

Influence of Temperature on Opsonization and Phagocytosis of Staphylococci

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The effect of incubation temperatures of 41, 37, and 4°C on phagocytosis was investigated using human neutrophils and [³H]thymidine-labeled staphylococci. Depressed phagocytosis was observed at 41 and 4°C. At 41°C diminished staphylococcal uptake resulted from decreased attachment of bacteria to leukocytes; the inhibitory effect at 4°C was secondary both to decreased opsonization and to reduced attachment to leukocytes. In contrast to the findings with normal serum, opsonization with heat-inactivated serum appeared to be relatively intact at 4°C. By incubating samples in lysostaphin, it was determined that the process of bacterial ingestion as well as that of attachment was adversely affected by incubation temperatures of 41 and 4°C.

The outcome of the encounter between staphylococci and phagocytic cells is dependent upon the integrity of four separate but related processes. For effective phagocytosis, the bacteria must be opsonized by serum factors, attached to receptors on the phagocytic membrane, internalized (ingestion), and finally killed (4, 8). Using macrophages and sheep erythrocytes, other investigators have developed *in vitro* assay systems that allow several of these processes to be studied separately (7). Lowered incubation temperature has been frequently used in such systems for this purpose (3).

In the studies reported here, human neutrophils and [³H]thymidine-labeled staphylococci were used to determine the effects of low temperature (4°C) on the processes of opsonization, attachment, ingestion, and bacterial killing. As diminished staphylococcal uptake had been previously observed at elevated temperature (6), the effect of 41°C on these processes was also studied.

MATERIALS AND METHODS

Bacterial strains. *Staphylococcus aureus* Wood 46 and *Staphylococcus epidermidis* Her, an isolate from a patient with bacterial endocarditis, were used.

Radioactive labeling. A 0.1-ml portion of an overnight culture of bacteria was inoculated into 10 ml of Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) containing 0.02 mCi of [*methyl*-³H]thymidine (specific activity, 6.7 Ci/mmol; New England Nuclear, Boston, Mass.). After 18 h of growth at 37°C, the bacteria were washed three times in phosphate-buffered saline (PBS), pH 7.4. A final bacterial concentration of 5×10^8 colony-forming units (CFU) per ml of PBS was obtained using a spectrophotometric method confirmed by pour plate colony counts.

Leukocytes. Forty milliliters of blood was drawn from healthy donors in a syringe containing 200 U of heparin. The erythrocytes were sedimented for 1 h in 6% dextran 70 (Cutter Laboratories, Berkeley, Calif.) in normal saline (10 ml of blood/3 ml of saline). The leukocyte-rich plasma was withdrawn and centrifuged at $160 \times g$ for 5 min. The resulting pellet was washed twice in heparinized saline (10 U of heparin/10 ml of saline). Using a standard method, total and differential leukocyte counts were performed. The final leukocyte pellet was resuspended to a final concentration of 10^7 polymorphonuclear leukocytes per ml of Hanks balanced salt solution with 1% gelatin (HBSS).

Bacterial opsonization. One-milliliter portions of normal serum, pooled from five healthy donors, were kept frozen at -70°C. Shortly before use, portions were thawed and diluted to a concentration of 10% in HBSS. Heat-inactivated serum was prepared by heating portions at 56°C for 1 h and then diluting to a concentration of 10% in HBSS. In some experiments, the diluted serum was added to the bacterial and leukocyte suspensions directly, after which the phagocytosis assays were performed. In other experiments, bacteria were first incubated with the serum for 30 min at temperatures of 41, 37, and 4°C followed by centrifugation at $1,600 \times g$ at 4°C and resuspension in HBSS. The bacteria were then added to the leukocytes.

Phagocytosis mixtures. Mixtures of 1.5 ml of leukocyte suspension, serum, and bacteria were prepared in plastic tubes (12 by 75 mm; Falcon Plastics, Oxnard, Calif.) in a volume ratio of 5:4:1. The final bacteria-polymorphonuclear leukocyte ratio was approximately 10:1. In those experiments in which opsonized bacteria were added to the leukocytes, the same final volume and bacteria-leukocyte ratio were used. In all experiments the leukocytes were preincubated for 1 h at the desired assay temperature before the phagocytosis mixtures were constituted. During the incubation and assay periods, the re-

spective leukocyte suspensions and phagocytic mixtures were tumbled at 10 rpm in a rotating rack (Fisher Roto-Rack; Fisher Scientific Co., Chicago, Ill.). In one series of experiments, leukocytes were incubated for 1 h in undiluted normal serum in place of HBSS at 37 and 41°C followed by centrifuging at $160 \times g$ and washing the leukocyte pellets with HBSS.

