Effect of Erythrocyte Ingestion on Macrophage Antibacterial Function

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Received 15 December 1982/Accepted 17 March 1983

Individuals with sickle cell anemia are subject to serious infections caused by a number of bacteria, including Salmonella species and Staphylococcus aureus. It has been suggested that in sickle cell anemia, extensive erythrophagocytosis by macrophages may interfere with their antibacterial function and thereby predispose to infection. As a means of investigating this possibility, we evaluated the effects of erythrocyte ingestion on the killing of Salmonella typhimurium by peritoneal macrophages and of S. aureus by alveolar macrophages. Monolayers of rabbit macrophages were exposed to erythrocytes or latex particles immediately before and during bacterial challenge. Erythrophagocytosis markedly inhibited intracellular killing of S. typhimurium by peritoneal macrophages (bacterial survival was 181% of control) and of staphylococci by alveolar macrophages (bacterial survival was >200% of control). Exposure to latex particles depressed the bactericidal activity of alveolar macrophages to a lesser degree. Next we investigated the possibility that erythrophagocytosis inhibits oxidative bactericidal mechanisms in macrophages. Hexose monophosphate shunt activity was stimulated by erythrocyte ingestion. However, zymosan-induced superoxide generation and chemiluminescence were suppressed by erythrocytes. Furthermore, a cell-free (hypoxanthine-xanthine oxidase) system for chemiluminescence generation was also depressed in the presence of erythrocytes (intact or lysate) or by purified hemoglobin. These studies reveal that erythrophagocytosis inhibits macrophage antibacterial function, probably because of interactions between erythrocyte components and reactive products of phagocyte oxygen metabolism. This host defense abnormality may predispose to bacterial infection in certain hemolytic anemias.

Bacterial infections are a major complication of sickle cell anemia (SCA) (3). In the evaluation of this predisposition to infection, considerable attention has been focused on the role of the reticuloendothelial system, especially the spleen, in sickle cell disease. Defects of splenic macrophage function in SCA (28) and of hepatic and splenic macrophage (reticuloendothelial system) function in experimental hemolytic anemia (22) have been reported. Among the major functions of reticuloendothelial macrophages are removal and inactivation of bacteria circulating in the blood (9, 24). The concept that marked erythrophagocytosis by reticuloendothelial macrophages may interfere with these antibacterial functions and subsequently predispose to systemic infection in SCA has been proposed (10, 22). Among the organisms which produce serious infection in SCA are Salmonella species (3, 18) and Staphylococcus aureus (6, 7). Thus, we examined the effects of erythrocyte (RBC) ingestion on bactericidal activity against these organisms and on oxidative metabolism in macrophages.

MATERIALS AND METHODS

Bacteria. Clinical isolates of Salmonella typhimurium and S. aureus were stored at −70°C. Radiolabeled organisms (S. typhimurium or S. aureus) were prepared by inoculating $10^9$ organisms ($10^{-9}$ dilution of frozen stock culture) into 10 ml of Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) containing 10 μCi of [methyl-$^3$H]thymidine (20 Ci/mmol; New England Nuclear Corp., Boston, Mass.) per ml. These bacteria were cultured at 37°C for 5 h in a shaking water bath, washed, and utilized in the studies described below.

Collection of alveolar and peritoneal macrophages. New Zealand male rabbits weighing 2 to 3 kg were anesthetized with sodium thiopental and exsanguinated before collection of cells.

(i) Alveolar macrophages. Cells from the normal lower respiratory tract were collected by lavage with sterile 0.15 M saline containing heparin (10 U/ml), penicillin (100 U/ml), and streptomycin (100 μg/ml) (3, 19, 25).
(ii) Peritoneal macrophages. Three days after intra- peritoneal injection of thioglycolate or glycerogen, elicited cells were collected by lavage with the saline solution described above.

