Phagocytosis and killing of staphylococci by human polymorphonuclear and mononuclear leucocytes

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SUMMARY The phagocytosis and killing of 3H-thymidine-labelled Staphylococcus aureus by polymorphonuclear leucocytes (PMNs) and monocytes (MNs) obtained from 50 healthy donors were evaluated. In addition, extracellular factors that might influence phagocytosis and killing were studied. The method described gave highly reproducible results. No significant difference was observed in the phagocytic and killing functions of a single donor's PMNs and MNs when studied several times in one day and longitudinally over a period of 1-12 weeks for six donors tested. Likewise, no significant difference in uptake and killing was observed when bacteria were opsonised with sera from 11 different normal donors. When Staph. aureus opsonised with normal serum was added to the leucocytes in a ratio of 10:1 leucocyte, the uptake by PMNs and MNs from 50 donors after 20 minutes' incubation was 85% ± 7 standard deviation (SD) (range 75-98%) and 69% ± 11 SD (range 54-90%), respectively. The rate of uptake by MNs in the first three minutes of the assay period was only 60% of that by PMNs.

The phagocytosis and killing of staphylococci by leucocytes serve as an important defence mechanism against infection. Patients with defects in these phagocytic cells suffer from recurrent and severe infections, not infrequently with a fatal outcome (Baehner and Johnston, 1972; Cline, 1973; Davis et al., 1968; Goldman and Th'ing, 1973; Quie, 1973; Stossel, 1974; Quie et al., 1976).

Two morphologically different phagocytic cell populations are found in human peripheral blood — polymorphonuclear leucocytes (PMNs) and monocytes (MNs). Both are derived from precursor cells in the bone marrow. The MNs are capable of further differentiation into macrophages of the reticuloendothelial system (Cohn, 1968; van Furth, 1970; van Furth et al., 1972).

Davis et al. (1968) and Rodey et al. (1969) presented evidence that there were no differences in phagocytic activities between PMNs and MNs, but, more recently, studies from Cline and Lehrer (1968), Root et al. (1972), Steigbigel et al. (1974), and Peterson et al. (1977) have revealed that monocytes do not have the same phagocytic capacity (attachment and ingestion) as neutrophils. According to Steigbigel et al. (1974), the rate of killing once the bacteria had been ingested was similar for both leucocyte populations. Peterson et al. (1977), however, found that the rate of killing by MNs was slower than that by PMNs. This slower rate of killing appeared to be secondary to slower ingestion by MNs of attached bacteria.

As has been pointed out by Territo and Cline (1977), normal PMN and MN phagocytic function and the effect of serum opsonins on phagocytosis have to be defined before PMNs and MNs from patients with recurrent infections can be studied. Extracellular and bacterial factors that might influence these processes must be standardised. In this study the phagocytic and killing capacities of PMNs and MNs from 50 normal donors were evaluated, and the influences on these capacities of different bacterial cultural conditions, of varying bacteria to leucocyte ratios, and of different sera were investigated. The phagocytic and killing functions were also studied longitudinally in blood from six donors.

Normal values for the rates of phagocytosis and killing have been defined and the phagocytosis and
killing functions of PMNs and MNs from patients with recurrent infections can now be compared with these normal values.

Material and methods

Bacterial Strains and Radioactive Labelling

Staphylococcus aureus Ev (a clinical isolate) was used throughout the study. Several colonies from blood agar plates were inoculated into 5 ml Mueller-Hinton broth (Difco, Detroit, Mich, USA) containing 0-02 mCi thymidine-methyl-³H (specific activity 5 Ci/mmol, Amersham, Bucks, UK). After 18 hours' growth at 37°C the bacteria were washed three times in phosphate-buffered saline (PBS), pH 7-4, and adjusted to a final bacterial concentration of 2-5 × 10⁸ cfu/ml using a spectrophotometric method confirmed by pour plate colony counts.

