated that when preopsonization was performed with 10% heat-inactivated serum instead of fresh serum, fewer bacteria were killed (Table 1, column C). Thus, preopsonization with fresh serum not only gave a higher level of ingestion than was seen with inactivated serum but also led to more effective killing of opsonized bacteria during the ingestion phase.

To investigate the effect of opsonization on the killing of microorganisms during ingestion in more detail, bacteria preopsonized with various concentrations of fresh serum were incubated with granulocytes for 15 or 30 min in the presence and absence of phenylbutazone (Tables 2 and 3). After preopsonization of *S. aureus* with 0.1, 0.5, and 1.0% serum and *E. coli* with 0.1% serum, almost all ingested bacteria were viable after 15 and 30 min of incubation in the absence of phenylbutazone, which means that no killing had occurred.

To make certain that, after phagocytosis of bacteria preopsonized with 0.1 to 5% serum, the number of bacteria determined after lysis of the granulocytes really represented ingested bacteria, phagocytosis was performed in the presence of 0.2 M sodium fluoride, a drug which inhibits phagocytosis (15). The number of viable cell-associated bacteria was only about 10 to 30% of that found after phagocytosis without this drug (Tables 2 and 3). Sodium fluoride did not affect the viability of the granulocytes. The results indicated that the number of viable cell-associated bacteria represented mainly intracellular bacteria.

Ingestion of both species of bacteria preopsonized with serum in higher concentrations led to much lower recoveries of viable ingested bacteria after incubation in the absence of phenylbutazone (Tables 2 and 3), which indicated that the amount of opsonins present on the bacteria influenced the killing during ingestion of the microorganisms. The lowest concentration of serum giving hardly any killing during ingestion differed for *S. aureus* and *E. coli*.

**Effect of opsonins on intracellular killing of microorganisms.** To find out whether the effect of preopsonization of bacteria with various concentrations of serum also influences intracellular killing after ingestion has been completed,

TABLE 1. *Recovery of viable cell-associated bacteria after phagocytosis*[a]

| Microorganism | Preopsonized with: | Duration of phagocytosis (min) | (A) No. of viable cell-associated bacteria after ingestion in the absence of phenylbutazone ($\times 10^5$) | (B) No. of viable cell-associated bacteria after ingestion in the presence of phenylbutazone[b] ($\times 10^5$) | | (C) Killing during the ingestion phase: $\frac{B-A}{B} \times 100$ (%) |
|---|---|---|---|---|---|---|
| *S. aureus* | 10% fresh serum | 15 | 7.3 ± 1.0 | 32.0 ± 6.0 | $P < 0.02$ | 78.1 |
| | | 30 | 9.7 ± 0.7 | 45.0 ± 6.0 | | 78.4 |
| | 10% inactivated serum | 15 | 15.0 ± 4.0 | 18.0 ± 2.0 | $P < 0.05$ | 16.6 |
| | | 30 | 22.0 ± 2.0 | 31.0 ± 6.0 | | 29.0 |
| *E. coli* | 10% fresh serum | 15 | 2.5 ± 1.3 | 30.0 ± 4.0 | $P < 0.02$ | 91.6 |
| | | 30 | 1.4 ± 1.3 | 37.0 ± 5.0 | | 96.2 |
| | 10% inactivated serum | 15 | 18.0 ± 2.0 | 21.0 ± 3.0 | $P < 0.05$ | 14.2 |
| | | 30 | 21.0 ± 6.3 | 24.0 ± 6.0 | | 12.5 |

[a] All experiments were performed three times. Phagocytosis was performed at a bacteria-to-cell ratio of 1:1, and the number of viable cell-associated bacteria per $5 \times 10^6$ granulocytes was determined after lysis of the granulocytes.

[b] Phenylbutazone concentration of 2 mg/ml.

TABLE 2. *Effect of opsonins present on S. aureus on the killing during ingestion*[a]