Macrophage monolayers. Lower respiratory tract or peritoneal cells were washed and resuspended in tissue culture medium 199 (TC 199) (BBL) containing gentamicin (25 μg/ml) and 15% normal heat-inactivated rabbit serum (NRS). Two milliliters of cell suspension (approximately 5 x 10^6 cells) was placed in 35-mm plastic tissue culture dishes (Falcon Plastics, Oxnard, Calif.) and incubated for 2 h at 37°C in 95% air-5% CO₂ (19). Nonadherent cells were removed by washing. Total and differential cell counts, as well as determinations of cell viability, were performed as previously described (5, 19).

Exposure of macrophages to RBC or latex particles. Macrophage monolayers were exposed to the following: RBC or latex particles in TC 199-NRS or to normal medium alone (control) for 60 min. RBC and latex particles were added to the dishes in a ratio of 10:1 with macrophages.

RBC used in various phases of the study included formalinized sheep cells (Difco Laboratories, Detroit, Mich.), glutaraldehyde-treated rabbit cells, and sheep RBC coated with immunoglobulin G antibody to these cells (sheep EA) (Cordis Laboratories, Miami, Fla.). Latex particles (1.1 or 5.7 μm in diameter) were obtained from Dow Chemical Co., Indianapolis, Ind. Assays of macrophage bactericidal activity. Exposure of macrophage monolayers to the above preparations occurred 1 h before and during challenge with radiolabeled bacteria (5 x 10^8 S. typhimurium or 1 x 10^8 S. aureus) in 1 ml of TC 199-NRS without antibiotics.

\(^3\)H-labeled S. typhimurium or S. aureus was added to monolayers of alveolar or peritoneal macrophages for 1 h. Relatively small numbers of S. typhimurium were used to minimize the number of noningested, extraphagocytic organisms. In experiments with S. aureus, lysostaphin (5 U/ml) was added to the culture medium after 45 min of incubation to degrade extraphagocytic staphylococci. At this point, some macrophage monolayers were lysed, appropriately diluted, and cultured on bacteriological media for enumeration of organisms or placed in NCS solubilizer (Amersham Corp., Arlington Heights, Ill.) and added to vials containing toluene-POPOP (2,5-diphenyloxazole)-POPOP [1,4-bis-(5-phenyloxazolyl)benzene] before determination of radioactivity in a liquid scintillation counter (Beckman LS-350 liquid scintillation system). In studies with S. typhimurium, the remaining monolayers were washed and incubated for an additional 60 min before quantitation of intracellular organisms and radioactivity.

Calculations of bacterial ingestion and intracellular killing by macrophages were performed as previously described (15, 19).

Assays of macrophage oxidative metabolism. The influence of erythropagocytosis on oxidative metabolism in alveolar macrophages was examined.

HMPS activity. Alveolar macrophages were washed and then resuspended at a concentration of 2 x 10^6 cells per ml in phosphate-buffered saline (pH 7.2) containing 15% NRS. Four milliliters of macrophage suspension (8 x 10^6 cells) was placed in 60-ml glass vials with or without opsonized zymosan (1 mg/ml), sheep EA (2 x 10^6/ml), or 1.1-μm-diameter latex particles (2 x 10^6/ml). Control vials lacking either macrophages or phagocytic particles were established. A 0.5-μCi amount of β-[1-^14\(^C\)]glucose (New England Nuclear) was added to each vial. Production and release of ^14\(^CO₂\), reflecting hexose monophosphate shunt (HMPS) activity, was determined in the following manner. Microcentrifuge tubes containing hydroxyurea (New England Nuclear), which traps CO₂, were suspended from the rubber stoppers which closed the vials. Incubation was carried out for 2 h at 37°C, after which 1 ml of N H₂SO₄ was injected through each stopper to terminate the experiment and facilitate release of CO₂. After an additional 30-min period, the center wells were transferred to scintillation vials containing toluene-POPOP-POPOP and counted in a Beckman LS-350 scintillation counter. Results were expressed as nanomoles of glucose converted to ^14\(^CO₂\) (21).