Leucocytes

Blood was drawn into heparinised syringes (10 U heparin/ml blood) from healthy volunteers aged 18-69 (mean 28) years who were not taking any medication. PMNs and MNs were prepared using a method modified from that of Böyum (1968) in which the erythrocytes were allowed to settle for one hour in 6% dextran “70” (Fluka AG, Buchs, Switzerland) in normal saline (10 ml blood, 3 ml saline). The leucocyte-rich plasma was withdrawn and centrifuged at 160 g for 5 minutes. The pellet was resuspended in Eagle’s Minimal Essential Medium (MEM, without penicillin). Eight millilitres of this suspension was carefully layered onto 3 ml Ficoll-Isopaque (Ficoll, Pharmacia, Uppsala, Sweden; Isopaque, Nyegaard, Oslo, Norway). After centrifugation at 200 g for 30 minutes the mononuclear cell layer was removed with a Pasteur pipette. These cells were placed in a sterile plastic tube to which MEM was added in a volume ratio of 1:1. To obtain pure PMNs the remaining MEM was withdrawn and discarded, and 0-87% NH₄Cl in sterile water was added to the tube containing the red blood cell-PMN pellet, which was then rotated for 10 minutes at room temperature. After centrifugation for 10 minutes at 160 g the PMN pellet was washed twice more with 0-87% NH₄Cl to lyse the remaining red blood cells. Both the PMN and mononuclear cell preparations were resuspended finally in Hank’s Balanced Salt Solution containing 0-1% gelatin (HBSS), and total and differential counts were performed.

The final leucocyte pellets were adjusted to a concentration of 5 × 10⁶ PMNs and 5 × 10⁶ MNs/ml HBSS. Contamination of the PMN preparation by MNs and of the mononuclear cell preparations by PMNs was evaluated by Wright’s stained smears and never exceeded 1%.

Opsonins and Bacterial Opsonisation

Sera were collected from 11 normal donors and were stored both individually and as a serum pool. Serum was also obtained from a patient (Ev), with Staph. aureus bacteraemia. All sera were kept frozen in 1-0 ml aliquots at −70°C. Shortly before use the aliquots were thawed and diluted to the desired concentration in HBSS.

Three-tenths of a millilitre of each bacterial suspension was placed in plastic tubes (12 × 75 mm, Falcon, Oxnard, California, USA) and incubated for 30 minutes with 1-2 ml serum, followed by centrifugation at 1600 g for 15 minutes, the supernatants being discarded and the bacterial pellets resuspended in 1-5 ml HBSS.

Phagocytosis Mixtures and Determinations of Phagocytosis and Killing

Phagocytosis and killing assays were performed using a slight modification of a previously described method (Verhoef et al., 1977b). Into five polypropylene vials (Biovials, Beckman, Chicago, Ill, USA) 0-2 ml of the appropriate leucocyte suspension was added to 0-2 ml of the opsonised bacterial suspension. These phagocytosis mixtures were shaken at 150 rpm at an angle of 30° in a water-bath at 37°C. To determine leucocyte-associated radioactivity, biovials were removed after 3, 10, and 20 minutes' incubation and were placed in an ice-bath, and 2-5 ml ice-cold PBS was added. The vials were then centrifuged at 160 g for 5 minutes (at 4°C), and the leucocyte pellets were washed twice with ice-cold PBS to remove non-leucocyte-associated bacteria. The final leucocyte pellets, containing leucocyte-associated bacteria, were solubilised into 2-5 ml scintillation liquid (toluene containing fluor-alloy TLA [Beckman] and 20% Biosolve-3 [Beckman]) and counted in a liquid scintillation counter (Mark II, Nuclear-Chicago, Chicago, Ill, USA).

To determine total radioactivity (representing leucocyte-associated plus non-leucocyte-associated bacteria), to one biovial 2-5 ml ice-cold PBS was added. This biovial was then centrifuged at 2000 g for 15 minutes and the pellet was resuspended in 2-5 ml scintillation liquid. To determine the colony-forming units (cfu) which represented the viable leucocyte-associated bacterial population after 20 minutes' incubation, 2-5 ml ice-cold PBS was added to the fifth biovial. This vial was then centrifuged at 160 g for 5 minutes (4°C) and the leucocyte pellet
was washed twice with ice-cold PBS. The final leucocyte pellet was then disrupted by vigorous mixing in 2.5 ml sterile distilled water. After appropriate dilution, 20 μl samples were pour-plated in nutrient agar, and colonies were counted after 48 hours' incubation at 37°C. The initial number of cfu added to the biovials at time zero was determined spectrophotometrically. The percent of the total bacterial population that was leucocyte-associated at a given sampling time (% uptake) was calculated using formula 1:

\[
1 \text{ % uptake} = \frac{\text{dpm in leucocyte pellet}}{\text{dpm in total bacterial pellet}} \times 100.
\]