| Serum concn during opsonization (%) | Duration of phagocytosis (min) | (A) No. of viable cell-associated *S. aureus* after ingestion in the absence of phenylbutazone ($\times 10^5$) | (B) No. of viable cell-associated *S. aureus* after ingestion in the presence of phenylbutazone[b] ($\times 10^5$) | Killing during the ingestion phase: $\frac{B-A}{B} \times 100$ (%) | (C) No. of viable cell-associated *S. aureus* after ingestion in the presence of NaF ($\times 10^5$) | % of viable noningested bacteria: C/A $\times$ 100 |
|---|---|---|---|---|---|---|
| 0.1 | 15 | 4.4 ± 2.8 | 4.1 ± 3.0 | −7.3 | 0.9 ± 0.8 | 20.4 |
| | 30 | 10.5 ± 8.2 | 11.3 ± 7.2 | 7.7 | 1.7 ± 1.3 | 16.2 |
| 0.5 | 15 | 8.0 ± 7.8 | 9.3 ± 5.6 | 13.9 | 1.3 ± 2.0 | 16.3 |
| | 30 | 20.0 ± 10.0 | 18.5 ± 11.2 | −8.0 | 2.3 ± 1.7 | 11.5 |
| 1.0 | 15 | 7.9 ± 7.4 | 8.6 ± 8.6 | 8.1 | 1.7 ± 2.3 | 21.5 |
| | 30 | 18.0 ± 8.0 | 23.0 ± 6.0 | 21.7 | 2.7 ± 2.1 | 15.0 |
| 2.5 | 15 | 4.4 ± 3.6 | 26.0 ± 14.0 | 83.1 | 1.4 ± 1.3 | 31.8 |
| | 30 | 9.0 ± 7.0 | 40.0 ± 8.0 | 77.5 | 2.9 ± 2.7 | 32.2 |
| 5.0 | 15 | 5.2 ± 4.0 | 33.0 ± 3.0 | 84.3 | 1.3 ± 2.1 | 25.0 |
| | 30 | 8.4 ± 6.0 | 43.0 ± 8.0 | 80.4 | 2.7 ± 0.3 | 32.1 |

[a, b] See Table 1.

TABLE 3. *Effect of opsonins present on E. coli on the killing during ingestion*[a]

| Serum concn during opsonization (%) | Duration of phagocytosis (min) | (A) No. of viable cell-associated *E. coli* after ingestion in the absence of phenylbutazone ($\times 10^5$) | (B) No. of viable cell-associated *E. coli* after ingestion in the presence of phenylbutazone[b] ($\times 10^5$) | Killing during the ingestion phase: $\frac{B-A}{B} \times 100$ (%) | (C) No. of viable cell-associated *E. coli* after ingestion in the presence of 0.2 M NaF | % of viable noningested bacteria: C/A $\times$ 100 |
|---|---|---|---|---|---|---|
| 0.1 | 15 | 1.9 ± 2.0 | 2.1 ± 3.1 | 9.5 | 0.3 ± 0.6 | 15.8 |
| | 30 | 2.8 ± 2.6 | 3.1 ± 2.6 | 9.7 | 0.4 ± 0.7 | 14.3 |
| 0.5 | 15 | 6.0 ± 3.2 | 8.3 ± 2.0 | 27.7 | 0.6 ± 1.0 | 10.0 |
| | 30 | 7.0 ± 6.0 | 11.0 ± 3.0 | 36.4 | 0.5 ± 0.9 | 7.1 |
| 1.0 | 15 | 2.3 ± 2.4 | 14.5 ± 3.0 | 84.2 | 0.7 ± 0.6 | 30.4 |
| | 30 | 3.3 ± 2.0 | 18.0 ± 6.0 | 81.7 | 0.9 ± 1.2 | 27.2 |
| 2.5 | 15 | 5.3 ± 5.3 | 25.0 ± 7.0 | 78.8 | 0.7 ± 0.9 | 7.1 |
| | 30 | 2.1 ± 1.0 | 33.0 ± 6.0 | 93.7 | 0.5 ± 0.8 | 23.8 |
| 5.0 | 15 | 3.0 ± 0.6 | 32.0 ± 6.0 | 90.7 | 0.6 ± 1.0 | 20.0 |
| | 30 | 1.6 ± 1.7 | 39.0 ± 14.0 | 95.9 | 0.3 ± 0.6 | 18.9 |

[a, b] See Table 1.

intracellular killing was measured after 15 min of ingestion. This interval was chosen to obtain sufficient ingestion for accurate measurement of killing, even for bacteria opsonized with the lowest serum concentrations (Tables 2 and 3). Incubation of granulocytes containing ingested bacteria in HBSS for 60 min at 37°C resulted in intracellular killing only if the bacteria had been preopsonized with at least 1% serum (Fig. 1). Bacteria preopsonized with 10% heat-inactivated serum and ingested by granulocytes were not killed when the latter were reincubated in HBSS. With 10% fresh serum, the number of viable intracellular bacteria was reduced by about 90% after incubation of granulocytes containing bacteria preopsonized with various concentrations of fresh serum (Fig. 1). It is clear that the degree of opsonization of the microorganisms affected the intracellular fate of these bacteria after ingestion had been completed and also that extracellular serum was required to obtain maximal measurable intracellular killing.

