

# Hemoglobin and *Escherichia coli*, a Lethal Intraperitoneal Combination

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Intraperitoneal injection into mice of approximately  $8 \times 10^6$  washed cells of *Escherichia coli* suspended in a lysate of washed human red blood cells or an aqueous solution of crystalline hemoglobin was lethal. *E. coli* suspended in washed intact erythrocytes, whole blood, plasma, or saline was innocuous. Fractionation of non-hemoglobin proteins from hemoglobin in lysates showed that only hemoglobin promoted a lethal infection. Overwhelming intraperitoneal growth of *E. coli* was attained in about 12 hr in lethal infections. The polymorphonuclear leukocytic response was ineffective against this rapid growth. The lethal mechanism is hypothesized to center on a unique role for free hemoglobin in inhibiting peritoneal absorption and stimulating an intraperitoneal exudate which supports luxuriant bacterial growth. Death is attributed to a lethal intoxication from bacterial endotoxins. This role for hemoglobin involves neither enhanced bacterial virulence nor lowered host resistance, and it would be of importance not only in peritonitis but also in problems where hemolysis and infection coexist.

A hitherto unsuspected role for hemoglobin in promoting bacterial infections is emerging from studies of experimental bacterial peritonitis (9, 10, 17, 18). In 1961, Davis and Yull (7) postulated that a specific synergism occurred in mixtures of *Escherichia coli* and red blood cells which were lethal when injected intraperitoneally into healthy animals, whereas neither the bacteria nor blood alone were lethal. They established that the synergism occurred in mixtures containing  $10^8$  bacteria per ml and 4 g of hemoglobin per 100 ml and suggested that the mixture influenced host resistance (22). As the concentration of hemoglobin was decreased, the mortality of test animals diminished, and death did not occur in its absence. Crystalline hemoglobin was equally effective in the synergism; derivatives of the hemin moiety of the molecule had lesser effect; the globin moiety and lipids of red cells were without effect (8).

Initially, we viewed this synergism in relationship to our previous investigations of bacteria and clostridial exotoxins in the lethality of strangulation fluids. We demonstrated that sterile, non-lethal ultrafiltrates of strangulation fluids from humans, dogs, and rats killed healthy mice with fewer *E. coli* than was possible with saline suspensions of the same organism (4). We called the synergistic phenomenon enhanced bacterial virulence, a term which focuses attention primarily on the role of bacteria in the suspensions without

immediately requiring identification of any virulence-enhancing factor or its mode of action. Injection of *E. coli* suspended in whole blood or washed red cells was harmless, but when hemoglobin was released from red blood cells, it could serve as a bacterial virulence-enhancing factor at concentrations greater than 2 g per 100 ml (3). Virulence was occasionally demonstrated at concentrations as low as 0.2 g per 100 ml, but not at 0.07 g per 100 ml. In addition, enhanced virulence was demonstrated with six strains of *E. coli*, with *Proteus* species, and with *Pseudomonas aeruginosa*, but not with bacteroides. This report confirms our previous finding that hemoglobin released from red cells enhances bacterial virulence but that hemoglobin within the intact red cell does not. This study also demonstrates that nonhemoglobin proteins within the red cells are innocuous and correlates the lethal outcome for mice with intraperitoneal bacterial multiplication.

## MATERIALS AND METHODS

Citrated whole blood (480 ml of human blood plus 120 ml of citric acid, trisodium citrate, dextrose solution, formula B), rejected by the blood bank, was processed so that the following fractions were available for use as a diluent in preparing suspensions of *E. coli*: (i) whole blood, (ii) plasma (obtained following centrifugation of blood), (iii) red blood cells (washed three times with saline solution), and (iv) lysed red cells (obtained when washed cells were resuspended in

distilled water, frozen, and thawed; the stromal fraction was not removed). All centrifugations were conducted at  $1,900 \times g$  for 20 min at 5 C. Sterile technique was used throughout these procedures. All fractions and specimens were tested for sterility before use.

Erythrocyte enzymes and proteins amounted to approximately 7% of the hemoglobin content of lysates and were separated from hemoglobin by the diethylaminoethyl (DEAE) cellulose adsorption procedure of Hennessey et al. (13). After the nonhemoglobin protein fraction was desorbed from the DEAE cellulose by treatment with 0.5 M KCl solution, it was desalted by passage through a column of Sephadex G-25 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) with 0.5 M NaCl solution. This protein fraction, more dilute than the starting material, was concentrated by dialysis against Aquacide (Calbiochem, Los Angeles, Calif.) for 2 to 4 hr.