The leucocyte-associated bacterial population that was viable at 20 minutes' incubation was calculated according to formula 2:

\[
2 \text{ % viable leucocyte-associated bacteria} = \frac{\% \text{ cfu in leucocyte pellet}}{\% \text{ uptake}} \times 100
\]

where the denominator was obtained from formula 1 and the numerator was derived using formula 3:

\[
3 \text{ % cfu in leucocyte pellet} = \frac{\text{cfu in leucocyte pellet}}{\text{cfu at time zero}} \times 100.
\]

In most experiments the bacteria:leucocyte ratio was approximately 10:1. To test the maximum uptake capacity of PMNs and MNs, bacteria, opsonised in 5% pooled serum, were concentrated 10, 15, 20, and 40 times to obtain a bacteria:leucocyte ratio of 100:1, 150:1, 200:1, and 400:1.

Control experiments, as outlined by Stossel (1975), showed that (1) as the bacteria:leucocyte ratio was increased, the rate of uptake was saturated; (2) there was no uptake at 'zero' time (samples processed immediately after constituting the phagocytosis mixtures); and (3) there was essentially no uptake under ice-bath conditions, all of which indicated that bacterial ingestion by leucocytes was being measured.

Results

BACTERIAL PHAGOCYTOSIS AND KILLING

BY PMNS AND MNs

Before the phagocytic function of PMNs and MNs was studied, the effect of certain extracellular factors on phagocytosis had to be determined. Firstly, the reproducibility on the assay had to be established. The uptake of Staph. aureus Ev opsonised in 5% pooled serum by PMNs and MNs from the same donor was determined fivefold on one day. The results are given in Figure 1. No significant differences in uptake were observed in the five assays.

Fig. 1 Phagocytosis of Staph. aureus Ev by PMNs and MNs obtained from a single donor and assayed fivefold (● MNs; ○ PMNs).

The percentage of leucocyte-associated bacteria after 20 minutes' incubation with PMNs varied from 83 to 90% and with MNs from 58 to 74%. The effect of using bacteria grown in five separate cultures on phagocytosis was also studied. No significant difference in uptake of bacteria from the five cultures was found (Fig. 1). To study the effect of using serum from different donors on uptake and killing, bacteria were opsonised with a 5% concentration of sera from 11 normal donors (Fig. 2). Only minimal differences in the rate of phagocytosis were observed. After 20 minutes the mean percentage of leucocyte-associated bacteria was 86% ± 6 (SD). The percentage of viable leucocyte-associated
bacteria after 20 minutes' incubation was 16% ± 4 (SD). When HBSS instead of serum was used for opsonisation, essentially no phagocytosis occurred (less than 5% uptake).

After the reproducibility of the assay had been determined the phagocytic capacities of PMNs and MNs from 50 healthy donors were determined (Fig. 3, shown as mean percentage with extremes). As can be seen, 85% ± 7 SD (range 75-98%) and 69% ± 11 SD (range 54-90%) of the bacteria were taken up by the PMNs and MNs respectively. For six donors PMN and MN function was studied again after an interval of 1-12 weeks (Table 1). No significant differences were detected when PMNs and MNs from the same donor were retested.

**EFFECT OF BACTERIAL INOCULUM SIZE ON PHAGOCYTOSIS AND KILLING BY PMNS AND MNs**

The above assays were carried out at a bacteria:leucocyte ratio of 10:1. To study the maximum uptake of Staph. aureus Ev by PMNs and MNs, bacteria were concentrated after opsonisation and added to PMNs in a bacteria:PMN ratio of 10:1, 100:1, 150:1, 400:1, and to MNs in a bacteria:MN ratio of 10:1, 100:1, and 150:1 (Fig. 4). As shown, the maximum number of bacteria taken up by one PMN was 232 and by one MN 53 bacteria.