**Effect of extracellular serum on intracellular killing of catalase-positive microorganisms.** To investigate the stimulation of intracellular killing by extracellular serum further, the killing assay was performed (after a 3-min period of phagocytosis of bacteria preopsonized with 10% fresh serum, at a bacteria-to-granulocyte ratio of 1:1) with various concentrations of fresh serum. This interval is long enough for the ingestion of sufficient bacteria to permit accurate measurement of the intracellular killing and short enough to ensure that the number of bacteria killed during the ingestion phase is almost negligible (12). Incubation of granulocytes containing *S. aureus* or *E. coli* with various concentrations of extracellular serum resulted in a decrease in the number of viable intracellular bacteria that was correlated with the concentration

of the extracellular serum (Fig. 2). This decrease was greatest at extracellular serum concentrations of 5% or more for *S. aureus* and 2.5% serum or more for *E. coli* (Fig. 2).

Incubation of granulocytes containing ingested bacteria after phagocytosis at a bacteria-to-granulocyte ratio of 10:1 instead of 1:1 gave almost no decrease in the number of viable intracellular bacteria during incubation in the absence of serum (*S. aureus*, 26.1%; *E. coli*, 31.6%). In the presence of 10% extracellular serum, the killing indices at 60 min amounted to 75.2 and 86.9% for *S. aureus* and *E. coli*, respectively.

Thus, it is clear that the intracellular killing of microorganisms by granulocytes depended on the presence of extracellular serum.

**Effect of extracellular serum on the intracellular killing of catalase-negative microorganisms.** To find out whether the intra-

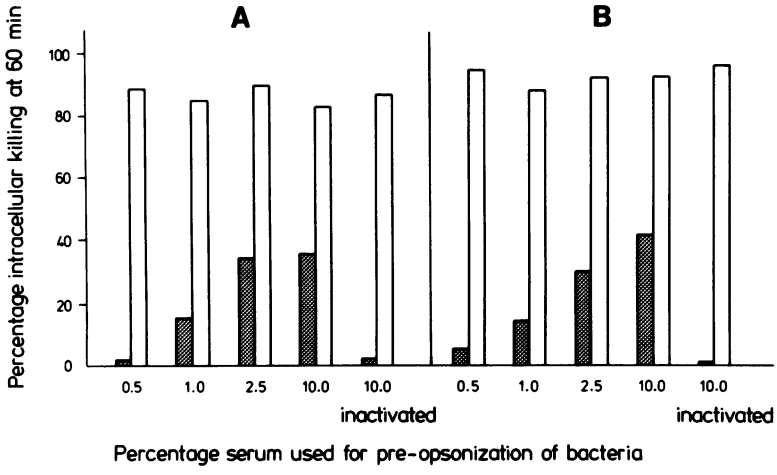


FIG. 1. *Effect of preopsonization on intracellular killing in the presence of 10% extracellular serum (open columns) and in the absence of serum (stippled columns). Phagocytosis was performed with microorganisms preopsonized as indicated for 15 min at 37°C at a bacteria-to-granulocyte ratio of 1:1. (Mean values of three experiments.) A, S. aureus; B, E. coli.*

