

# ANNALS OF SURGERY

Vol. 157

April 1963

No. 4



## Synergistic Toxicity of Gram-negative Bacteria and Free Colloidal Hemoglobin \*

MARTIN S. LITWIN,\*\* M.D., CARL W. WALTER,\*\*\* M.D.,  
PETER EJARQUE,† M.D., EDWARD S. REYNOLDS,†† M.D.

*From the Departments of Surgery and Pathology, Harvard Medical School,  
and the Peter Bent Brigham Hospital, Boston*

INFUSION of contaminated blood, though infrequent, is recorded in clinical case reports.<sup>1, 3, 5-9, 19, 20, 22, 23</sup> Because of the bacteriostatic properties of freshly collected whole blood and the practice of storing blood at 4.0° C., quantitatively significant contamination is usually with psychrophilic, gram-negative bacteria. These bacteria often do not grow at room temperature or

37° C., but flourish and multiply during refrigeration.<sup>3, 20, 22, 23</sup>

During infusion of the bacteria-laden blood, Whitby's *transfusion catastrophe* occurs. Alarming objective signs are violent chills with extreme vasodilatation, nausea, vomiting, and abdominal pain with bloody diarrhea. Within one to six hours the circulatory collapse and shock phase may appear. Response to vasoconstrictors is usually transitory. Death soon ensues.

It has been postulated that the toxicity of contaminated blood is due to an endotoxin elaborated in the bacterial cell.<sup>1, 4</sup> However, patients receiving such blood often have untoward reactions soon after the infusion is begun; and the number of bacteria administered is well below the minimal lethal dose.<sup>12, 13</sup>

This study attempted to reproduce the conditions of storage of bacterially contaminated blood, to study the biologic reactions of infusion of such a contaminated suspension, and to define the cause of these severe reactions.

\* Submitted for publication June 15, 1962.

\*\* Senior Surgical Resident, Peter Bent Brigham Hospital.

\*\*\* Surgeon, Peter Bent Brigham Hospital, Clinical Professor of Surgery, Harvard Medical School.

† Former Surgical Research Fellow, Harvard Medical School, and Assistant in Surgery, Peter Bent Brigham Hospital.

†† Teaching Fellow in Pathology, Harvard Medical School.

Read before the American Association of Blood Banks, Cincinnati, Ohio, November 21, 1958.

Supported by Research Grant H-1491(C4) from the National Institutes of Health, USPHS, and Contract W-49-007-MD-497 from the Office of the Surgeon General.

### Methods

Mongrel dogs ranging in weight from 10 to 17 kg. were anesthetized with pentobarbital sodium, 20 mg./kg., given intravenously. Groups of animals were injected intravenously with various combinations of fresh, compatible whole dog blood, colloidal hemoglobin, and sublethal doses of *Escherichia freundii*,# a gram-negative psychrophilic bacterium known to have caused a severe transfusion reaction in man.

Colloidal hemoglobin was prepared by lysing fresh, washed, dog red blood cells in the smallest quantity of sterile, distilled water in which lysis would be complete. Occasionally the concentration was so great that precipitation occurred. The lysed blood was centrifuged at 2,000 g. for 30 minutes to obtain a supernate for injection. Hemoglobin concentration was determined on each batch of lysed cells by conversion of an aliquot to cyanomethemoglobin for spectrophotometric comparison with a known standard at 540 m $\mu$ . All whole blood given was crossmatched with that of the recipient; incompatible specimens were discarded.

Pathologic examination was done on all animals that died. In animals that survived the initial injection period, representative specimens were sacrificed and examined five days after injection.

### Results

**Group 1: Effect of Infusion of Sublethal Doses of Gram-negative Bacteria.** Ten dogs were injected intravenously with 4.0 ml. of a saline suspension (washed slant) containing  $4 \times 10^8$  *E. freundii*. On the basis of body weight, this has been found to be a sublethal dose of gram-negative bacteria.<sup>12,13</sup> The animals survived these injections without apparent sequelae. Pathologic examination done on five animals after sacrifice on the fifth postinjection

day showed only occasional subserosal hemorrhages of the small bowel and a few subendocardial and subepicardial hemorrhages. The kidneys appeared normal.