Superoxide anion generation. Macrophage monolayers were washed and then covered with 3 ml of TC 199 containing 60 μM ferricytochrome C but lacking phenol red. Identical incubations were run in the presence or absence of opsonized zymosan (2 mg/ml), sheep EA (10^7 cells per ml), or both zymosan and EA. Some assay mixtures contained superoxide dismutase (SOD) (50 μg/ml) in addition to one of the stimuli. Controls included cell-free dishes incubated with each of the above reaction mixtures. All experiments were performed in duplicate.

These preparations were incubated for 90 min at 37°C in a CO₂ incubator. Supernatants were decanted and immediately placed in an ice bath to stop the reaction. After centrifugation at 500 x g for 12 min at 4°C, the optical density of the supernatants was determined at 550 nm in a Carey 219 spectrophotometer (Varian, Houston, Tex.), using the cell-free mixtures as blanks. Reduced cytochrome c was determined by using the equation E₅₅₀ = 2.1 x 10^4 M⁻¹ cm⁻¹ (20).

Macrophage monolayer cell counts were determined for the various experimental groups. Cells were counted from culture dishes with 1 ml of EDTA (0.007 M) in saline, combined with cells recovered from the supernatant centrifugation step above, and counted in a hemacytometer (5). Macrophage smears from each experimental group were prepared and examined microscopically for quantitation of zymosan and RBC ingestion.

Chemiluminescence. Phagocytosis is associated with the emission of light (chemiluminescence) as a consequence of interactions between released oxygen reactive products and cellular or extracellular chemical components. To examine the influence of erythropagocytosis on this phenomenon, the following studies were performed. All procedures were carried out in the dark. Alveolar macrophages (5 x 10^6) in 5 ml of TC 199-5% NRS were placed in dark-adapted plastic scintillation vials and allowed to incubate at 37°C for 1 h to establish a monolayer. Sheep EA or latex particles (1.1 μm), in a concentration of 10^6 particles per ml, were added to some vials, and incubation at 37°C was continued for 30 min. Luminal (10^-6 M) was added to each vial (1). The vials were placed in the scintillation counter (operated in the off-coincidence mode), and baseline counts were obtained. Opsonized zymosan (2 mg) was added to the vials, which were counted at frequent intervals until chemiluminescence peaked and returned toward baseline. Controls included vials...
without cells, vials without phagocytic particles, and
vials to which SOD had been added. Chemilumines-
cence was expressed as counts per minute at each time
point.
Cell-free (hypoxanthine-xanthine oxidase) chemilumi-
nescence. The consequences of erythrocyte, RBC lys-
ate, or hemoglobin presence on light emission during
the reaction between hypoxanthine and xanthine oxidi-
dase were studied. All procedures were performed in
the dark. Samples (2 ml each) of a solution containing
hypoxanthine, $1.5 \times 10^{-4}$ M, and disodium(ethylene-
dinitril)tetraacetate, $10^{-4}$ M, in 0.05 M potassium-
phosphate buffer were placed in dark-adapted scintilla-
tion vials ([7]). Luminol ($2.5 \times 10^{-4}$ M) was added
to each vial. Sheep EA, human RBC, human RBC lysate
(all at $10^7$ cells/ml), or purified human hemoglobin,
obtained by Sephadex G-200 column chromatography,
were added to some vials. The vials were then placed
in a scintillation counter (operated in the off-coinci-
dence mode), and baseline counts were obtained.
Xanthine oxidase (1.25 mU/ml) was added to the vials,
which were counted in continuous 12-s cycles until
chemiluminescence peaked and returned toward base-
line. Controls included vials without xanthine oxidase,
vials without hypoxanthine, and vials to which SOD
had been added.

Statistical analysis of data. Differences between ex-
perimental groups were evaluated by means of Stu-
dent's $t$ test, using a Tektronix 4051 computer.