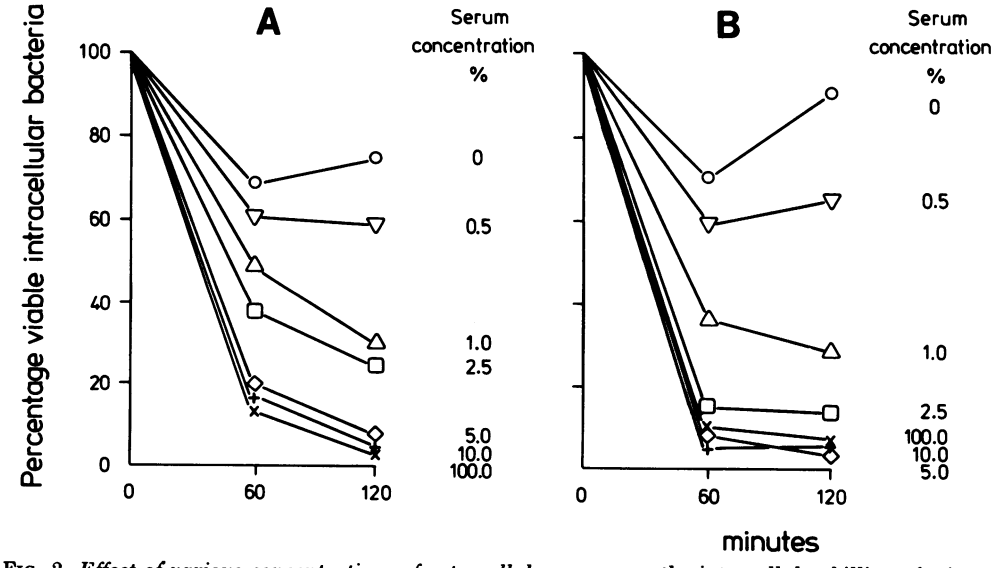


FIG. 2. *Effect of various concentrations of extracellular serum on the intracellular killing of microorganisms after ingestion for 3 min at a bacteria-to-granulocyte ratio of 1:1. (Mean values of three experiments.) A, S. auerus; B, E. coli.*

cellular killing of streptococci by granulocytes also requires stimulation by extracellular serum, the intracellular killing of *S. pneumoniae* and *S. pyogenes* by granulocytes was studied. Incubation of granulocytes containing ingested *S. pneumoniae* or *S. pyogenes*, after 3 min of phagocytosis of streptococci preopsonized with 10% serum and at a bacteria-to-granulocyte ratio of 1:1, showed that the decrease in the number of viable intracellular bacteria was greatest when 10% serum was present extracellularly (Table 4). The corresponding decrease in the absence of extracellular serum was significantly smaller ($P < 0.001$). These results indicate that the granulocytes killed catalase-negative microorganisms to a certain extent but that maximal killing required stimulation by extracellular serum.

**Effect of immunoglobulins on intracellular killing by granulocytes.** To find out which serum factors stimulate intracellular killing by granulocytes, the killing assay was performed in the presence of various concentrations of heat-inactivated serum. Incubation of granulocytes containing ingested *S. aureus* or *E. coli* led to a dose-related decrease in the number of viable intracellular bacteria which was greatest in the presence of 5% heat-inactivated serum or more (Fig. 3). Incubation of such granulocytes in the presence of 10% heat-inactivated serum adsorbed with *S. aureus* or *E. coli* to remove specific opsonic antibodies gave killing indices similar to those obtained with 10% heat-inactivated serum (Fig. 3). Thus, the intracellular killing of microorganisms was stimulated by heat-stable as well as heat-labile serum factors but not by specific opsonic antibodies.

To determine which heat-stable serum factors are able to stimulate intracellular killing, the killing-stimulatory activity of immunoglobulins was assessed. Since almost no differences were found in the course of intracellular killing of *S. aureus* and *E. coli* by granulocytes, all subsequent experiments were performed after ingestion of *S. aureus* at a bacteria-to-granulocyte ratio of 1:1. Incubation of granulocytes containing ingested *S. aureus* in the presence of various concentrations of IgM gave no increase in intracellular killing compared with the killing obtained during incubation with medium alone (Table 5). Incubation of granulocytes containing bacteria in the presence of extracellular IgG resulted in a concentration-related intracellular

TABLE 4. *Effect of extracellular serum on the intracellular killing of streptococci by granulocytes*[a]

| Incubation in the presence of: | Intracellular killing (%) at 60 min of: | |
|---|---|---|
| | *S. pneumoniae* | *S. pyogenes* |
| 10% serum | 99.9 ± 0.1 | 98.7 ± 0.5 |
| 10% inactivated serum | 96.0 ± 1.6 | 96.8 ± 1.2 |
| HBSS | 70.1 ± 5.7 | 81.8 ± 6.9 |

[a] All experiments were performed three times.