**Group 2: Effect of Sequential Infusion of Gram-negative Bacteria and Compatible, Fresh Whole Dog Blood.** Five dogs were injected intravenously with 4.0 ml. of a saline suspension of *E. freundii* as in Group 1. One hour later 200 ml. of fresh, compatible whole dog blood was infused. All survived without apparent sequelae. Pathologic examination done after sacrifice on the fifth postinjection day showed only occasional visceral hemorrhages as in Group 1. The kidneys appeared normal.

**Group 3: Effect of Sequential Infusion of Gram-negative Bacteria and Sterile, Colloidal Hemoglobin.** Five dogs were injected intravenously with 4.0 ml. of a saline suspension of *E. freundii* as in Group 1. One hour later they were injected with a solution of fresh, sterile, colloidal hemoglobin, 4.0 Gm./kg. During the first postinjection day, three of the dogs were lethargic and refused to eat or drink; they passed several loose, bloody stools. The urine was colored dark red by large amounts of hemoglobin.

All animals survived these injections. Gross and microscopic pathologic examination of the tissues after sacrifice on the fifth postinjection day revealed subserosal and submucosal hemorrhages throughout the viscera, most marked in the small intestine. There was congestion of the liver, spleen, and lungs; the reticuloendothelial stem cells were filled with hemoglobin. Renal tubules contained occasional heme casts, but tubular cell architecture was normal.

**Group 4: Effect of Sequential Infusion of Compatible, Fresh Whole Blood and Gram-negative Bacteria.** Eight animals were injected with 200 ml. of fresh, compatible whole dog blood. One hour following this injection they were injected with a 4.0 ml. saline suspension of *E. freundii* as in the previous experiments.

# P. C. No. 118, courtesy of Dr. Margaret Pittman, National Institutes of Health, Bethesda, Maryland.

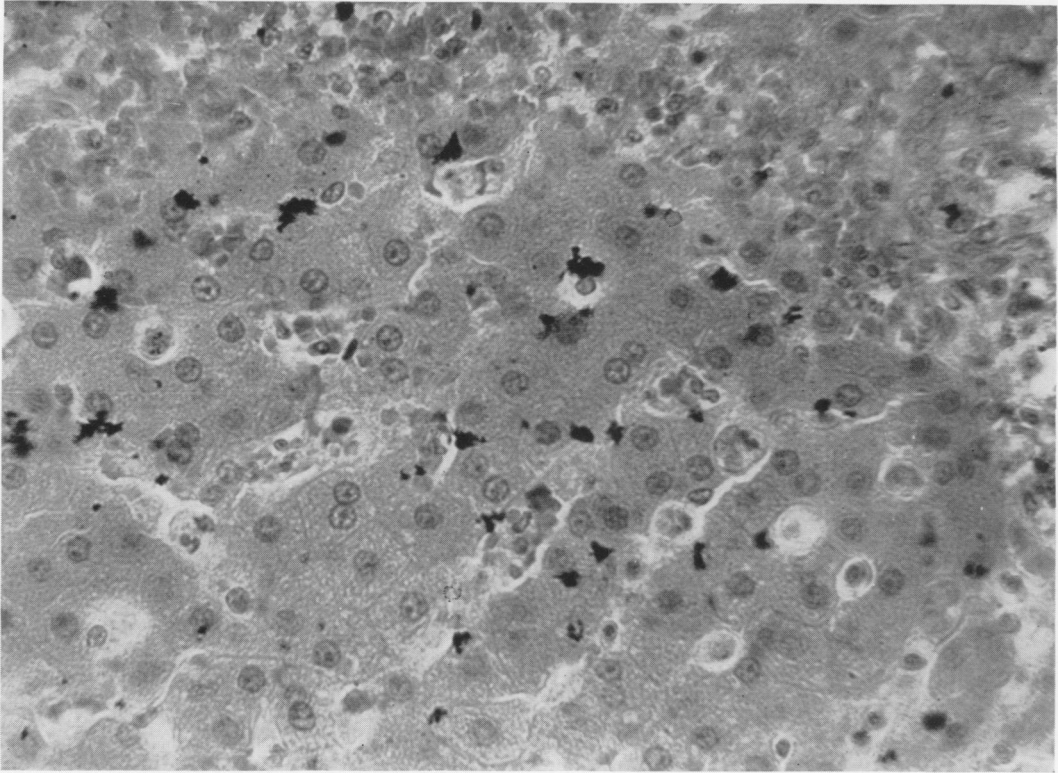


FIG. 1. Section from the liver of an animal that died after receiving sequential infusion of fresh colloidal hemoglobin, 4.0 Gm./kg., and then *E. freundii*,  $4 \times 10^8$  organisms. Note the reticuloendothelial cells filled with colloidal hemoglobin ( $\times 450$ ).