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### Table 1 Percentage uptake of Staph. aureus by PMNs from six different donors determined on different days

<table>
<thead>
<tr>
<th>Uptake at (min)</th>
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<td>3</td>
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<td>10</td>
<td>76</td>
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<td>20</td>
<td>78</td>
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<tr>
<td>% viable*</td>
<td>9</td>
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<tr>
<td>% % of viable leucocyte-associated bacteria at 20 minutes.</td>
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| nt = not tested.

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**Fig. 3** Phagocytosis of Staph. aureus Ev by PMNs and MNs from 50 different donors: ○ PMNs (mean % of uptake with extremes); ● MNs (mean % of uptake with extremes). % of viable leucocyte-associated bacteria at 20 min: 12 ± 7 (SD) (PMNs); 24 ± 12 (SD) (MN s).

**Fig. 4** Phagocytosis of Staph. aureus Ev opsonised with 5% pooled human serum by PMNs (A) and by MNs (B) at different leucocyte:bacteria ratios.
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When bacteria were opsonised with immune serum and added to PMNs in a high bacteria:PMN ratio, the rate of phagocytosis was nearly twice as fast as that of bacteria opsonised with normal serum (Fig. 5).

Fig. 5 Phagocytosis by PMNs of Staph. aureus Ev opsonised with 5% immune serum at different PMN: bacteria ratios.

The percentages of viable leucocyte-associated bacteria after 20 minutes' incubation with PMNs and MNs at different bacteria-inoculum sizes are given in Table 2. When bacteria opsonised with normal human serum were incubated with PMNs at a ratio of 10 bacteria:1 PMN, the percentage of viable leucocyte-associated bacteria was 11%. When a ratio of 400 bacteria:1 PMN was used 16% of leucocyte-associated bacteria were viable. Regardless of the number of bacteria used, more than 80% of the bacteria ingested by or attached to the PMNs were killed. The number of viable MN-associated bacteria was higher. When a ratio of 10 bacteria:1 MN was used about 27% of the MN-associated bacteria were viable. When the bacteria-inoculum size was 15 times greater, 37% of MN-associated bacteria were viable. Thus, although the killing by MNs was less efficient than by PMNs, killing by MNs was also independent of the number of bacteria ingested.

**EFFECT OF CONCENTRATION OF LEUCOCYTES ON PHAGOCYTOSIS BY PMNS AND MNS**

In the experiments described above $5 \times 10^6$ leucocytes/ml were used. To study the effect of the concentration of PMNs and MNs required for effective phagocytosis, PMNs, MNs, and bacteria were diluted in HBSS before the phagocytosis mixtures were constituted; the bacteria:leucocyte ratios were held constant at 10:1.

As can be seen in Fig. 6, lowering the concentration of PMNs and MNs decreases the rate of phagocytosis. The effect of lowering the concentration of leucocytes on bacterial uptake was more pronounced for MNs than for PMNs.

![Fig. 6 Phagocytosis of Staph. aureus Ev by PMNs (A) and MNs (B) with varying numbers of leucocytes. In all cases bacteria were added to the leucocytes in a bacteria:leucocyte ratio of 10:1.](image_url)
Discussion