Hemoglobin was determined by the cyanmethemoglobin method, employing Acuglobin (Ortho Pharmaceutical Corp., Raritan, N.J.), and protein was determined by the biuret method (12). All hemoglobin-containing fractions were diluted to contain 10 g of hemoglobin per 100 ml and averaged 99.8 mg of protein per ml. The hemoglobin-free protein fraction was adjusted to contain the same concentration of protein and was sterilized by filtration (Millipore Corp., Bedford, Mass.), as was the companion hemoglobin solution. Twice crystallized human hemoglobin (Mann Research Laboratories, New York, N.Y.) was purchased. Aqueous suspensions containing 10 g of hemoglobin per 100 ml were too dense to allow sterilization by filtration. Although they were not filtered, they were sterile.

A strain of *E. coli*, isolated from a hospital wound infection and previously studied (3, 4), was used. Cells were harvested by centrifugation from overnight cultures incubated at 37 C on a rotary platform shaker in 50 ml of Trypticase Soy Broth (BBL), washed twice with saline solution, and adjusted to read 56% transmittance at 610 m $\mu$  in a Coleman Junior spectrophotometer. The mean number of bacteria in these suspensions was  $1.6 \times 10^9$  per ml (range:  $0.9 \times 10^9$  to  $3.3 \times 10^9$  per ml). For assays of virulence enhancement, each suspension was diluted 1:100 with saline solution or the test material.

All test substances were injected intraperitoneally at a dosage of 20 ml per kg of body weight into female, random-bred, albino mice of the CD-1 strain (Charles River Mouse Farm, Inc., Wilmington, Mass.). The mice were observed for 72 hr, although death usually occurred at 24 hr. Groups of 6 to 12 mice were used for all studies. Each test substance (without added bacteria) was injected into two mice as a control. Although syringes with 26-gauge needles were used for most injections, it was necessary to use larger gauge needles, and extreme care was taken in filling and discharging syringes when studying washed erythrocytes to avoid depositing lysed red cells intraperitoneally.

Intraperitoneal populations of bacteria and leukocytes, following injection of *E. coli* suspended in test substances, were studied in groups of 12 mice. Two

mice were killed immediately following injection and after 2, 5, 12, 18, and 24 hr. The technique of Cohn (6) was used to collect aspirates from the peritoneal cavity. Standard hemocytometer chamber techniques were used to count and differentiate the polymorphonuclear leukocytes in freshly harvested peritoneal aspirates from each mouse. A sample of each aspirate was also serially diluted in saline, and Trypticase Soy Agar pour plates were prepared in duplicate and incubated at 37 C, and colonies were counted. The pour-plate technique was also used to study the in vitro multiplication of 1:100 dilutions of *E. coli* in each of the test substances.

## RESULTS

All test substances without added bacteria were innocuous for mice. The virulence of *E. coli* was not enhanced by whole human blood, plasma, or washed erythrocytes. Conversely, the lysates of the washed erythrocytes did enhance bacterial virulence. The nonhemoglobin protein fraction of lysed erythrocytes did not enhance bacterial virulence, and the ability of the lysate to enhance virulence was retained in the hemoglobin solution separated by the adsorption procedure. The identity of hemoglobin as the virulence-enhancing factor was verified with a solution prepared from crystalline human hemoglobin (Table 1).

The growth of bacteria suspended in blood and hemoglobin was investigated in vitro and intraperitoneally. *E. coli* did not multiply in saline, but it was able to undergo several divisions in vitro in other nonlethal suspending media. However, when mice were challenged with *E. coli* suspended in whole blood, plasma, or washed erythrocytes, viable bacteria in the peritoneal cavity decreased and the polymorphonuclear leukocytes increased (Fig. 1). In whole blood suspensions, these changes were gradual, but they occurred rapidly in plasma and washed erythrocytes. Control suspensions of *E. coli* in saline were continuously destroyed, but the phagocytic response was not different from that in the other nonlethal suspensions (Fig. 2A).

TABLE 1. Influence of human blood and fractions on the virulence of suspensions of *Escherichia coli*

Suspending medium	Virulence <sup>a</sup>
Whole blood.....	0/12
Plasma.....	0/10
Washed erythrocytes.....	0/32
Lysate of erythrocytes.....	35/42
Erythrocyte nonhemoglobin protein fraction from lysate.....	0/12
Hemoglobin fraction from lysate.....	9/18
Crystalline hemoglobin.....	21/24
Saline solution.....	3/28

<sup>a</sup> Number of mice dead/number challenged.