All animals survived without apparent sequelae. Postmortem examination done on three animals after a sacrifice on the fifth postinjection day showed only occasional small-bowel subserosal hemorrhages and subendocardial or subepicardial hemorrhages. The kidneys were normal.

**Group 5: Effect of Sequential Infusion of Fresh, Colloidal Hemoglobin and Gram-negative Bacteria.** Ten animals were injected with fresh, sterile, colloidal hemoglobin solution, 4.0 Gm./kg. One hour later they were injected with a saline suspension (washed slant) of *E. freundii*, 4.0 ml., containing  $4 \times 10^8$  organisms.

Of these animals all died within 12 hours following injection of the bacteria. The clinical picture was constant. All became progressively cold and cyanotic. Vomiting and bloody diarrhea usually supervened

about five hours following injection; and the animals died quietly eight to 24 hours after injection of the bacteria, never having regained consciousness.

Pathologic examination revealed many subserosal and submucosal hemorrhages throughout the viscera, most marked in the small bowel. The large-bowel lumen was distended with gas and large amounts of bloody, watery fluid. There was marked passive congestion of the liver, spleen, and lungs. Examination of sections taken from these organs and stained, using a modification of Mallory's reaction for iron, showed the reticuloendothelial stem cells to be filled with hemoglobin.

A microscopic section taken from the liver of one of these animals is shown in Figure 1. The kidneys in all animals showed moderate to severe cortical necrosis. The

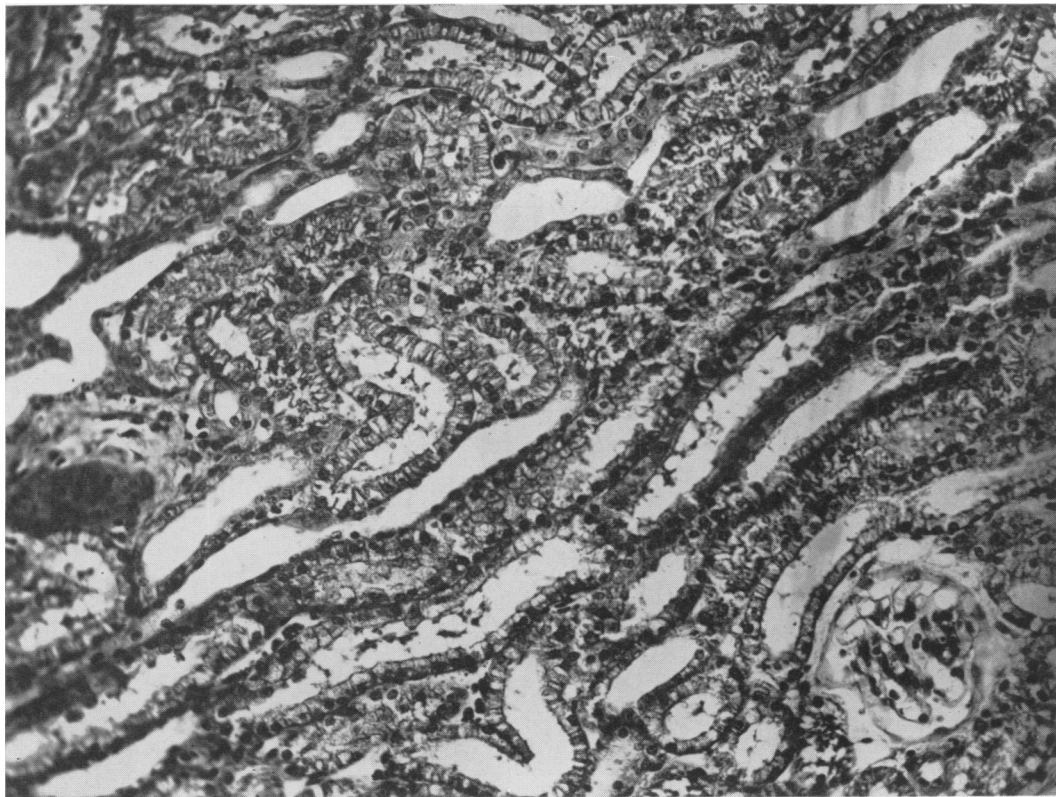


FIG. 2. Section from the kidney of an animal that died after receiving sequential infusion of fresh colloidal hemoglobin, 4.0 Gm/kg., and then *E. freundii*,  $4 \times 10^8$  organisms. There is only moderate tubular degeneration with vacuolization and granularity of the cytoplasm and occasional migration of the cell nucleus from its basilar position ( $\times 200$ ).