Determination of neutrophil bacterial uptake and killing. Immediately after the phagocytosis mixtures were constituted, duplicate 5- μ l samples were taken for plating and determination of the total CFU added to the mixture at time zero. To determine the leukocyte-associated bacterial population, duplicate 100- μ l samples were taken from the phagocytosis mixtures with an Eppendorf pipette at 3-, 10-, and 20-min intervals and placed in 3 ml of cold PBS in polypropylene vials (Bio-Vials; Beckman Instruments, Inc., Chicago, Ill.). The vials were centrifuged for 5 min at $160 \times g$ at 4°C, and the leukocyte pellets were washed twice with ice-cold PBS. The final leukocyte pellets were disrupted with 2.5 ml of sterile distilled water. After vigorous mixing, samples were taken with a 5- μ l Eppendorf pipette for plating in nutrient agar and determination of CFU after an 18-h incubation at 37°C. These CFU represented the viable leukocyte-associated bacteria. The total leukocyte-associated population of bacteria (alive and dead) was determined by centrifuging the final suspensions at $1,600 \times g$ for 15 min and solubilizing the pellets in 2.5 ml of scintillation liquid (toluene containing fluoralloy [Beckman] and 20% Biosolve-3 [Beckman]) and counting in a liquid scintillation counter (Beckman LS-250). To determine the total bacterial counts per minute (representing leukocyte-associated plus non-leukocyte-associated bacteria), duplicate 100- μ l samples were taken at the end of the assay period, placed in 2.5 ml of water, and centrifuged at $1,600 \times g$ for 15 min. The pellets were resuspended in scintillation liquid and counted. An average of the duplicate values was used in all calculations.

The percentage of the total bacterial population that was leukocyte associated at a given sampling time (% uptake) was calculated using the formula:

$$\% \text{ uptake} = \frac{\text{cpm in leukocyte pellet}}{\text{cpm in total bacterial pellet}} \times 100 \quad (\text{i})$$

The leukocyte-associated bacterial population that was viable at a given sampling time was calculated using the formula:

$$\% \text{ viable leukocyte-associated bacteria} = \frac{\% \text{ CFU in leukocyte pellet}}{\% \text{ uptake}} \times 100 \quad (\text{ii})$$

where the denominator was obtained from formula (i) and the numerator was derived using formula (iii):

$$\frac{\text{CFU in leukocyte pellet at sampling time}}{\text{CFU at time 0}} \times 100 \quad (\text{iii})$$

In one series of experiments, samples were placed in PBS alone and in PBS containing 1 μ g of lysostaphin per ml (Schwarz/Mann, Orangeburg, N.Y.) in order to lyse the extracellular population of bacteria, including those bacteria which were attached to but not ingested by the leukocytes. The lytic activity of this enzyme proceeds at a slower rate at 4 than at 37°C. Therefore, in assays carried out at 4°C samples were incubated for 1 h at 4°C before centrifuging and washing the leukocytes; in assays at 37°C samples were incubated for 30 min at 37°C. Bacterial uptake was calculated as outlined above. The difference between the percentage of uptake calculated from samples placed in PBS alone and samples placed in PBS containing lysostaphin was considered to be representative of the attached bacterial population. To confirm lysostaphin activity, control mixtures which contained the same concentration of bacteria and serum but no leukocytes were also sampled into PBS and PBS containing lysostaphin and incubated at 37 and 4°C, as outlined above, and after centrifugation at $1,600 \times g$ the bacterial pellets were suspended in scintillation liquid and counted.

Experiments comparing the effect of different temperatures were carried out simultaneously at the different temperatures and were repeated on at least three separate days with leukocytes from different normal donors.