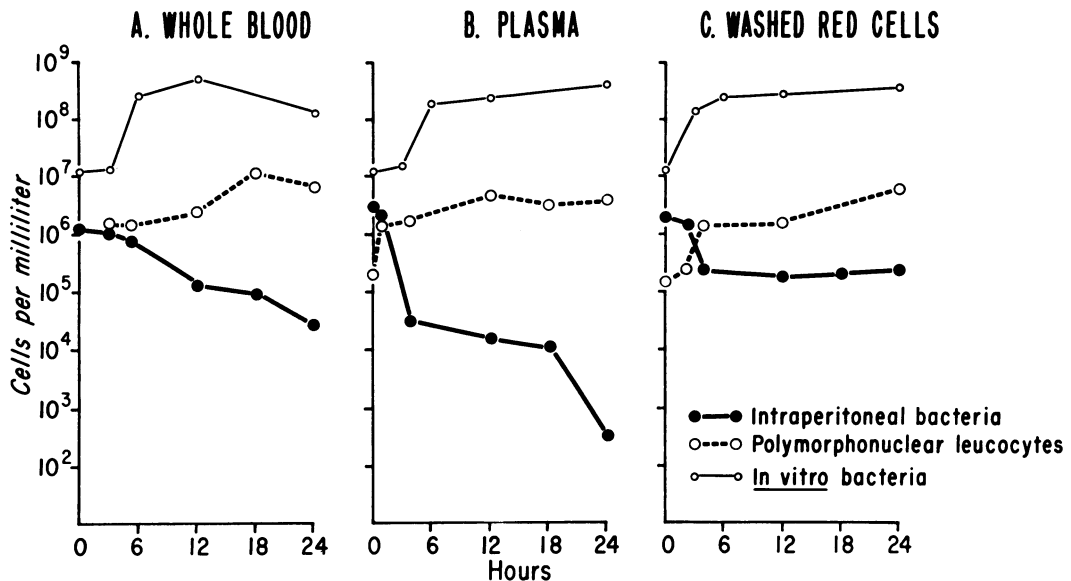


FIG. 1. Influence of whole blood, plasma, and washed red cells on the intraperitoneal response of mice to *Escherichia coli* and on in vitro bacterial growth.

In contrast, *E. coli* suspended in lysates of washed erythrocytes and in an aqueous solution of crystalline hemoglobin grew rapidly in the mouse peritoneal cavity and attained maximal numbers of bacteria in about 12 hr (Fig. 2B, C). The polymorphonuclear leukocytic response, however, was similar to that in the nonlethal systems. The lysate also supported luxuriant growth in vitro, but the hemoglobin solution did not.

#### DISCUSSION

These results confirm the previously described requirement for free hemoglobin (3) and demonstrate that the hemoglobin in lysates of washed erythrocytes is involved in virulence enhancement but that nonhemoglobin proteins are not. Overwhelming intraperitoneal growth of *E. coli* occurs in aqueous hemoglobin and in lysates of washed erythrocytes, and injected mice die. The lysate supports the in vitro growth of *E. coli* because of nonhemoglobin constituents of erythrocytes, since aqueous pure hemoglobin is bactericidal (Fig. 2B, C). Accordingly, it may be hypothesized that intraperitoneal growth of *E. coli* occurs at the expense of nutrients whose accumulation in the peritoneal cavity is dependent on the presence of free hemoglobin. This was not the case when *E. coli* was suspended in whole blood, plasma, washed erythrocytes, or saline.

The ability of mice to resist infection and survive was directly related to the influx of

phagocytic polymorphonuclear leukocytes into the peritoneal cavity (Fig. 1 and 2A). Similar relationships have been reported by Cohn (6) in studies of intraperitoneal infection with staphylococci. However, the polymorphonuclear responses to *E. coli* suspended in lysate or in hemoglobin were ineffective against overwhelming intraperitoneal multiplication by the microorganism.

Although we have described the end result of the activity of *E. coli* suspended in hemoglobin solution as enhanced bacterial virulence, altered bacterial virulence may not be involved. A wide variety of substances are known to potentiate intraperitoneal invasiveness of bacteria. These include endotoxins (1); bile (4); polysaccharides, proteins, and gastric mucin (15); whole bacterial cells, bacterial cell wall preparations, and cellular constituents (16). Adjuvants, such as gastric mucin, are known to aid production of lethal intraperitoneal infections by coating bacteria and protecting them from peritoneal phagocytosis (15). We have presented evidence that bacteria multiply rapidly intraperitoneally in the presence of hemoglobin, but we do not know if the role of hemoglobin is that of an adjuvant. In recent studies with rats and dogs, Filler and Sleeman reported that hemoglobin inhibits peritoneal clearance and destruction of bacteria by defense mechanisms of the hosts and that depressed peritoneal absorption is correlated with lethality (9, 18). Peritoneal exudation during the specific inhibition of peritoneal absorption by solutions