reticuloendothelial cells are filled with hemoglobin particles. Figures 2 and 3 show sections of kidney taken from two of these animals. While some presented a somewhat milder picture of tubular degeneration on microscopic section, all consistently and clearly demonstrated changes indicative of moderate to severe acute tubular necrosis or acute cortical necrosis.

**Group 6: Effect of Simultaneous Infusion of Fresh, Colloidal Hemoglobin and Gram-negative Bacteria.**  $4 \times 10^8$  *E. freundii* (washed slant) in 4.0-ml. saline suspension were added to a solution of fresh, colloidal hemoglobin containing 4.0 Gm./kg. hemoglobin in relation to the animal to be injected. Five animals were injected with this solution, each over a one-

hour period. All died within 12 hours after injection. The clinical signs and symptoms and the postmortem pathologic findings were essentially the same as those for Group 5.

#### In Vitro Experiments

A. To demonstrate increased fragility of contaminated red blood cells, osmotic fragility studies were done on normal whole blood (human) collected in ACD solution and intentionally inoculated with bacteria grown from routine cultures of blood collected from our donor clinic and with the previously described *E. freundii*.

Five hundred ml. of sterile, fresh whole blood were taken in routine fashion from a normal male (M.S.L.) and divided into

four equal portions in plastic transfusion bags. Sterility was demonstrated by culture both on blood agar and in thioglycolate broth at 7.0°, 25° and 37° C. for 72 hours. Immediately after the initial culture was taken, an inoculum of one of the following contaminants was injected into one of the blood packs: 1) *Staphylococcus aureus*, nonhemolytic; 2) *Pseudomonas aeruginosa*; and 3) *E. freundii* (P. C. No. 118). The fourth pack served as a control. All were stored at 7.0° C. The osmotic fragility of the red blood cells was determined at various intervals up to 24 days.

The results of these tests are shown in Figures 4 and 5. The blood contaminated with *Ps. aeruginosa* retained essentially the same fragility as the sterile control sample; however, the cell fragilities of both were

slightly increased over the initial control levels. Although the *Staph. aureus* was noted to be nonhemolytic on blood agar in 48-hour culture, nevertheless, on the twenty-fourth day of storage, fragility of the red cells, of which it was a contaminant, was twice that of the control specimen done at the same instant and five times the fragility done on the tenth day.

In Figure 5 the results of the fragility tests done on the cells contaminated with *E. freundii*, the organism which has caused a *transfusion catastrophe*, are shown. The marked differences in the fragilities of the red blood cells of the contaminated specimen and the control specimens are obvious. Culture on blood agar revealed this organism to be moderately hemolytic at 48 hours.

