Role of Serum in the Intracellular Killing of Staphylococci in Rabbit Monocytes

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ABSTRACT

SHAYEGANI, MEHDI G. (U.S. Veterans Administration Hospital, Philadelphia, Pa.), AND STUART MUDD. Role of serum in the intracellular killing of staphylococci in rabbit monocytes. *J. Bacteriol.* 91:1393-1398, 1966.—Although some intracellular killing occurs in rabbit monocytes with heated normal serum or even in monocytes washed three times with Hanks' solution and with staphylococci not exposed to serum, efficient killing of coagulase-positive *Staphylococcus aureus* cells in the mononuclear phagocytes of rabbits is shown to require heat-labile components of serum. The effect of serum in promoting phagocytosis and intracellular killing may be exhibited either by presensitization of the staphylococcal cells before contact with leukocytes or by the presence of serum in the phagocytic system. Under any conditions studied the rate of intracellular killing of *S. aureus* is very slow.

Coagulase-positive *Staphylococcus aureus* cells, after ingestion by both polymorphonuclear and mononuclear phagocytes, are more slowly and less completely rendered noncultivable than are other pyogenic cocci (1-3, 6, 8, 9, 11-18). This relative refractoriness to intracellular killing in all probability is a significant factor in the tendency of staphylococcal infections to become chronic and recurrent. In the present study, we define somewhat more precisely the relation of thermostable and thermolabile components of serum to the phagocytosis and intracellular survival of staphylococci in rabbit mononuclear phagocytes.

MATERIALS AND METHODS

Staphylococci. Coagulase-positive strain 18 Z of *S. aureus* was used. The characteristics of this strain have been described previously (2). Strain Copenhagen, received through the kindness of J. L. Strominger, was also used. As the method used showed the intracellular fate of these two strains in monocytes from rabbits to be similar, all data represented in this paper were obtained with strain 18 Z. Bacterial suspensions were made from 24-hr cultures grown in Trypticase Soy Broth and washed twice with saline.

Rabbit exudate monocytes. Mononuclear exudate cells were induced by the intraperitoneal injection of 30 ml of mineral oil 4 to 5 days prior to the collection of the monocytes. The peritoneal cavity was washed with Hanks' solution containing heparin (1:20,000), either by bleeding the rabbit to death and opening the peritoneal cavity or by injecting the Hanks' solution into the peritoneal cavity and then with-

drawing the exudate, using a 30- to 50-ml syringe and an 18-gauge needle. In the latter method the rabbits can be kept alive for future experiments. The monocytes were washed twice with Hanks' solution.

Serum. Blood was obtained from rabbits by cardiac puncture and was allowed to clot. The serum obtained was either used fresh and unheated (FS) or heated at 56 C in a water bath for 30 min (HS). In all cases the serum was used not later than 1 to 2 hr after bleeding.

Infection of rabbits by live staphylococci. Cultures of *S. aureus* 18 Z grown in Trypticase Soy Broth for 18 hr were washed twice with saline. A dose of 10^9 live organisms was injected intradermally into each side of the rabbits at 1-week intervals for 5 weeks. The first injection usually developed large nonpurulent necrotic lesions which healed after about 1 week. Subsequent injections produced small localized lesions. At 1 week after the last injection, the leukocytes of rabbits and their autologous fresh and heated sera were used.

Experimental procedure. Amounts of 1.8 ml of the washed leukocytes suspended in Hanks' solution were placed in each of two siliconized Wassermann tubes, and either fresh or heated serum was added in a final concentration of 10%. Washed staphylococci in a ratio of 1 or 2 per leukocyte were then added. The tubes were placed in a roller drum (about 30 rev/min) in a 37 C incubator. The counts of viable total, and cell-associated staphylococci, staphylococci in the centrifugal supernatant fluid, and leukocytes were determined by methods described previously (3, 13).

For sensitization with serum prior to phagocytosis, 24-hr-old cultures of staphylococci in 5 ml of Trypticase Soy Broth were washed twice with saline.

Amounts of 1 ml of fresh rabbit serum and 1 ml of heated serum from the same rabbit were added to separate tubes containing the washed sedimented bacteria. The mixtures were rotated in an incubator at 37 C for 5 min and then were washed in the cold three times.