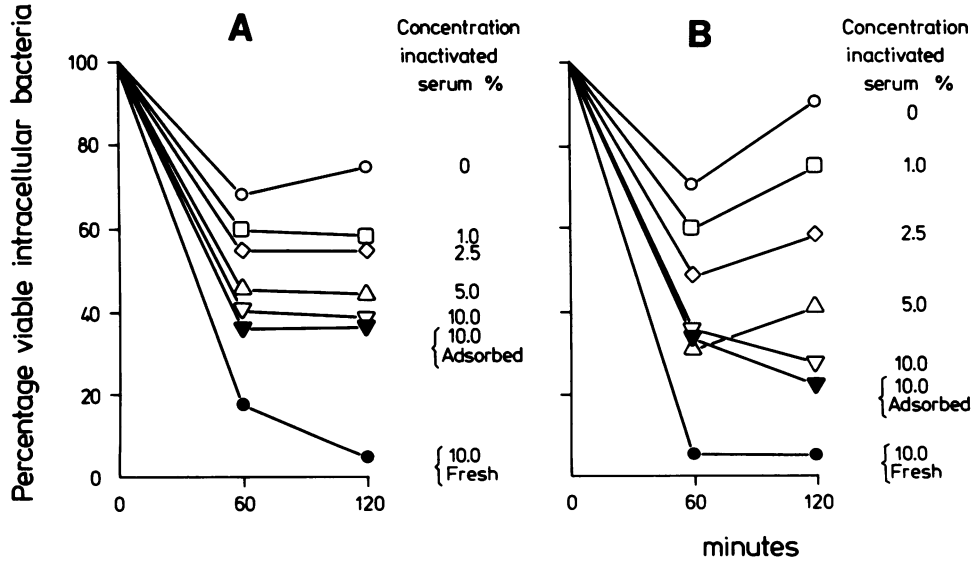


FIG. 3. *Effect of various concentrations of extracellular heat-inactivated serum and 10% heat-inactivated serum, adsorbed with the microorganisms under study to remove specific antibodies, on the intracellular killing of microorganisms after ingestion for 3 min at a bacteria-to-granulocyte ratio of 1:1. (Mean values of three experiments.) A, S. aureus; B, E. coli.*

killing index up to a maximum (with 400 μg of IgG or more per ml) close to that obtained in the presence of 5 to 10% heat-inactivated serum (containing about 600 to 1,200 μg of IgG/ml). Incubation of similar granulocytes in the presence of each of the four IgG subclasses gave intracellular killing similar to that in the presence of heat-inactivated serum when IgG1 or IgG2 was present; IgG4 stimulated intracellular killing only slightly, and IgG2 was not stimulatory at all (Table 5).

When pFc fragments of IgG were used instead of IgG, the killing index was the same as for IgG (Table 5). Incubation with $(Fab^1)_2$ fragments of IgG did not result in stronger stimulation of the intracellular killing than was obtained with medium alone (Table 5). Thus, IgG was able to stimulate the intracellular killing of *S. aureus* by granulocytes, most probably by interaction of the Fc part of the molecule with the Fc receptor in the cell membrane.

**Effect of the complement system on intracellular killing.** To find out whether the stronger stimulation of intracellular killing by fresh serum compared with inactivated serum is due to an extra stimulatory effect of factors of the complement system, we assessed the effect on intracellular killing of various sera with one or both pathways of complement activation blocked. Incubation of granulocytes containing *S. aureus* in the presence of either 10% fresh serum chelated with 10 mM EDTA, C3-deficient serum, or B-deficient serum chelated with 10 mM EGTA and supplemented with 2.5 mM $MgCl_2$, with both the classical and alternate pathways of complement activation impaired in all three cases, gave killing indices at 60 min that did not differ significantly from the index obtained in the presence of 10% heat-inactivated serum (Table 6). Incubation in the presence of sera in which the classical complement pathway was blocked (i.e., 10% serum chelated with 10 mM EGTA supplemented with 2.5 mM $MgCl_2$ or 10% serum deficient in C4) gave killing indices lying between those obtained in the presence of fresh and heat-inactivated serum (Table 6). Incubation in the presence of two sera in which the alternate pathway of complement activation was blocked (sera deficient in factor D or B) also led to an intermediate decrease in the numbers of viable intracellular bacteria, i.e., values between those obtained with fresh and heat-inactivated serum (Table 6). To investigate the involvement of C3 in the intracellular killing, C3-deficient serum was restored by adding purified C3 and used as a stimulator of intracellular killing. Under these conditions, intracellular killing was the same as in the presence of fresh serum (Table 6). Thus, the intracellular killing of microorganisms by granulocytes was stimulated by extracellular complement components. Since both the classical and the alternate path-

TABLE 5. *Effect of immunoglobulins on the intracellular killing of S. aureus by human granulocytes*[a]