RESULTS

Effect of temperature on staphylococcal uptake by neutrophils. The effect of temperatures of 41, 37, 22, and 4°C on leukocyte uptake of *S. aureus* Wood 46 and *S. epidermidis* Her was studied by preincubating each component of the phagocytosis mixture (leukocytes, bacteria, and normal serum) separately at these respective temperatures for 1 h prior to constituting the phagocytosis mixtures. Figure 1 represents the results using *S. aureus* Wood 46. After a 20-min incubation, 71% of the bacteria were leukocyte associated in the assay performed at 37°C compared with 55, 24, and 4% in assays carried out at 22, 41, and 4°C, respectively. Similar results were obtained using *S. epidermidis* Her. No significant difference in bacterial uptake was found when incubation temperatures of 32 and 37°C were compared (data not presented). Comparable results were obtained when leukocytes were preincubated in serum and in HBSS. Although leukocytes which had been incubated at 4°C phagocytized staphylococci normally in assays carried out at 37°C, leukocytes incubated at 41°C continued to show diminished phagocytic capacity at 37°C (data not shown).

Effect of temperature on staphylococcal opsonization. To study whether the diminished bacterial uptake observed at 41 and 4°C

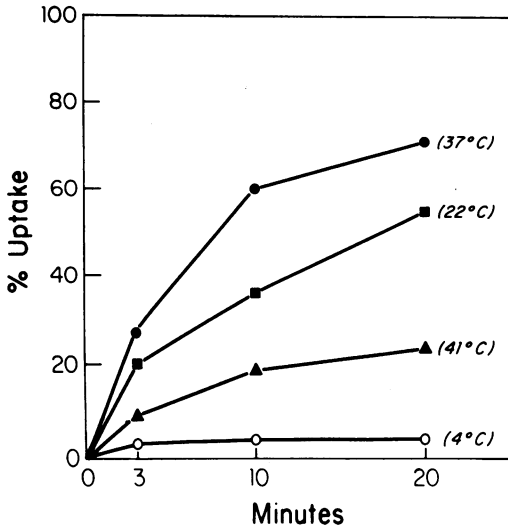


FIG. 1. Effect of temperature on neutrophil uptake of *S. aureus* Wood 46. Leukocytes, bacteria, and normal serum were incubated separately for 1 h at 41°C (▲), 37°C (●), 22°C (■), and 4°C (○) before constituting the phagocytosis mixtures and performing assays at these respective temperatures.

was due in part to decreased opsonization at these temperatures, bacteria were incubated with normal serum at 41, 37, and 4°C prior to being added to the leukocytes. After centrifugation at 4°C, the bacterial pellets were resuspended in HBSS. Bacteria were also opsonized in the same manner in heat-inactivated serum. Leukocytes were then added, and assays were performed at 37°C. Figure 2 shows the results of this experiment using *S. epidermidis* Her. Uptake of bacteria which had been opsonized with normal serum at 37 and 41°C was similar, but there was depressed phagocytosis of bacteria opsonized with normal serum at 4°C. In contrast, phagocytosis of bacteria opsonized with heat-inactivated serum at 37 and 4°C was similar. Whereas 86% of bacteria opsonized with normal serum at 37°C were leukocyte associated after 20 min, only 66% of bacteria opsonized at 4°C were taken up. However, uptake of staphylococci opsonized with normal serum at 4°C was significantly better than that of bacteria opsonized with heat-inactivated serum at 37°C (66 and 36%, respectively). Similar results were obtained when *S. aureus* Wood 46 was studied and when leukocytes from three different donors were used. Apparently, complement-dependent opsonic activity was diminished at 4°C but immunoglobulin G opsonic activity was intact at this temperature.

Effect of lysostaphin on measurement of staphylococcal uptake. In an attempt to determine the number of bacteria attached to but not ingested by the leukocytes and to study the effect of temperature on the processes of attachment and ingestion, samples were taken from phagocytosis assays performed at 41, 37, and 4°C and incubated in both PBS and in PBS containing lysostaphin.

When equal concentrations of *S. aureus* Wood 46 opsonized with normal serum at 37°C were added to leukocytes that had been incubated for 1 h at 41, 37, and 4°C, diminished uptake was observed at 41 and 4°C as described above. Treatment of the samples with lysostaphin resulted in a decrease of measured leukocyte-associated bacteria (counts per minute) at all three temperatures (Fig. 3). The effect of lysostaphin was greater at 41 and 4°C. At 37°C, lysostaphin treatment of the samples reduced the measured uptake at 20 min by 17% of the total, compared with a reduction of approximately 60% at 41 and 4°C. Similar results were obtained using polymorphonuclear leukocytes from three different normal donors. Samples from control mixtures containing the same concentration of bacteria in normal serum without leukocytes showed 86 and 53% loss of centrifugeable (1,600 × g) radioactivity after incubation at 37 and 4°C, respectively. When bacteria were added to leukocytes in the absence of an opsonic source, less than 10% of the bacteria were leukocyte associated after