RESULTS

Peritoneal monocytes from young normal and infected rabbits. The intracellular survival of *S. aureus* within peritoneal exudate monocytes from young normal rabbits (5 to 6 weeks old, approximately 1,400 g in weight) was tested in the presence of fresh and heated autologous serum. The results of such an experiment are shown in Fig. 1, and the mean values derived from seven rabbits are shown in Fig. 2. Fresh serum promotes active phagocytosis within the first 30 min; with the heated serum, the phagocytosis occurs more slowly. Subsequent intracellular killing was reduced in the presence of heated serum when compared with that observed in the presence of fresh serum. Some microscopic studies at zero time and 30 min also were performed. Smears were prepared and stained with Wright's stain. Quantitative counts of 100 to 200 monocytes were made to determine the number of monocytes containing organisms and the number of staphylococci per 100 monocytes. The phagocytosis

results of the stained smears essentially confirmed those shown in the graphs.

The same young rabbits used in the above experiments were infected with live staphylococci as described in Materials and Methods. After the second and fifth infections, their monocytes were tested by use of the method described. The results were similar to those found prior to infection. Monocytes from older normal rabbits (6 to 8 months old) were also tested, and results were again similar to the above.

Statistical analyses of variance of the data from seven young and seven older normal rabbits and from three rabbits infected twice and five rabbits infected five times did not differ by amounts conventionally regarded as statistically significant. However, in each of these groups of rabbits, more intracellular killing occurred in the presence of fresh serum than with heated serum (level of significance 90 to 99%).

Role of serum in the intracellular fate of staphylococci in monocytes from normal rabbits. A series of experiments were done to attempt to define more exactly the locus of action of the serum.

Staphylococci presensitized with fresh and heated normal serum as described in Materials and Methods were rotated with monocytes from

FIG. 1. Intracellular fate of staphylococci in monocytes from a young normal rabbit in the presence of autologous fresh and heated serum.

FIG. 2. Mean values of experiments on the intracellular fate of staphylococci in monocytes from seven young normal rabbits in the presence of autologous fresh and heated serum.

the same rabbits. The counts of viable monocytes in these experiments in the absence of serum in the medium for the 3-hr period of study were not significantly different from those in experiments done in the presence of serum. Figure 3 represents the mean values from four experiments. Presensitization with fresh serum is obviously more effective in promoting phagocytosis and intracellular killing than presensitization with heated serum.

In other experiments, both presensitized and unsensitized staphylococci were rotated with macrophages in the presence of fresh or heated serum. Figure 4 gives the mean results obtained from four experiments. When no fresh serum was used, phagocytosis and intracellular killing lagged behind the three systems in which fresh serum was present at some stage. However, the three systems in which fresh serum was used do not differ greatly from one another.

Infection of monocytes in the absence of added serum by packing the cells and bacteria. Monocytes from normal rabbits were washed three times with Hanks' solution. The sedimented monocytes were resuspended in about 1 ml of Hanks' solution containing staphylococci, in a ratio of 5 to 10 cocci per monocyte, in a conical centrifuge tube. The monocytes were packed by centrifugation at $250 \times g$ for 4 min, and then were kept in a water bath at 37°C for 15 min, to permit phagocytosis without the addition of serum. The supernatant fluid was decanted, and the sediment was resuspended in 40 ml of Hanks' solution and centrifuged at $50 \times g$ for 4 min at approximately 4°C . This procedure was repeated

twice more to remove as many extracellular staphylococci as possible. Infected monocytes were distributed in three siliconized Wassermann tubes. One tube received 10% fresh serum in Hanks' solution, the second received 10% heated serum, and the third tube received only Hanks' solution. The experiments were then carried out as usual.

In this series of experiments, stained smears of infected monocytes at zero time were prepared. It was found that 70 to 90% of the monocytes contained staphylococci. The viable counts of monocytes under all these conditions were similar. The results of such an experiment are shown in Fig. 5. It is interesting to observe that, without serum added in the system, intracellular killing of staphylococci occurred which was comparable to that which occurred when heated serum was present in the medium. This suggested that monocytes are capable of killing intracellular organisms to some degree even when the bacteria have not had contact with serum. Despite the reduction of the number of extracellular cocci by slow centrifugation, the number was high enough to permit the continuous phagocytosis of extracellular bacteria by monocytes. This gives more phagocytosis and intracellular killing of cocci in the presence of fresh serum.