| Incubation of granulocytes containing ingested *S. aureus* with: | Concn (μg/ml) | Intracellular killing at 60 min (%) | *n* | $P_1$ | $P_2$ | $P_3$ |
|---|---|---|---|---|---|---|
| HBSS | | 36.1 ± 18.2 | 15 | | <0.001 | <0.01 |
| 10% fresh serum | | 83.6 ± 13.4 | 15 | <0.001 | | <0.001 |
| 10% inactivated serum | | 59.8 ± 10.0 | 8 | <0.01 | <0.001 | |
| IgM | 125 | 38.7 ± 17.6 | 3 | NS | <0.001 | <0.05 |
| | 250 | 30.3 ± 16.3 | 3 | NS | <0.001 | <0.01 |
| | 500 | 42.3 ± 19.5 | 3 | NS | <0.001 | <0.1 |
| IgG | 50 | 35.3 ± 17.6 | 3 | NS | <0.001 | <0.01 |
| | 100 | 38.9 ± 12.0 | 3 | NS | <0.001 | <0.05 |
| | 200 | 41.1 ± 13.6 | 3 | NS | <0.001 | <0.05 |
| | 400 | 59.7 ± 13.1 | 4 | <0.05 | <0.01 | NS |
| | 800 | 58.0 ± 12.7 | 4 | <0.05 | <0.01 | NS |
| pFc fragments of IgG | 50 | 48.6 ± 11.3 | 4 | NS | <0.001 | NS |
| | 100 | 60.3 ± 15.3 | 4 | <0.05 | <0.01 | NS |
| | 200 | 58.9 ± 18.2 | 4 | <0.05 | <0.01 | NS |
| $(Fab^1)_2$ fragments of IgG | 200 | 35.9 ± 15.7 | 4 | NS | <0.001 | <0.01 |
| | 400 | 37.6 ± 10.3 | 4 | NS | <0.001 | <0.01 |
| | 800 | 40.3 ± 11.3 | 4 | NS | <0.001 | <0.05 |
| IgG1 | 500 | 62.6 ± 16.2 | 4 | <0.05 | <0.01 | NS |
| IgG2 | 500 | 38.3 ± 18.0 | 4 | NS | <0.001 | <0.05 |
| IgG3 | 500 | 59.8 ± 15.0 | 4 | <0.05 | <0.01 | NS |
| IgG4 | 500 | 45.3 ± 14.1 | 4 | NS | <0.001 | <0.05 |

[a] After phagocytosis at a bacteria-to-granulocyte ratio of 1:1. *n*, Number of experiments; $P_1$, compared with killing in the presence of HBSS; $P_2$, compared with killing in the presence of fresh serum; $P_3$, compared with killing in the presence of heat-inactivated serum. NS, Not significant ($P \geq 0.1$).

ways of complement activation are involved in this stimulation, a stimulatory effect of C3b is indicated.

**Participation of Fc and complement receptors in the stimulation of intracellular killing.** Since intracellular killing is stimulated by IgG and probably also by C3b, an interaction of these proteins with their respective receptors in the granulocyte membrane is likely. To investigate this point, the intracellular killing of *S. aureus* by granulocytes was measured after modulation of the membrane receptors.

Incubation of granulocytes containing ingested *S. aureus* with 1 mg of pronase per ml for 20 min at 37°C, followed by three washes to remove the excess pronase, gave cells with a decreased number of C receptors (Table 7), whereas the percentage of Fc receptor-positive granulocytes remained constant (Table 7). Incubation of these pronase-treated granulocytes for an additional 60 min with 10% fresh serum lowered the killing index relative to that of untreated cells, whereas incubation with 10% heat-inactivated serum gave an index comparable to that obtained in control experiments in which granulocytes containing ingested *S. aureus* were pretreated with HBSS (Table 7).

A second method was also used to alter the number of Fc and C3b receptors, i.e., incubation of the granulocytes with a rabbit antigranulocyte serum for 30 min at 37°C, which resulted in covering of the cell membrane and a diminished

TABLE 6. *Involvement of the classical and alternate pathways of complement activation on the intracellular killing of S. aureus by granulocytes*[a]

| Incubation of granulocytes containing ingested *S. aureus* with: | Intracellular killing at 60 min (%) | *n* | $P_1$ | $P_2$ | $P_3$ |
|---|---|---|---|---|---|
| HBSS | 36.1 ± 18.2 | 15 | | <0.001 | <0.01 |
| 10% fresh serum | 83.6 ± 13.4 | 15 | <0.001 | | <0.001 |
| 10% inactivated serum | 59.8 ± 10.0 | 8 | <0.01 | <0.001 | |
| 10% fresh serum chelated with 10 mM EDTA | 52.0 ± 13.3 | 4 | <0.1 | <0.001 | NS |
| 10% C3-deficient serum | 52.5 | 2 | | | |
| 10% C3-deficient serum + 100 μg of C3/ml | 85.0 | 2 | | | |
| 25% B-deficient serum chelated with 10 mM EGTA + 2.5 mM $MgCl_2$ | 53.2 ± 13.0 | 4 | <0.1 | <0.001 | NS |
| 10% fresh serum chelated with 10 mM EGTA + 2.5 mM $MgCl_2$ | 69.3 ± 19.3 | 4 | <0.01 | NS | NS |
| 10% C4-deficient serum | 66.3 ± 17.2 | 4 | <0.01 | <0.01 | NS |
| 25% D-deficient serum | 75.6 ± 16.0 | 4 | <0.001 | NS | <0.1 |
| 25% B-deficient serum | 66.8 ± 17.2 | 4 | <0.01 | <0.1 | NS |

[a] See Table 5.