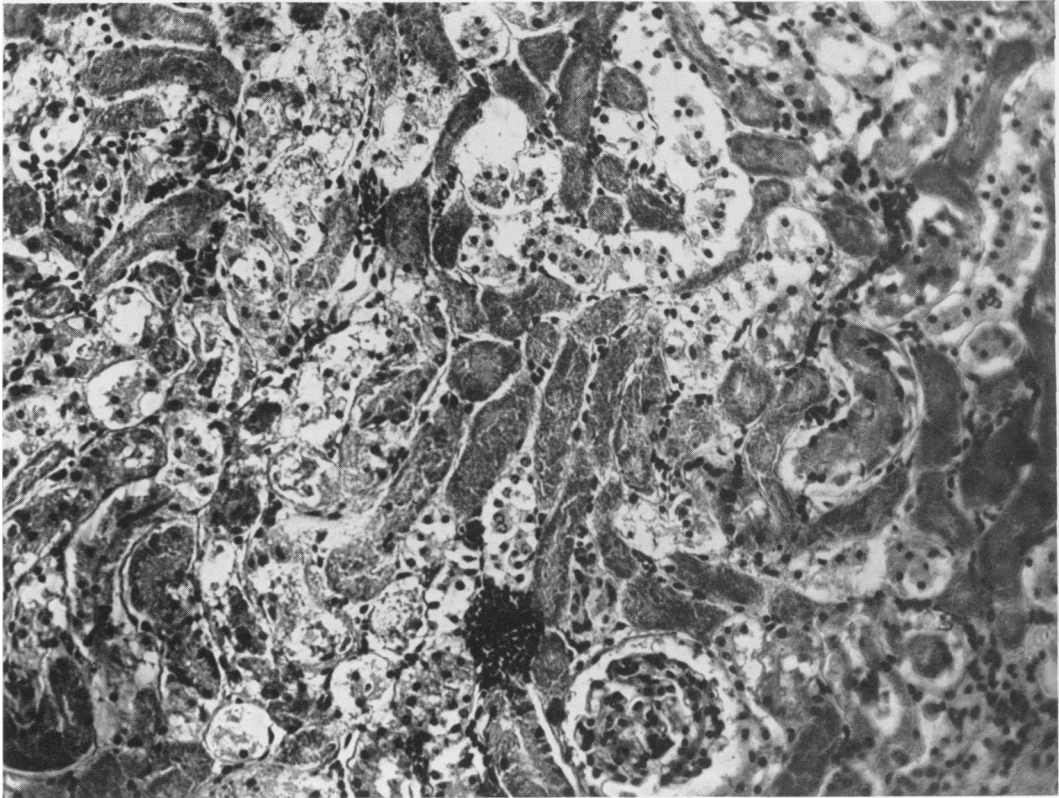


FIG. 3. Section of the kidney of an animal that died after receiving sequential infusions of fresh colloidal hemoglobin, 4.0 Gm/kg., and the *E. freundii*,  $4 \times 10^8$  organisms. Acute cortical necrosis was present. There is a complete loss of normal cellular architecture, sloughing of the tubular cells, and disruption of the basement membrane of the tubules ( $\times 200$ ).

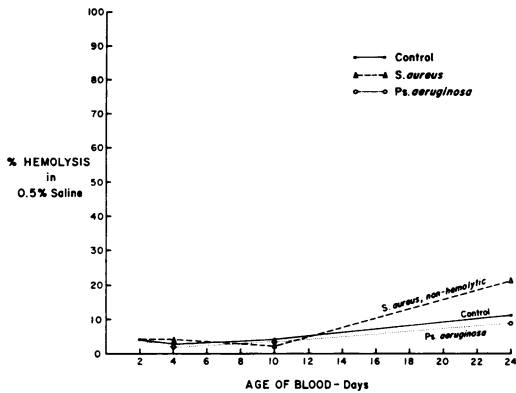
OSMOTIC FRAGILITY OF RED CELLS  
IN CONTAMINATED BLOOD

FIG. 4. Osmotic fragility of red cells in contaminated blood in 0.5% saline. The cells of the blood contaminated with *P. aeruginosa* retained essentially the same fragility as the sterile control sample. In the sample contaminated with nonhemolytic *S. aureus*, fragility measured on the 24th day of storage was twice that of the control specimen done at the same instant and five times the fragility done on the tenth day.

B. During our studies on the fragility of contaminated red blood cells, it was noted that the supernatant plasma, and occasionally the red cells, of the specimens contaminated with *E. freundii* were light brown in color. Spectrophotometric analysis of this material was therefore done. Measurements were done on a Coleman Jr. spectrophotometer. The spectrophotometric absorption curves for light were plotted between the wave lengths of 400 and 700  $m\mu$  for both this material and normal, reduced hemoglobin. Absorption patterns are shown in Figure 6. The curve presented is consistent with that of acid hematin in aqueous solution. pH of this plasma ranged between 4.5 and 5.5.

### Discussion

Hemoglobin in solution is a colloidal suspension with particles measuring  $64 \times 55 \times 50 \text{ \AA}$ .<sup>10</sup> When released free into the circulation, it is either excreted in the urine or phagocytosed and broken down in the reticuloendothelial system.<sup>11</sup> Beeson<sup>2</sup> and

Good, Thomas, and Smith<sup>16, 21</sup> have demonstrated that various colloidal substances taken up by the reticuloendothelial cells may produce effects interfering with normal protective functions and markedly impair an animal's general defense against bacterial toxin. These substances include trypan blue, colloidal carbon, saccharate of iron, and thorotrast.