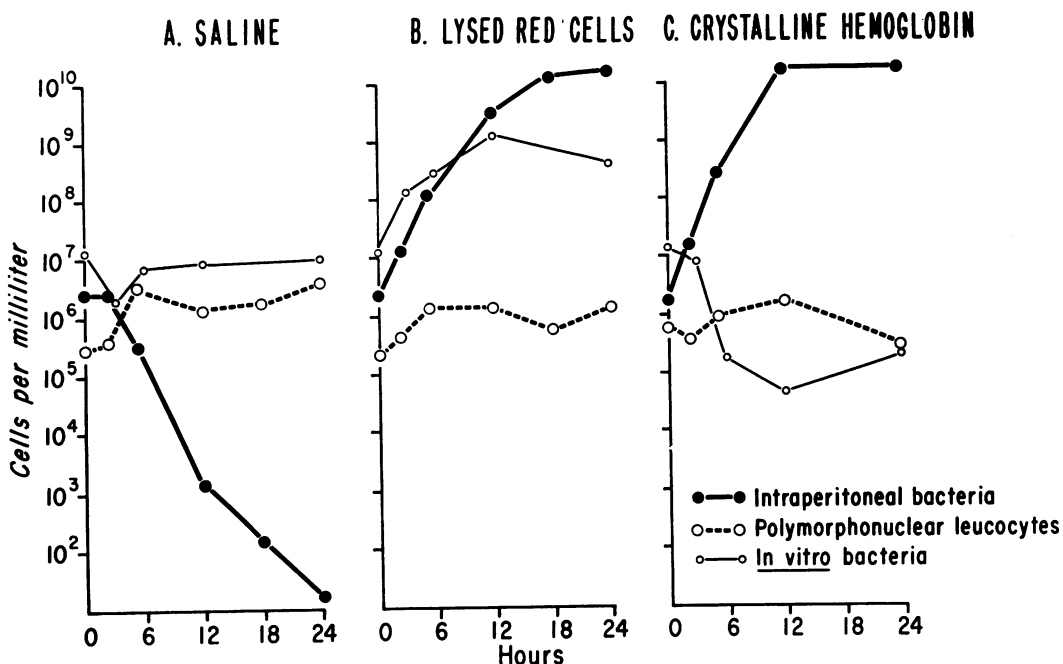


FIG. 2. Influence of saline solution, lysed red cells, and an aqueous solution of crystalline hemoglobin on the intraperitoneal response of mice to *Escherichia coli* and on in vitro bacterial growth.

of hemoglobin is unknown and remains to be studied. The role of absorbed hemoglobin in lowering host resistance to intraperitoneal bacterial infection also requires study.

The antibacterial properties of blood reside in a variety of important serum and cellular constituents (2). Those of hemoglobin are attributed to both the porphyrin (20, 23) and globulin (14, 21) moieties of the molecule. Nevertheless, when the numbers of *E. coli* used in these studies were suspended in whole blood, plasma, and washed erythrocytes and were incubated in vitro, the inoculum was able to divide several times. Thus, the intraperitoneal decline of viable bacteria occurred despite the ability of these substances to promote growth in vitro. The saline suspensions of *E. coli* did not multiply in vitro, and their continuous decline intraperitoneally reflects the ability of the mouse to overcome infection. It is possible that the lesser bacterial decline (Fig. 1A, C) indicates some intraperitoneal multiplication in addition to in vivo destruction.

The postulated specific inhibition of peritoneal absorption by hemoglobin (9, 18), along with the hypothesized production of an exudate promoting luxuriant bacterial growth, could facilitate intraperitoneal growth, opposed only by inadequate local defense mechanisms, so that numbers of bacteria capable of overwhelming the host with a lethal intoxication due to bacterial endotoxins

would be reached (11). This mechanism would necessitate neither enhanced bacterial virulence nor lowered host resistance. In addition, unabsorbed hemoglobin might also contribute to intraperitoneal bacterial growth by virtue of its classical function in oxygen transport (19), as well as by supplying iron which stimulates bacterial growth and inhibits nonspecific antibacterial systems in serum (5). A role for hemoglobin in enhancing bacterial growth in vivo and hastening death is of importance not only in peritonitis but also in problems where hemolysis and infection coexist.

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