TABLE 7. *Participation of Fc and C3b receptors in the stimulation of intracellular killing of S. aureus*[a]

| Treatment of granulocytes containing *S. aureus* with: | Granulocytes with Fc receptors | | Granulocytes with C3b receptors | | Intracellular killing at 60 min in presence of: | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | HBSS | | | 10% serum | | | 10% inactivated serum | | |
| | % | *n* | % | *n* | % | *n* | $P_1$ | % | *n* | $P_2$ | % | *n* | $P_3$ |
| HBSS | 80.1 ± 5.4 | 6 | 60.0 ± 14.0 | 4 | 35.8 ± 13.3 | 10 | | 78.8 ± 10.3 | 10 | | 55.8 ± 15.1 | 10 | |
| 1 mg of pronase/ml | 75.0 ± 7.3 | 4 | 0 | 4 | 30.3 ± 15.3 | 4 | NS | 44.6 ± 17.2 | 4 | <0.001 | 47.8 ± 13.1 | 4 | NS |
| 10% inactivated rabbit anti-granulocyte serum | 1 ± 1 | 4 | 1 ± 0.5 | 4 | 29.9 ± 14.2 | 4 | NS | 39.0 ± 10.0 | 4 | <0.001 | 33.1 ± 9.3 | 4 | <0.05 |
| 10% inactivated rabbit serum | 78.3 ± 7.2 | 3 | 55.3 ± 8.2 | 3 | 38.3 ± 18.2 | 3 | NS | 84.2 ± 15.3 | 3 | NS | 54.6 ± 14.3 | 3 | NS |

[a] After phagocytosis at a bacteria-to-cell ratio of 1:1, the granulocytes containing ingested bacteria were incubated with HBSS, 1 mg of pronase/ml, 10% antigranulocyte serum, or 10% inactivated rabbit serum for 20 min at 37°C, followed by three washes. $P_1$ compares the killing indices in the presence of HBSS with the killing index in the presence of HBSS after HBSS treatment; $P_2$ compares the killing indices in the presence of 10% serum with the killing index in the presence of 10% serum after HBSS treatment; $P_3$ compares the killing indices in the presence of 10% heat-inactivated serum with the killing index in the presence of 10% heat-inactivated serum after HBSS treatment. Other details as in Table 5.

number of detectable Fc and C3 receptors. Incubation of these antigranulocyte serum-treated granulocytes containing ingested bacteria in the presence of heat-inactivated or fresh serum gave a lower killing index at 60 min. In control experiments in which the granulocytes were treated with heat-inactivated normal rabbit serum, the killing indices were similar to those after pretreatment with HBSS (Table 7).

Although both treatments (pronase and antigranulocyte serum) used to modulate the number of Fc and C3b receptors in the cell membrane are nonspecific, so that other functions may also be affected, these findings indicate that optimal intracellular killing of microorganisms by granulocytes required normally functioning Fc and C3b receptors.

## DISCUSSION

Most of the published studies on the ingestion and intracellular killing of microorganisms by phagocytic cells have shown that IgG and C3b are the most important serum factors for maximal phagocytosis (7, 8, 10). These findings were confirmed by the present study, in which bacteria preopsonized with fresh serum, i.e., with IgG and C3b, were ingested to a higher degree than bacteria preopsonized with heat-inactivated serum, i.e., with IgG. In a previous study, we showed that without opsonization, ingestion does not occur (15). The main conclusion to be drawn from the present study is that these serum proteins with opsonic properties not only mediate ingestion but also are obligatory for optimal killing of microorganisms by granulocytes. This effect on intracellular killing was found to be twofold in that both the degree of opsonization and the kind of opsonin present on the surface of the bacteria affected the killing of these bacteria during the ingestion phase, and extracellular IgG and C3b were obligatory for maximal intracellular killing of ingested microorganisms.