In this study, the phagocytic and killing functions of PMNs and MNs from 50 healthy donors were evaluated. In addition, extracellular factors that might influence phagocytosis and killing were studied. The method used, in which uptake and killing of $^3$H-thymidine-labelled Staph. aureus were measured, gave highly reproducible results. No significant variation was observed in the phagocytic and killing functions of a single donor's PMNs and MNs when studied several times in one day and longitudinally over a period of 1 to 12 weeks for the six donors tested. Likewise, no significant difference in uptake and killing was observed when bacteria were opsonised with sera from 11 different donors. When Staph. aureus was added to the leucocytes in a ratio of 10 bacteria:1 leucocyte, for the 50 donors tested the uptake by PMNs and MNs after 20 minutes' incubation was 85% ± 7 SD (range 75-98%) and 69% ± 11 SD (range 54-90%), respectively. The rate of uptake by MNs in the first three minutes of the assay period was only 60% of that of PMNs. These results support the conclusions of other investigators (Cline and Lehrer, 1968; Root et al., 1972; Steigbigel et al., 1974; Peterson et al., 1977), namely, that the phagocytic capacity of MNs is less than that of PMNs. However, whereas Steigbigel et al. (1974) found that only 10% of Staph. aureus added to MNs and 45% of Staph. aureus added to PMNs were taken up after one hour's incubation, we found a significantly higher uptake by both leucocyte populations after 20 minutes' incubation. This discrepancy is most likely explained by methodological differences. In our assay phagocytosis mixtures were incubated in separate biovials. One biovial was incubated for 3, one for 10, and another for 20 minutes. In these biovials the percentage of uptake by leucocytes was determined. In other assay systems the phagocytic mixture was incubated in a tube, and samples were drawn at different times from that tube. We found, however, that when samples were drawn from a plastic tube after 20 minutes, results were sometimes influenced by the MNs sticking to the sides of the tube, even when the tubes were coated with silicon. Occasionally this was also seen after 30 minutes' incubation with PMNs. It is possible that the low uptake of Staph. aureus by MNs and the variation in the function of MNs of different donors observed by Steigbigel et al. (1974) were due to MNs sticking to the sides of the tubes (van Furth and van Zwet, 1973).

Other investigators (Steigbigel et al., 1974; Peterson et al., 1977) have found that once bacteria have been phagocytised, the majority are rapidly killed by both PMNs and MNs. In this study, 76% ± 12 (SD) of the MN-associated bacterial population were killed after 20 minutes' incubation compared with 88% ± 7 (SD) of the PMN-associated bacterial population. There was little difference in the killing capacities of MNs and PMNs among the 50 individual donors tested. When bacteria were added to leucocytes in different ratios it was shown that a single PMN could ingest approximately 230 Staph. aureus compared with only 50 by a single MN. These numbers are higher than previously reported (Craig and Sutter, 1966; Peterson et al., 1976). However, in our experiments, bacteria were opsonised in 5% pooled serum before concentration of the bacteria to obtain a higher inoculum, instead of opsonising different concentrations of bacteria in the same serum concentration and then adding these bacteria to the leucocytes.

Although a saturation point of phagocytosis was reached as the bacteria:leucocyte ratio was increased, the percentage of cell-associated bacteria that was killed by the PMNs and MNs remained more or less constant, suggesting that the major limiting factor in leucocyte 'handling' was the phagocytic capacity of these cells rather than their bactericidal capacity.

The slower uptake of opsonised bacteria by MNs and the lower maximum capacity of MNs presumably reflect fewer and/or less efficient receptor mechanisms on these cells compared to PMNs (Peterson et al., 1977; Verhoef et al., 1977a). Opsonisation in immune serum enhanced the maximum uptake of Staph. aureus by leucocytes. It is possible that when bacteria are opsonised with immune serum more receptors for the Fc fragment of the IgG molecule on the leucocyte membrane were utilised for attachment. It has previously been shown that Staph. aureus opsonised in non-immune serum were taken up by PMNs mainly through the receptor for C3b on the leucocyte membrane (Verhoef et al., 1977a). It is also possible that metabolic differences between MNs and PMNs rather than differences in receptors account for the lower phagocytic capacity of MNs. Sagone et al. (1976) showed that the metabolism of MNs differs from that of PMNs. They presented evidence that superoxide dismutase added to the incubation mixture of bacteria and MNs did not result in increased hexosemonophosphate shunt activity in response to phagocytosis.

As it was found that lowering the number of PMNs and MNs while at the same time keeping the bacteria:leucocyte ratio constant had a significant effect on bacterial uptake, especially by MNs, it is obviously essential to keep the number of leucocytes constant when studying the phagocytic capacities of leucocytes from different donors.
In a recent editorial (Lancet, 1976) it was stressed that leucocyte functions should be tested in patients with infectious diseases who have an unduly severe or protracted course or when infection becomes recurrent or chronic in the absence of predisposing conditions such as hypogammaglobulinaemia. The phagocytosis and killing assay evaluated in this study provided reliable and reproducible results and could therefore be used for testing leucocytes from patients with such disorders.

References


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