Particles of hemoglobin are about one half the size of those of thorotrast and twice the size of those of saccharate of iron. We have been able to demonstrate free colloidal hemoglobin in the reticuloendothelial cells of the liver, lung, and spleen after intravenous administration. When free, colloidal hemoglobin was given to animals intravenously after the administration of gram-negative organisms, survival was constant; however, when the same number of bacteria was given *after* the hemoglobin was administered, death followed in all cases. If injection of the same number of bacteria was either preceded by or followed by the administration of type-specific red blood cells, all animals survived. From these experiments it would appear that

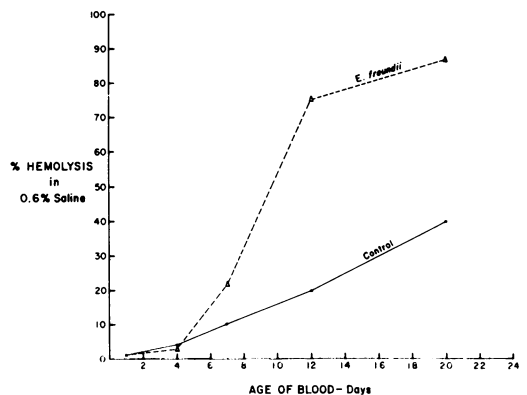
OSMOTIC FRAGILITY OF RED CELLS  
IN CONTAMINATED BLOOD

FIG. 5. Osmotic fragility of red cells in contaminated blood in 0.6% saline. The marked differences in the fragilities of the red cells of the specimen contaminated with *E. freundii*, an organism known to have caused a *transfusion catastrophe*, and the control specimen are obvious.

hemoglobin blocks the reticuloendothelial system and serves to potentiate otherwise sublethal doses of gram-negative, bacterial toxin.

During the infusion of 21-day-old blood, as much as 30 per cent of infused red cells may be lysed within 24 hours,<sup>14</sup> even though compatibility has been demonstrated by Coombs' crossmatch.<sup>16, 24</sup> This is often unavoidable and is due to damage done the cells by mechanical and osmotic trauma at time of collection, increasing fragility as the cells grow older, or undetected antibodies.<sup>15, 17, 24</sup> In the patient who is hypovolemic with good renal function, mild hemoglobinemia is usually of minor import; however, when large amounts of endotoxin are present in the blood stream, effects of this noxious polysaccharide may be magnified greatly. While the group of animals to which we administered gram-negative bacteria and then free hemoglobin uniformly survived the injections, they became acutely ill to varying degrees. It was obvious that had they been debilitated as after a major operative procedure, this added insult would have caused their demise. Administration of fresh, type-specific whole blood had no significant, untoward effects on any of the animals to which it was given.

When transfusions must be given to an acutely ill patient with gram-negative septicemia and shock, the relative age of the red cells becomes of paramount importance. The release of only small quantities of free hemoglobin may serve to interfere partially with the function of the reticuloendothelial system, and the effects of the gram-negative toxin will be intensified. While the maintenance of adequate blood pressure is of utmost importance in patients with severe hemorrhage, the synergistic action of these two agents must be remembered. Viable fresh and compatible red cells should always be used when gram-negative infection is suspected. In situations of this sort, where the patient

ABSORPTION CURVE OF BLOOD CONTAMINATED WITH GRAM-NEGATIVE PSYCHROPHYLIC BACTERIA

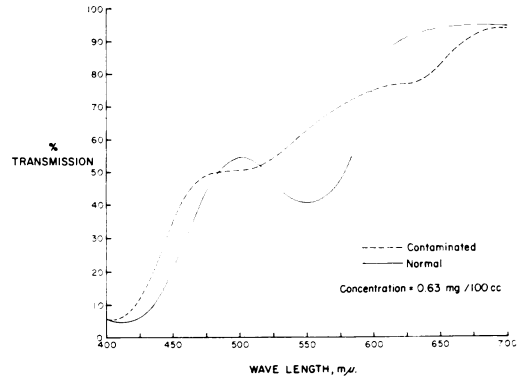


FIG. 6. Spectrophotometric absorption curve of plasma of blood contaminated with gram-negative psychrophilic bacteria. Two bands between 400 and 700 millimicrons is typical of acid hematin. Plasma was light brown in color.

may be so critically ill as to be unable to withstand even the slightest physiologic insult, administration of packed red cells from which the plasma, containing small amounts of free colloidal hemoglobin, has been removed may be of benefit. The administration of cells previously washed with saline and then repacked will serve to remove the most fragile cells and so prevent their *in vivo* lysis and subsequent phagocytosis of released hemoglobin by the reticuloendothelial system.