RESULTS
Monolayers of alveolar or peritoneal macro-
phages contained $2 \times 10^6$ to $3 \times 10^6$ cells. The
mean $\pm$ standard error of the mean (SEM) num-
ers of cells on the culture dishes were $2.33
\pm 0.14 \times 10^6$ alveolar macrophages and $2.17 \pm
0.19 \times 10^6$ peritoneal macrophages. Assays of
macrophage antibacterial function were per-
formed after exposure of cells to TC 199-NRS
containing RBC or latex particles or to the
culture medium alone. Macrophages readily in-
gested the particles to which they were exposed.
At least 90% of these cells ingested three or
more RBC during 1 h of exposure. A similar
percentage of macrophages ingested latex parti-
cles during the same period of time.

Intracellular bactericidal activity. (i) S. typh-
murium. Monolayers of peritoneal macrophages
were exposed to aldehyde (formaldehyde or glutaraldehyde)-treated RBC for 60 min, follow-
ing which the monolayers were washed and
immediately challenged with S. typhimurium.
RBC ingestion by peritoneal macrophages did
not significantly inhibit subsequent phagocytosis
of S. typhimurium. However, erythrophagocy-
tosis by these macrophages did inhibit intra-
cellular killing of ingested bacteria. Thus, the
survival of intracellular S. typhimurium in peri-
toneal cells exposed to RBC was increased at 1
and 2 h after bacterial ingestion as compared with
controls (Fig. 1). Phagocytosis of latex
particles failed to alter either the ingestion or
intracellular inactivation of S. typhimurium by
peritoneal macrophages.
(ii) S. aureus. The influence of RBC or latex
exposure on the inactivation of staphylococci by
alveolar macrophages was examined. In these
experiments, lysostaphin was added to the mac-
rophage culture medium at the conclusion of the
bacterial phagocytosis period to eradicate extra-
phagocytic organisms. This procedure elimi-
nated the possibility that survival and growth of
extracellular organisms might complicate calcu-
lations of intracellular bacterial survival.

Ingestion of erythrocytes or latex particles
had little effect on the phagocytosis of staphylo-
cocci by alveolar macrophages. Organisms ($1.73
\pm 0.25 \times 10^7$) were phagocytized by macro-
phages exposed to erythrocytes, as opposed to
$2.07 \pm 0.25 \times 10^7$ bacteria for control macro-
phages. As in the studies with S. typhimurium,
phagocytosis of aldehyde-treated RBC or sheep
EA interfered with the ability of macrophages to
kill S. aureus. To a lesser extent, latex ingestion
also inhibited macrophage bactericidal activity
(Fig. 2).

Macrophage oxidative metabolism. Killing of
bacteria by phagocytes is largely mediated
through reactive products of oxidative metab-
olism (2, 23). Thus, the possibility that erythro-
phagocytosis inhibits macrophage bactericidal

![Graph](http://iai.asm.org/)
function by interfering with oxidative killing mechanisms was considered.

(i) HMPS activity. Stimulation of oxygen uptake and HMPS activity occurs in association with phagocytosis of bacteria and various other particles (2). In these studies, the abilities of zymosan, sheep EA, and latex ingestion to stimulate HMPS activity in alveolar macrophages was determined. Phagocytosis of zymosan caused a threefold increase in glucose oxidation by the HMPS in macrophages, whereas RBC ingestion led to a smaller but definite increase (approximately 50%) in HMPS activity (Table 1).

TABLE 1. HMPS activity in alveolar macrophages exposed to phagocytic particles

<table>
<thead>
<tr>
<th>Incubation conditions (2 h)</th>
<th>Glucose oxidized to CO₂ via HMPS (nmol/8 × 10⁶ cells/2 h)²</th>
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<tbody>
<tr>
<td>Macrophages + zymosan</td>
<td>382.48 ± 38.24 (11)⁶</td>
</tr>
<tr>
<td>Macrophages + sheep EA</td>
<td>191.68 ± 25.60 (10)⁵</td>
</tr>
<tr>
<td>Macrophages + latex</td>
<td>150.23 ± 18.40 (10)⁴</td>
</tr>
<tr>
<td>Macrophages alone</td>
<td>125.96 ± 17.62 (12)</td>
</tr>
</tbody>
</table>

² Values are mean ± SEM; number of experiments in parentheses.