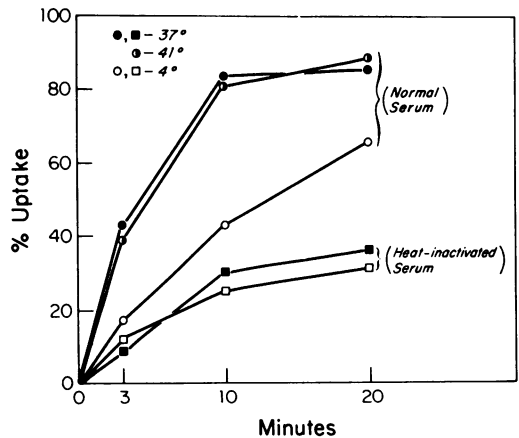


FIG. 2. Effect of temperature on opsonization of *S. epidermidis* Her. Bacteria were incubated for 30 min with normal serum at 41°C (○), 37°C (●), and 4°C (○) and with heat-inactivated serum at 37°C (■) and 4°C (□). After centrifuging at 4°C the bacteria were resuspended in HBSS, leukocytes were added, and assays were performed at 37°C.

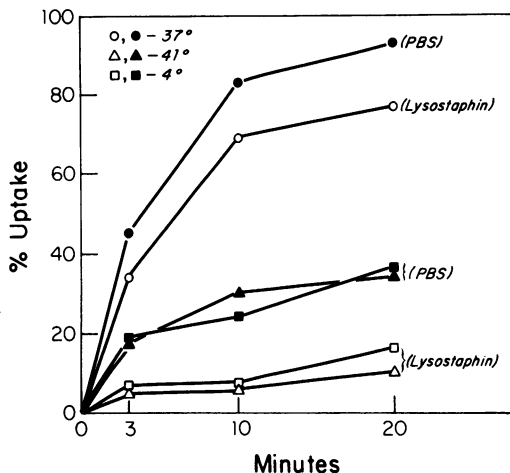


FIG. 3. Effect of lysostaphin on measurement of staphylococcal uptake. Equal concentrations of *S. aureus* Wood 46 were incubated with normal serum at 37°C for 30 min. After centrifuging, the bacteria were resuspended in HBSS, and leukocytes that had been incubated for 1 h at 37, 41, and 4°C were added. Samples were taken from the 37°C assay and incubated in PBS (●) and in PBS containing lysostaphin (○), from the 41°C assay and incubated in PBS (▲) and in PBS containing lysostaphin (△), and from the 4°C assay and incubated in PBS (■) and in PBS containing lysostaphin (□).

washing the leukocyte pellets, indicating that lysostaphin was not reducing measured uptake by an effect on non-leukocyte-associated extracellular bacteria.

Effect of temperature on bacterial killing. To determine the effect of elevated incubation temperature (41°C) on neutrophil killing of staphylococci, studies in which samples were taken from disrupted leukocyte pellets for determination of viable bacteria were performed. Figure 4 represents the findings of this study using *S. aureus* Wood 46 and *S. epidermidis* Her. When the killing capacity of leukocytes that had been incubated for 1 h at 41°C was compared with that of leukocytes incubated at 37°C, a significantly greater percentage of bacteria remained viable when associated with the leukocytes incubated at 41°C. Similar results were obtained using leukocytes from two other donors; a 1.5- to 2-fold greater number of bacteria were viable when associated with leukocytes preincubated at 41°C than with leukocytes preincubated at 37°C.

DISCUSSION

Both low (4°C) and elevated (41°C) temperatures were found to significantly inhibit the

uptake of an *S. aureus* and an *S. epidermidis* strain by human neutrophils. When the three components of the phagocytosis mixture (neutrophils, bacteria, and serum) were incubated separately for 1 h before performing the phagocytosis assay, there was essentially no bacterial uptake at 4°C. Incubation at 41°C reduced bacterial uptake by over 50% when assayed at 20 min.