Opsonization of *S. aureus* or *E. coli* with serum at concentrations as low as 0.1 to 0.5% resulted in ingestion of the bacteria, but this amount of opsonin was too low to induce the granulocytes to kill these bacteria during the ingestion phase or afterwards, i.e., during reincubation of granulocytes containing ingested bacteria in HBSS. A minimal serum concentration for opsonization, amounting to 2.5% for *S. aureus* and 0.5% for *E. coli*, was necessary to induce killing of these bacteria. The numbers of bacteria killed by granulocytes during the ingestion phase after preopsonization with fresh serum and with heat-inactivated serum showed that the latter gave almost no killing of bacteria. These findings indicate that when C3b is present on the bacteria as an opsonin, more bacteria are killed by the granulocytes during ingestion. From these experiments, it is not clear whether this killing occurs at the cell surface or intracellularly.

The conclusion that opsonins present on the bacterial surface affect the killing of these microorganisms is in accordance with the findings of Menzel and co-workers, who assessed the killing of *E. coli* in monolayers of polymorphonuclear leukocytes and showed that the effect of C3b on killing occurs mainly during the ingestion phase, when the bacteria become attached to the granulocyte surface (2, 19). This enhancement of killing by C3b is probably due to activation of the $O_2$-dependent killing mechanisms operating at the cell surface (19). Yamamura and Valdimarsson (32) showed that C3b is not essential for the ingestion of *Candida albicans* but participates in the phagocytic killing of these microorganisms. Li and Mudd found that although optimal ingestion of *S. aureus* by human polymorphonuclear leukocytes was achieved when the bacteria were opsonized with heat-inactivated serum, opsonization with fresh serum was required for maximal killing (18). Solberg and co-workers also described an effect of bacteria-bound IgG and complement on the bactericidal activity of human granulocytes (24, 25), but because their method measures ingestion and intracellular killing simultaneously, a definite conclusion cannot be drawn from their results. Other authors (7, 8, 10, 17) have also claimed an effect of immunoglobulins on the intracellular killing of microorganisms, but these effects proved to be due mainly to suboptimal opsonization of the bacteria, resulting in defective ingestion and intracellular killing.

The second conclusion, i.e., that the presence of extracellular serum is mandatory to obtain maximal killing of ingested bacteria, could be drawn because the method used made it possible to study the intracellular killing of ingested microorganisms independent of the ingestion phase (12, 13, 30). Maximal killing of ingested catalase-positive and -negative microorganisms is only obtained when granulocytes containing ingested bacteria are incubated in the presence of fresh serum. In the presence of heat-inactivated serum, the decrease in the number of viable intracellular bacteria was intermediate between the values obtained in the presence of serum and of HBSS. The stimulatory activity of heat-inactivated serum may be attributed to the interaction between IgG and the Fc receptor on the granulocyte membrane. This conclusion is based on the similarity between the stimulation of intra-

cellular killing by heat-inactivated serum and by IgG preparations, especially IgG subclasses IgG1 and IgG3, the only subclasses for which granulocytes carry Fc receptors (11, 20). Furthermore, pFc fragments of IgG showed stimulatory activity, whereas $(Fab^1)_2$ fragments were inactive in this respect, and intracellular killing was reduced in the presence of heat-inactivated serum after modulation of the number of Fc receptors on the granulocyte membrane by a rabbit anti-granulocyte serum.

The higher level of intracellular killing in the presence of fresh serum compared with heat-inactivated serum is very probably due to stimulation by C3b generated via both the alternate and classical pathways of complement activation. In all likelihood, the stimulation of the killing process by C3b involves an interaction with the C3b receptors on the granulocyte membrane, because treatment with pronase or anti-granulocyte serum to modify the number of C3b receptors on the cells also affected intracellular killing in the presence of fresh serum.

The mechanisms by which serum factors stimulate the killing process is still unknown. Stimulation due to triggering of the $O_2$-dependent bactericidal mechanisms in the granulocyte membrane is possible (5, 6), as is a mechanism by which the stimulus is transferred by a kind of shuttle process (27, 28) to the phagolysosome, where it exerts its effect.

The biological relevance of the finding that extracellular stimulation of granulocytes and monocytes (13, 14, 16) by IgG and C3b is indispensable for optimal intracellular killing of bacteria is obvious. During an infection, granulocytes, monocytes, and plasma proteins enter the site of inflammation, where the immunoglobulins and complement components act as opsonins to facilitate the ingestion of bacteria. However, as shown in this and previous studies (13, 14, 16), these proteins are also necessary for optimal intracellular killing by granulocytes and monocytes. It is conceivable that in certain stages of a disease extracellular stimulation by serum factors is impaired, which would result in defective killing at the site of inflammation and thus in prolonged infection. Indications that such a mechanism is likely have been supplied by studies on monocyte function during the acute state of certain diseases and during a period of clinical remission; although ingestion was normal under both conditions, killing was impaired during the acute state (9, 29).

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