The toxicity of acid hematin has been described. We were able to demonstrate the presence of this lethal substance in the plasma of blood transfusions contaminated with gram-negative, psychrophilic bacteria. It was not present in quantities large enough to account for ensuing death; however, from other experiments it would seem that large-enough quantities were present to account for any subsequent development of acute renal tubular degeneration.<sup>18</sup>

The role of acid hematin in the production of acute tubular necrosis in dogs has been studied extensively.<sup>18</sup> This material alone has not caused the development of tubular necrosis; however, even in small quantities in the presence of renal vasocon-

striction, dehydration, or renal ischemia, it has consistently led to acute renal failure. Under conditions of severe stress, the kidney is being maximally affected by anti-diuretic hormone and is in a physiologic state of water retention. If shock with diminished blood pressure and decreased renal perfusion is present, then renal tubular oxygenation will be at a minimum and local renal tissue acidosis may be present. When a small intravenous load of free colloidal hemoglobin in the form of excessively fragile red cells or hemoglobin-containing plasma is superimposed on a setting of this sort and converted to acid hematin in the acidotic kidney, oliguria and renal failure may well ensue. The infusion of fresh, compatible whole blood, fresh packed red cells, or washed packed red cells is indicated.

In many of the reported cases of severe reaction or death following administration of contaminated transfusions, hemolysis or plasma discoloration were not present; in many others, there was marked plasma discoloration. In our *in vitro* study, where whole blood was contaminated with non-hemolytic bacteria, it was shown that, even though hemolysis did not occur, the red cells were excessively fragile. This was most likely due to a direct effect of the actively growing bacteria on the red cell membrane. In the case of the specimen contaminated with the *Ps. aeruginosa*, the increased fragility was probably due to age alone since the fragility of the red cells of the control specimen increased equally.

When a *transfusion catastrophe* has occurred and the infusion discontinued, proper bacteriologic examination must be carried out on the remaining specimen if the offending organism is to be identified. Other studies<sup>25</sup> have shown that microscopic examination or routine culture at 37° C. will often fail to lead to identification of bacteriologic pathogens present. Obligatory or partial psychrophils may grow

out at either 4.0° or 20° C. when culture at 37° C. has shown no growth. If red cells remain, fragility studies may be done in order to determine the stability of the red cell membrane. By this method the chances of *in vivo* hemolysis occurring may be judged. Spectrophotometric analysis of both the patient's plasma and the supernatant plasma in any blood as yet not infused will aid not only in determining the amount of heme pigment present but also the type. Complete examination between wavelengths 400 and 700 millimicrons is desirable.

### Conclusions

The administration of a contaminated transfusion is fatal because of the following sequence of events:

The blood is collected from the donor and contaminated in some way. During storage under refrigeration, psychrophilic bacteria grow. If the bacteria are of the hemolytic type, the red cells become exceedingly fragile. Any free hemoglobin in the plasma is converted to acid hematin, a toxic substance. When the contaminated blood is administered, fragile cells are lysed, releasing free, colloidal hemoglobin. This is promptly taken up by the reticulo-endothelial system and serves to potentiate the toxicity of the sublethal numbers of gram-negative bacteria present. Bacterial toxin potentiation, together with small amounts of toxic acid hematin, serves to produce fatality unless the situation is recognized early and the transfusion discontinued.

These experiments stress the importance of using fresh, compatible blood for administration to patients in shock, particularly those suspected of having gram-negative bacteremia. Should there be obvious evidence of hemolysis or plasma discoloration in the blood to be administered, the specimen should be discarded.



### Acknowledgment

The authors are grateful to Dr. Francis D. Moore for helpful criticisms of this manuscript, and to Mr. John Rahilly for his expert assistance in preparing microscopic sections.