As the diminished bacterial uptake observed in the initial experiments could be explained by either decreased opsonization at 4 and 41°C and/or reduced attachment, a study was undertaken to determine the degree of opsonic activation at 4 and 41°C using bacterial uptake by leukocytes as a functional assay system. Whereas opsonization with normal serum was found to be intact at 41°C, diminished opsonization was observed at 4°C. In contrast to the findings with normal serum, opsonization with heat-inactivated serum appeared to be relatively intact at 4°C. Although opsonization at 4°C with normal serum was not optimal, uptake of bacteria so opsonized was significantly greater than that of bacteria which had been opsonized with heat-inactivated serum at 37°C. In a previous study, immunoglobulin G had been found to be the major heat-stable opsonic factor and the alternate complement system the primary heat-labile serum opsonic system for the two staphylococ-

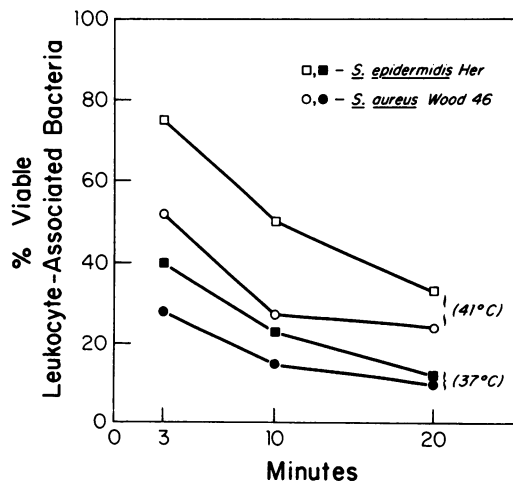


FIG. 4. Effect of temperature (41 and 37°C) on survival of leukocyte-associated bacteria. *S. aureus* Wood 46 was added to leukocytes that had been incubated for 1 h at 37°C (●) and 41°C (○). *S. epidermidis* Her was added to leukocytes that had been incubated for 1 h at 37°C (■) and 41°C (□). Normal serum was used as an opsonic source. Bacterial viability was determined as described in Materials and Methods.

cal strains used in these experiments (J. Verhoef et al., submitted for publication). These findings therefore suggest that whereas opsonization with immunoglobulin G is relatively normal at 4°C, activation of the alternative complement opsonic system proceeds more slowly at this temperature.

The decreased staphylococcal uptake observed at 4°C appeared to be due in part to decreased opsonization; however, the degree of inhibition appeared to be too great to be explained on this basis alone. As bacteria that had been opsonized with normal serum at 37°C were also poorly taken up when added to leukocytes at 4°C, diminished attachment seemed to be the major defect responsible for reduced uptake at lowered temperature. As opsonization was normal at 41°C, diminished attachment was responsible for the decreased phagocytosis observed at this temperature. Whereas the attachment defect observed at incubation temperatures of 4°C disappeared after warming the leukocytes to 37°C, the defect found at 41°C was relatively irreversible.

To separate the processes of attachment and ingestion, samples from the phagocytosis mixtures containing *S. aureus* Wood 46 were incubated in lysostaphin prior to washing and differential centrifugation of the leukocytes. This muralytic enzyme was used to lyse the attached bacterial population, and thereby measured bacterial uptake would primarily represent the ingested population of bacteria. After incubation with lysostaphin, the measured staphylococcal uptake was lower at all assay temperatures. Whereas a majority of the leukocyte-associated bacteria were removed after lysostaphin treatment of samples taken from the 41 and 4°C assays, less than 20% of the bacteria were removed by lysostaphin treatment of samples from the 37°C assay. These results suggest that although bacterial attachment was the process most significantly affected by abnormal temperature, the process of ingestion is inhibited as well.

When compared with assays performed at 37°C, a greater percentage of leukocyte-associated bacteria remained viable when incubated with leukocytes exposed to 41°C. The de-

creased killing observed at this temperature was most likely due to the diminished ingestion capacity of leukocytes that had been preincubated at 41°C.

Whether these *in vitro* findings are relevant to leukocyte function in patients with high or prolonged fevers is difficult to judge considering the variables present in such patients. Other investigators, using different assay systems in which the leukocytes were not preincubated at elevated temperatures, have reported either a positive influence on phagocytosis of temperatures in the febrile range (2) or no significant effect (1, 5). The effect of elevated temperature on phagocytic function *in vivo* deserves further investigation.

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