To get phagocytosis without serum, the ratio of cocci per monocyte was necessarily higher, and the method used to remove extracellular organisms was not efficient. Other means for elimination of extracellular bacteria were tried without success. Streptomycin was used after phagocytosis in the process of slow centrifugation, but the time was not long enough to reduce

FIG. 3. Mean values of experiments on the intracellular fate of presensitized staphylococci in monocytes from normal rabbits in the absence of serum in the medium.

FIG. 4. Mean values of experiments on the intracellular fate of nonsensitized and presensitized staphylococci in monocytes from normal rabbits in the presence of autologous fresh and heated serum in the medium.

FIG. 5. Intracellular fate of staphylococci phagocytized in the absence of serum, when no serum, or fresh or heated serum, was used in the medium.

the number of the organisms, and when streptomycin was used during the 3-hr period of experiment the number of organisms dropped steadily and the result could not be interpreted well.

DISCUSSION

Altered function of monocytes from immune animals has been demonstrated in *Mycobacterium tuberculosis* (5), *Brucella abortus* (10), and *Listeria monocytogenes* (7). These organisms grow intracellularly in monocytes from normal animals, whereas monocytes from immunized subjects suppress the intracellular growth. Although most staphylococci are slowly destroyed in normal rabbit monocytes, some survive for a prolonged time.

In this report, monocytes from young and older normal rabbits and rabbits infected with live staphylococci were tested for their capacity for intracellular killing of staphylococci in the presence of autologous serum. The rates of destruction of bacteria were not significantly different during the 3-hr period of the experiment. Results of long-term experiments also are under investigation in our laboratory.

Fresh serum requirements for efficient intracellular killing of coagulase-positive staphylococci have been demonstrated in human (3, 4) and rabbit blood leukocytes (16). With heated serum, the extent of phagocytosis and killing was much lower except in normal rabbits and 5- to 6-month-old infants, in whom the killing effect found for heated serum was not significantly different from that for fresh serum (16).

In the present study, it was found that fresh serum promotes active phagocytosis and intracellular killing of staphylococci in peritoneal exudate monocytes from rabbits, but that with heat-inactivated serum the phagocytosis was slower and intracellular killing was reduced. In contrast to the pronounced difference between the effects of fresh and heated serum with monocytes, in blood leukocytes from the same normal rabbits we found no significant difference in the intracellular fate of staphylococci.

Experiments in which small amounts of fresh serum were added to heated serum in the system using monocytes from normal rabbits showed that the killing effect was restored. Staphylococci presensitized with fresh serum show more phagocytosis and intracellular killing in monocytes from normal rabbits than do heated serum-sensitized bacteria.

Experiments were performed in the hope of learning whether the conditioning effect of serum on staphylococci necessarily must occur before

phagocytosis, or whether serum entering the macrophages, for instance by pinocytosis, might condition the ingested bacteria for intracellular killing. We were unable to obtain an unambiguous answer to this question. [However, after this paper had gone to press, Berken and Benacerraf (J. Exptl. Med. 123:119-144, 1966) presented evidence that 7S γ_2 opsonin could be adsorbed and taken in by macrophages.]

It was also observed that monocytes are capable of destroying some intracellular staphylococci with no serum used in the system. This experiment is done by forcing washed monocytes to phagocytize the bacteria by a method of packing the thrice-washed cells and bacteria in the absence of serum. In observations with washed human blood leukocytes, Rogers and Melly reported (11) that two or more washings effectively removed the serum component promoting phagocytosis, and in our experience washed blood leukocytes and monocytes have not detectably phagocytized unsensitized staphylococci suspended in Hanks' solution.

In our investigations of the pathogenesis of staphylococcal infections, we are particularly interested in explaining their chronicity and tendency to recur. The relationship of staphylococcal cells and macrophages is obviously one critical factor in this connection. The fact that staphylococci are killed in monocytes, but only very slowly, suggests that these facultatively intracellular parasites may in some cases outlive the cells which have ingested them, and multiply extracellularly, thus setting up a steady state of smoldering infection.

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