### References

1. Andre, R., A. Germain and E. Polacco: Apropos of a Transfusion Complication Due to Bacterial Contamination of Preserved Blood by a Gram-negative Bacillus. *Bull. Soc. Med. Hop. Paris*, **75**:811, 1959.
2. Beeson, P. B.: Effect of Reticuloendothelial Blockade on Immunity to the Schwartzman Phenomenon. *Proc. Soc. Exper. Biol. & Med.*, **64**:146, 1947.
3. Borden, C. W. and W. H. Hall: Fatal Transfusion Reactions from Massive Bacterial Contamination of Blood. *New England J. Med.*, **245**:760, 1951.
4. Braude, A. I.: Transfusion Reactions from Contaminated Blood. *New England J. Med.*, **258**:1289, 1958.
5. Braude, A. I., J. P. Sanford, J. E. Bartlett and O. T. Mallery: Effects and Clinical Significance of Bacterial Contaminants in Transfused Blood. *J. Lab. & Clin. Med.*, **39**:902, 1952.
6. Braude, A. I., D. Williams, J. Siemienski and R. Murphy: Shocklike State Due to Transfusion of Blood Contaminated with Gram-negative Bacteria. *Arch. Int. Med.*, **92**:75, 1953.
7. Editorial: Alleged Fatal Blood Transfusion. *Brit. M. J.*, **2**:1277, 1953.
8. Editorial: A Transfusion Accident. *Lancet*, **2**:1204, 1953.
9. Editorial: Infected Blood Transfusion. *Brit. M. J.*, **2**:334, 1958.
10. Editorial: Hemoglobin Molecule. *Brit. M. J.*, **1**:1796, 1960.
11. Fairley, N. H.: The Fate of Extracorporeal Circulating Hemoglobin. *Brit. M. J.*, **2**:213, 1940.
12. Geller, P. and E. Jawetz: Experimental Studies on Bacterial Contaminants of Bank Blood. I. The Nature of the Toxicity of Contaminated Blood. *J. Lab. & Clin. Med.*, **43**:696, 1954.
13. Geller, P., E. R. Merrill and E. Jawetz: Experimental Studies on Bacterial Contaminants of Bank Blood. II. Observations on Bacteremia in Mice and the Influence of Antibiotics in Preventing Death. *J. Lab. & Clin. Med.*, **45**:943, 1955.
14. Gibson, J. G., R. D. Evans, J. C. Aub, T. Sack and W. C. Peacock: In Vivo Survival of Stored Red Cells: Radioiron. *J. Clin. Invest.*, **26**:715, 1947.
15. Gibson, J. G., W. P. Murphy, W. A. Scheitlin and S. B. Rees: The Influence of Extracellular Factors Involved in the Collection of Blood in ACD on Maintenance of Red Cell Viability During Refrigerated Storage. *Am. J. Clin. Path.*, **26**:855, 1956.
16. Good, R. A. and L. Thomas: Studies on the Generalized Schwartzman Reaction. II. The Production of Bilateral Cortical Necrosis of the Kidneys by a Single Injection of Bacterial Toxin in Rabbits Previously Treated with Thorotrast or Trypan Blue. *J. Exper. Med.*, **96**:625, 1952.
17. Goudsmit, R. and H. W. Krijnen: Increased Destruction of Compatible Red Cells. *Vox Sanguinis, Basel*, **2**:357, 1957.
18. Litwin, M. S., C. W. Walter and N. Jackson: Experimental Production of Acute Renal Tubular Necrosis. *Ann. Surg.*, **152**:1010, 1960.
19. McEntegart, M. G.: Dangerous Contaminants in Stored Blood. *Lancet*, **2**:909, 1956.
20. Pittman, M.: A Study of Bacteria Implicated in Transfusion Reactions and of Bacteria Isolated from Blood Products. *J. Lab. & Clin. Med.*, **42**:273, 1953.
21. Smith, R. T., L. Thomas and R. A. Good: Generalized Schwartzman Reaction. V. Intravenous Injection of Colloidal Iron or Carbon on Response of Rabbits to Meningococcal Toxin. *Proc. Soc. Exper. Biol. & Med.*, **82**:712, 1953.
22. Stevens, A. R., J. S. Legg, B. S. Henry, J. M. Dille, W. M. Kirby and C. A. Finch: Fatal Transfusion Reactions from Contamination of Stored Blood by Cold-growing Bacteria. *Ann. Int. Med.*, **39**:1228, 1953.
23. Stokes, E. J. and J. D. James: Effect of Temperature on Survival of Bacteria in Blood for Transfusion. *Brit. M. J.*, **2**:1389, 1957.
24. Tyminski, W.: Manifest and Latent Hemolysis of Preserved Blood. *Pol. Arch. Med. Wewnet*, **29**:511, 1959.
25. Walter, C. W., R. B. Kundsinn and L. N. Button: New Technic for Detection of Bacterial Contamination in a Blood Bank Using Plastic Equipment. *N. Eng. J. Med.*, **257**:364, 1957.