6 Significantly greater than controls (macrophages alone); P = 0.000001.

5 Significantly greater than controls (macrophages alone); P = 0.047.

4 Not significantly different from controls (macrophages alone); P = 0.366.

1. Latex phagocytosis induced only minor augmentation of glucose oxidation by the HMPS.

(ii) Superoxide generation. Next, the ability of alveolar macrophages to generate superoxide following a phagocytic stimulus, using zymosan or RBC or both, was determined. Zymosan ingestion stimulated the generation and release of superoxide into the extracellular medium (15.82 ± 2.31 nmol/10⁶ cells) (Fig. 3). Exposure of macrophages to sheep EA inhibited the release of superoxide associated with zymosan challenge. Ingestion of RBC alone caused no release of superoxide by macrophages.

(iii) Chemiluminescence. To examine the influence of erythropagocytosis on light emission, alveolar macrophages were exposed to RBC (sheep EA) or latex particles (1.1 μm) before phagocytic challenge with zymosan. The ingestion of RBC alone produced no chemiluminescence above background levels. However, exposure to RBC depressed the macrophage chemiluminescence response to zymosan (Fig. 4). The presence of latex particles had little influence on zymosan-induced light emission.

Cell-free (hypoxanthine-xanthine oxidase) chemiluminescence. The hypoxanthine-xanthine-oxidase reaction, which generates superoxide, is associated with light emission. This chemiluminescence is inhibited by superoxide dismutase. We examined the effects of intact or lysed RBC and of hemoglobin on this reaction. Sheep EA, fresh human RBC, and human RBC lysate inhibited chemiluminescence (Fig. 5). In a similar

FIG. 2. Effects of erythrocyte and latex exposure on alveolar macrophage bactericidal activity against S. aureus. Survival of intracellular staphylococci was determined 1 h after ingestion. Percent bacterial survival was calculated as described in the legend to Fig. 1. Results are expressed as the mean ± SEM (numbers of experiments in parentheses) for each group. Differences between experimental groups and controls are expressed as P values.

FIG. 3. Effects of exposure to zymosan, RBC (sheep EA), or both on superoxide generation by alveolar macrophages during a 90-min incubation period. Values are expressed as the mean ± SEM for each group. Numbers of experiments are in parentheses. The difference between zymosan and zymosan + RBC experimental groups is expressed by the stated P value.
manner, purified hemoglobin (fractioned on a Sephadex G-200 column) depressed the light emission associated with the hypoxanthine-xanthine oxidase reaction (data not shown).

DISCUSSION

Impaired macrophage function may account for certain host defense abnormalities observed in sickle cell disease. The present study evaluated the possibility that extensive erythrophagocytosis by macrophages, a well-documented phenomenon in SCA (3), might compromise the ability of these phagocytes to inactivate ingested bacteria. Our investigation revealed that erythrophagocytosis led to marked deterioration in the ability of peritoneal macrophages to kill S. typhimurium. These observations are consistent with those reported by Gill et al. (10), who noted depressed phagocytosis and killing of S. typhimurium by mouse peritoneal macrophages when organisms and either antibody-coated homologous RBC or intact heterologous RBC were simultaneously added to culture dishes.

S. aureus, which has been reported to cause excess pneumonia (6) and septicemia (7) in SCA, was employed in our studies of alveolar macrophage activity. In agreement with the S. typhimurium experiments, we found that erythrophagocytosis markedly diminished the capacity of macrophages to inactivate S. aureus.

Ingestion of latex particles also inhibited macrophage bactericidal activity against S. aureus to some extent. This suggests that particle ingestion per se may play a role in alteration of antibacterial function. Extensive phagocytosis of particles might interfere with subsequent degranulation of lysosomes and phagolysosome formation or function after bacterial ingestion.

That simple particle ingestion is not the entire explanation for RBC-mediated suppression of macrophage bactericidal activity is implied by a number of facts, including observations made in this study. For example, we found that RBC
Thus, exposure of alveolar macrophages to chemiluminescence indicative bacterial killing interferes with certain aspects of phagocyte function. Gladstone and Walton demonstrated that iron and hematin impair killing of staphylococci by rabbit polymorphonuclear leukocytes or by extracts of their lysosomes (11, 12, 31). This effect was believed due to inhibition of lysosomal cationic proteins. Other investigators found that hemoglobin in high concentrations decreased the bactericidal activity of human polymorphonuclear leukocytes in vitro (16, 33). Current evidence indicates that the killing of bacteria by phagocytes is largely mediated through reactive products of oxidative metabolism (2, 23). Therefore, it is of interest that iron will inactivate (reduce) intracellular H$_2$O$_2$ produced by granulocytes (21). In addition, exposure to RBC or hemoglobin inhibits the tumoricidal activity of mouse macrophages (32), a function at least partially dependent on H$_2$O$_2$ generation (26, 27).

In the present study, we examined the possibility that exposure to RBC might impair oxidative bacterial killing mechanisms in macrophages. Our results support this hypothesis. Thus, exposure of alveolar macrophages to RBC depressed release of superoxide and production of chemiluminescence during phagocytosis of zymosan. This curtailment of oxidative phenomena in macrophages could be due to direct suppression of the phagocytosis-induced respiratory burst, to the known presence of SOD in erythrocytes, or to interactions between other RBC components and oxygen reactive products. In the present study, we demonstrated that RBC ingestion by macrophages stimulates the oxidation of glucose by the HMPS pathway. This observation establishes that RBC do not directly inhibit the respiratory burst and increased uptake of oxygen associated with phagocytosis. Therefore, it appears that one or more RBC components interfere with the expression of oxygen metabolic product reactivity.

This inhibitory activity is undergoing further evaluation in our studies with cell-free oxidative reactions. In work presented here, we examined the effects of intact RBC, RBC lysate, and Sephadex-fractionated hemoglobin on the generation of chemiluminescence by the hypoxanthine-xanthine oxidase reaction. Each of these preparations reduced the production of chemiluminescence in the cell-free system. Furthermore, we have demonstrated that both Sephadex-fractionated hemoglobin and intact RBC interfere with the generation of superoxide and bactericidal activity in cell-free oxidative systems employing xanthine oxidase and appropriate substrates (unpublished data). Since the pu-

![Graph](image-url)

**FIG. 5.** Influence of sheep EA, human RBC, and human RBC lysate on the chemiluminescence generated by the hypoxanthine-xanthine oxidase reaction in the presence of luminol. Values are means ± SEM of experiments at each time point. Numbers of experiments are in parentheses. Differences between the experimental group without RBC (●) and all experimental groups with RBC (intact or lysate) are significant ($P < 0.05$) at each time point.
rifed hemoglobin preparations do not contain SOD, it appears that hemoglobin interacts di-
rectly with reactive products of oxidative metab-
olism and may thereby compromise oxidative bacterial mechanisms. In other words, the competition between hemoglobin and bacteria for oxygen reduction products might increase survival of the organisms.

It is entirely possible that erythrophagocytosis influences multiple macrophage functions. One of these macrophage functions is the production of complement system components (4, 8, 29, 30). Recently, we described deficiencies of the serum complement system, associated with decreased bactericidal and opsonizing activities for S. typhimurium, in persons with SCA (13, 14). In view of these findings, it is plausible that the consequences of marked erythrophagocytosis by macrophages in SCA might include decreased synthesis of certain complement factors, as well as reduced intracellular antibacterial activity. This unifying concept could explain the major host defense abnormalities, with subsequent predisposition to bacterial infection, in SCA and should be studied further.

ACKNOWLEDGMENTS

This work was supported by the Medical Research Service of the Veterans Administration.

We thank Katie Gilliam and Lynne Wilson for secretarial assistance.

LITERATURE CITED

tive pathway of complement activation by mouse perito-